BIOACTIVITY OF CAROTENOIDS FROM SHRIMP SHELL WASTE

By

SINDHU.S, M.F.Sc.

Thesis

Submitted in partial fulfillment of the requirement for the degree

DOCTOR OF PHILOSOPHY

IN

FISH PROCESSING TECHNOLOGY

Faculty of Fisheries

Kerala Agricultural University

2010

DEPARTMENT OF PROCESSING TECHNOLOGY

COLLEGE OF FISHERIES

PANANGAD, COCHIN-682 506

DECLARATION

I hereby declare that this thesis entitled **"BIOACTIVITY OF CAROTENOIDS FROM SHRIMP SHELL WASTE"** is a bonafide record of research work done by me during the course of research and that the thesis has not formed the basis for the award to me of any degree, diploma, associateship, or other similar title, of any other University or society.

Panangad **SINDHU.S**

20/03/2010 **2006-26-102**

CERTIFICATE

Certified that this thesis entitled "**BIOACTIVITY OF CAROTENOIDS FROM SHRIMP SHELL WASTE"** is a record of research work done independently by **Smt. SINDHU.S** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship, or associateship to her.

Panangad

20/03/2010

Dr.P.M.Sherief

(Chairman, Advisory Committee)
Professor & HOD
Department of Processing Technology,
College of Fisheries
Panangad, Kochi.

NAME AND DESIGNATION OF THE MEMBERS OF THE ADVISORY COMMITTEE/ EXAMINATION COMMITTEE

CHAIRMAN **SIGNATURE**

Dr. P. M. SHERIEF
PROFESSOR AND HOD
DEPARTMENT OF PROCESSING TECHNOLOGY,
COLLEGE OF FISHERIES,
PANANGAD, KOCHI

MEMBER

Sri. S. KRISHNAKUMAR
ASSISTANT PROFESSOR (SELN. GR.),
DEPARTMENT OF PROCESSING TECHNOLOGY,
COLLEGE OF FISHERIES,
PANANGAD, KOCHI.

MEMBER

Dr. SAJAN GEORGE,
PROFESSOR,
DEPARTMENT OF PROCESSING TECHNOLOGY,
COLLEGE OF FISHERIES,
PANANGAD, KOCHI.

MEMBER

Dr. DEVIKA PILLAI
ASSOCIATE PROFESSOR,
DEPARTMENT OF AQUACULTURE,
COLLEGE OF FISHERIES,
PANANGAD, KOCHI.

MEMBER

Smt. MALIKA. V
ASSISTANT PROFESSOR (SELN. GR),
DEPARTMENT OF MANAGEMENT STUDIES,
COLLEGE OF FISHERIES,
PANANGAD, KOCHI.

EXTERNAL EXAMINER

ACKNOWLEDGEMENTS

First and foremost let me express my profound gratitude to my beloved guide **Dr.P.M.Sherief**, Professor, Department of Processing Technology, without whose help this thesis would not have materialised. He has always been a pillar of support, initiating me into this topic, providing me not only guidance and advice but also timely help whenever needed. My sincere thanks also go to him for his meticulous correction of the manuscript.

I owe my deep sense of gratitude to **Dr. C. Mohanakumaran Nair**, Dean, College of Fisheries for permitting me to utilize all the facilities at the College of Fisheries, Panangad and for the moral support extended to me.

Dr.M.C.George, Associate Professor (Retd), Department of Processing Technology, College of Fisheries has always been very approachable and co-operative. I take this opportunity to thank him for all the help, guidance given to me especially in carrying out the animal studies which made this endeavour successful. **Sri. S. Krishnakumar**, Assistant Professor (Seln.Gr), Department of Processing Technology, College of Fisheries has always showed great interest in the progress of my work. I whole heartedly thank him for the goodwill extended to me. My profound thanks go to **Dr. Sajan George**, Professor, Department of Processing Technology, College of Fisheries for his conducive suggestions, advice and help in the conduct of my work and also in the final correction of the thesis.

I would like to thank **Dr. D. D. Nambudiri** , Former Dean (i/c), College of Fisheries for his genuine interest and the indispensable encouragement during the entire course of study.

I would like to thank **Dr. Devika Pillai**, Associate Professor, Department of Aquaculture, College of Fisheries for the motivation and hearty co-operation she extended to me in the course of my work.

The supervision of the statistical analysis and design of experiments for the thesis has been competently done by **Smt. Malika. V**, Assistant Professor (Seln.Gr), Department of Management Studies. My sincere gratitude goes to her for all the help given to me.

Dr. Lizy Behnan, Professor (Retd), Department of Processing Technology, College of Fisheries, has always been a constant source of help and care. I am greatly indebted to her for her careful attention and encouragement during the analytical works of my project.

I would also like to thank **Dr. T.V. Sankar**, Principal Scientist, Biochemistry Division, Central Institute of Fisheries Technology for his help in carrying out certain analytical works in their department.

My sincere thanks go to the library staff of College of Fisheries and CIFT for the unstinting help they have rendered me in the collection of data and research materials. The dedication of technical staff and non teaching staff, College of Fisheries has also helped in the successful conduct of the work and I sincerely thank them for this.

My friends have all been extremely supportive of me right from the beginning of this work. I would like my friends **Sophia, Manjusha, Manju**, **K.G, Vijayata** etc to know that it is their loyalty and belief in me that has contributed to the successful completion of this work. My friend **Anu** deserves special thanks for her unfaltering help and encouragement through out the course of study. I also wish to express my thanks to **Mr. A.M. Kareem**, Lab assistant, Biochemistry Lab for his kind and timely help extended to me during the course of my research work.

I would like to thank my beloved parents, in laws, my husband and my son for their sacrifices, encouragement and timely support in successfully completing my work.

I would like to thank the **Kerala Agricultural University** for awarding me a merit scholarship during the tenure of my work.

Sindhu.S

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

1. INTRODUCTION

The ocean is one of earth's most valuable natural resources. The sea is full of riches with a big treasure of living and non living resources. It provides food in the form of fish and shellfish, about 200 billion pounds are caught each year. Fisheries today provide about 16% of the world's total protein requirement with higher percentages occurring in developing nations. Fisheries is enormously important to the economy and well being of communities. The oceans have been fished for thousands of years and are an integral part of human society.

The ocean is an increasingly important source of bioactive substances with enormous potential for fighting disease. Marine natural products have attracted the attention of biologists and chemists the world over for the past five decades. As a result of the potential for new drug discoveries, marine natural products have attracted scientists from different disciplines, such as organic chemistry, bioorganic chemistry, pharmacology, biology and ecology. In recent years, many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs, etc (Donia and Hamann, 2003; Haefner, 2003). More recently, marine-derived microorganisms such as bacteria, fungi, cyanobacteria and other microalgae have also come into the focus of marine drug prospection. The search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites, many of which are endowed with pharmocodynamic properties (Fuesetani, 2000).

The low yields of compounds that are produced by many marine invertebrates and marine microorganisms have proven to be a severe obstacle for the development of promising compounds. Considering the potential of marine bioactive substances, and the avenues for developing potent new drugs and other useful products, a holistic approach is required to develop fish/ shellfish therapeutics, immunostimulants and other feed additives. Due to the structural complexity of most marine natural products total synthesis is not

feasible in many cases. Alternative strategies aiming at a sustainable supply of marine derived drugs are hence needed. One strategy which holds promises for the future is mass culture of potential organisms.

Although a wide and extensive speciation at all phylogenetic levels, from microorganisms to mammals are available as potential candidates for bioactive compounds, one of the biggest impediments in the development of drug candidates isolated from marine animals is the creation of a long-term, sustainable supply. Initial identification of bioactives from marine sources paved the way for utilization of huge amounts of fish processing waste. Following the identification of the potential to isolate new functional biomaterials from fish and shell fish, seafood processing waste acquired a commercial value as an industrial raw material. The recovery of these valuable components from the waste would not only improve the economy for seafood processors, but also would minimize the pollution potential of this industrial waste. Owing to their easy accessibility and economic aspects, many of these wastes can serve as a major source of medicinally useful products. Bioactives from cephalopod wastes such as antibacterial substance from accessory nidamental gland (ANG) of squid and cuttlefish; a peptidoglycan from cuttlefish ink showing antitumour activity were isolated (Sherief *et al.,* 2004). Cuttlefish liver oil showed antiatherogenic, anti-inflammatory and immunostimulant properties (Sophia, 2007). In addition, various fish and shell fish source materials such as skin, muscle, frame, bone and internal organs are presently utilized as a source to isolate a number of bioactive materials. Skin and proteins remaining on 'fish frame' serve as a source of bioactive peptides and internal organs are a source of enzymes (Kim *et al.,* 2008). Other seafood wastes such as shrimp shell waste has long been used for the production of chitin, chitosan and glucosamine hydrochloride with diverse biopotency. In addition to such compounds shrimp shell discards can be utilized for isolation of novel biopotent compounds. A recent innovation in fisheries and shrimp waste management is the scale/astaxanthin sorption process where a solid

waste like fish scales is used as a natural adsorbent for a carotenoid pigment astaxanthin from the seafood industry wastewater (Stepnowski *et al.*, 2004).

Shrimp processing is one of the major food industries in India. Shrimp processing waste is the single largest industrial fish waste in the country causing diverse environmental problems. Only 40% of the shrimp is edible and remaining 60 % account for the processing discards (Barratt and Montano,1986). An estimated 1.44 million tonnes of seafood waste is generated world wide every year, most of which is dumped in landfill or oceans. Most of these byproducts are not properly utilized. This waste finds very little practical application at present and is categorized as a major environmental contaminant. Since strict environmental restrictions are enforced disposal of shrimp processing waste has become a problem to processors. Effective utilization of the waste can resolve many of the environmental concerns facing the shell fish processors (Sahidi *et al.*, 1992). Hence proper technology has to be developed for the utilization of the waste for the production of materials which are beneficial to mankind.

Shrimp shell waste is a good economic source of protein, lipid, calcium carbonate, chitin and carotenoids. Utilisation of shrimp processing waste also provides ample scope for value addition and enhances its value as an agricultural commodity. Crustacean offal may also be used for the production of value added products such as flavourants, protein hydrolysate, chitin, chitosan and carotenoid pigments. Shrimp shell proteins are well balanced in amino acid composition and as such can be used as an excellent component of starter feed for animals and for the aquaculture industry (Sahidi *et al.*, 1992). Chitin, chitosan, glucosamine hydrochloride are some of the bioactives substances commercially produced from shrimp shell waste.

Prawn shell has the potential to do a lot more than create a fearsome smell in garbage bins. There is a growing interest in the functionality of natural colourants and pigments (Sang *et al.*, 2002). Natural products have long been

used as foods, fragrances, pigments, insecticides, medicines, etc (Joffe and Thomas, 1989).

In addition to the traditional uses, shrimp waste is one of the important natural sources of carotenoid (Shahidi *et al.*, 1998; Venugopal, 2008). Shrimp waste could be the cheapest raw material for carotenoid recovery, and later could be a better alternative to synthetic carotenoid. The carotenoid waste from Indian shrimps was found to vary from 35 to 153µg/g depending on the species, the major pigment being astaxanthin and its esters (Sachindra *et al.*, 2005a). Carotenoids are the most important among the numerous pigments that are found in nature. They provide, in nature, a wide variety of colours ranging from yellow to orange or red thus giving colour to aquatic life. Only phytoplankton, algae and plants produce carotenoids. Their native function in algae and plants are as light harvesting molecules and as antioxidants.

The major component of carotenoids of shrimp and crab shell backs were mono and diesters of astaxanthin (Shahidi *et al.*, 1992), a very potent antioxidant with some unique properties suitable for use as a drug or food supplement in the treatment of cardiovascular,immune,neurodegenerative diseases and cancer. Astaxanthin is a powerful quencher of singlet oxygen activity and a strong scavenger of oxygen free radicals. It is more effective than all others antioxidants known till date. These properties have caused great interest in this substance. Drugs from natural sources with these medicinal effects would be a boon to the areas of health and industry. Many of the synthetic medicines available for the treatment of these disorders have the risk of serious side effects and safer alternatives are essential. Effective utilization of shrimp shell waste will enhance its status as a biomedical research material, for development of natural medicine without side effects.

This present study is aimed at considering more useful purposes for this rotting waste. The novel extraction techniques raise the possibility of not only accessing a new source of astaxanthin but also finding an environment friendly use of thousands of tonnes of prawn waste discarded by seafood lovers around

the world each year. The present study is aimed at complete utilization of prawn waste or to “value add” the same. The work can claim impressive green credentials, the solvents can be recycled and the remnants of the shell can be used as water filling agents or to create a natural food coating, a substitute for petrochemical derived waxes.

Health promoting role of certain foods beyond their nutrional value have identified them as functional foods or neutraceuticals. As a whole, both physiological functional foods and neutraceuticals demonstrate many physiological cum health benefits (Schmidi, 1993; Defelice, 1995). This stinking waste can be used for extracting such a super food supplement, a powerful antioxidant which protect cells in human body from oxidative damage. Astaxanthin is permitted by FDA as a dietary supplement in dosages of 2 mg/day (Meland, 2008). It can be manufactured as the sole ingredient in capsule form or as an ingredient in a mixture with other antioxidants or vitamins. Its safety is well documented and has a proven protective role in aquatic life. It exhibits the same antioxidant properties in humans. Scientific studies suggest effects against most of the plagues of western society, diseases which sooner or later will affect most of all. It also crosses the blood brain barrier, which makes it available to the eye, brain and central nervous system to alleviate oxidative stress that contributes to ocular and neurodegenerative diseases such as Glaucoma and Alzheimer’s. Astaxanthin can act as a prophylactic and curative agent or even a panacea against various disease conditions. It is the most powerful antioxidant known till date and finds application in poultry feeds, aquaculture feeds, in cosmetics like anti ageing creams, lotions and other personal care products and even in health drinks. Almost all of the astaxanthin is contained in the shells and heads of prawn which are thrown away. The residue available after extraction of carotenoids can be used for production of chitin and chitosan, thus having an integrated approach for efficient utilization of shrimp waste (Khanfari *et al.,* 2007). Thus the generation of dual income from this cheap raw material will lead to a profitable waste utilisation strategy without causing environmental pollution.

The main objectives of the present study are

- To develop a suitable method for the optimum extraction of carotenoids from shrimp shell waste.
- To study the antioxidant activity of pigments extracted from shrimp shell.
- To investigate the anti-inflammatory, anticancer and other biological activities of the shrimp shell pigment.

The investigations carried out in this thesis to fulfill the objectives are:

- Qunatification of carotenoids
- Superoxide anion scavenging activity
- Inhibition of lipid peroxidation
- Hydroxyl radical scavenging activity
- Measurement of paw edema
- Assay of cardiac enzymes
- Thymocyte and bone marrow cell proliferation
- Measurement of antibody titre
- Alpha-naphthyl acetate esterase activity in bone marrow cells.
- Assay of direct cytotoxic activity *in vitro*
- Assay of antitumour activity *in vivo* (Ascite tumour model and solid tumour model.
- Assay of anticancer activity *in vivo* (MTT cell proliferation assay)

2. REVIEW OF LITERATURE

2.1. UTILISATION OF SHRIMP SHELL WASTE

Shrimp processing waste is the single largest industrial fish waste in the country causing diverse environmental problems. Only 40% of the shrimp is edible and remaining 60 % is discarded as shrimp shell waste. In addition to protein, lipid, calcium carbonate and chitin, shrimp shell is a good source of carotenoids especially astaxanthin, a very potent antioxidant suitable for use as a drug or food supplement in the treatment of cardiovascular, immune, neurodegenerative diseases and also cancer. Effective utilization of shrimp shell waste will enhance its status as a biomedical research material, for development of natural medicine without side effects.

This waste finds very little practical application at present and is categorized as a major environmental contaminant. Hence proper technology has to be developed for the utilization of the waste for the production of materials which are beneficial to mankind (Nair *et al.,* 2002). Utilisation of shrimp processing waste also provides ample scope for value addition and enhances its value as an agricultural commodity. This will lead to profitable waste utilisation technology without causing environmental pollution.

2.1.1. Chitin and Chitosan

Chitin is a natural biopolymer of anhydro-N-acetyl-D glucosamine which is the most important structural constituent of the exoskeletal material or shell of the crustaceans(Sen, 2005). On an average shrimp shell waste contains 15-20% chitin. Hence shrimp shell forms the most economic source of chitin (Synowiecki and Al-Khateeb, 2003). Several studies have been carried out in India to develop methods for utilizing shrimp shell waste as a source of chitin. The work done in Central Institute of Fisheries Technology, Kochi in this field is worth mentioning. Methods have been developed for the extraction of protein and chitin and for the conversion of chitin into chitosan (Radhakrishnan and Prabhu, 1971; Madhavan and Nair, 1974, 1975; Nair and Madhavan,

1975;Gopakumar, 1993; Gopakumar, 1997). Chitosan is the deacetylated form of chitin. Scientists of Central Food Technological Research Institute, Mysore have developed processes for chitosan production (Moorjani *et al.,* 1975; 1978). Chitosan is a versatile polymer with a wide range of potential uses or commercial applications. Extensive work has also been done on the industrial application of Chitosan (Prabhu *et al.,* 1976; Madhavan and Nair, 1977, Nair and Madhavan, 1984; Nair *et al*., 1987; Nair and Madhavan, 1989; Mathew *et al.,* 1989; Gopal *et al.,* 1991 Madhavan, 1992; Rao *et al*., 1994; Benny *et al.,* 1994). Heamostatic activity of Chitosan has been reported by Sambasivan (1992).

Low molecular weight chitin oligosaccharides and chitosan oligosaccharides (COSs) are physiologically functional molecules responsible for biological activities such as antitumour activity (Jeon and Kim, 2002) antibacterial activity (Jeon *et al.,* 2001; Park *et al.,*2004a), and immunoenhancing activity (Tsukada *et al.,*1990). Heterochitosan oligosaccharides, those derived from partially deacetylated chitosans, can also be considered as promising biomaterials to positively affect some adverse biological events such as cancer (Jeon and Kim,2002) free radical generation(Park *et al.,*2003a; Je, *et al.,*2004), hypertension (Park *et al.,*2003b) and blood coagulation (Park *et al.,*2004b).

2.1.2. Glucosamine Hydrochloride

Hydrolysis of chitin with concentrated HCl gives glucosamine hydrochloride. Glucosamine is now widely used as a dietary supplement particularly for aged people to cure osteoarthritis. It helps to regenerate and rebuild cartilage, and to maintain healthy functioning of mobile joints. (Gopakumar, 1993; Nair *et al.,* 2002; Sen, 2005). Methods for the production of glucosamine hydrochloride have been developed by CIFT, Kochi and (National Chemical Laboratary (NCL), Pune.

2.1.3. Feed

2.1.3.1. Aquaculture Feed

Crustacean meals that are made from shrimp and crab waste are generally formulated into aquaculture rations, but not in large amounts as they are low in protein (Rutledge, 1971; Spinelli *et al.,* 1974). Shrimp wastes are generally rich in carotenoid pigments especially astaxanthin, a major pigment in marine crustaceans, which can be used as a pigment improver in aquaculture feeds(Wilkie, 1972; Omara-Alwala *et al*., 1985; Guillou *et al*., 1995; Whathene *et al.,* 1998). A number of authors have reported on the use of crustacean waste as a carotenoid source for the pigmentation of freshwater and marine fish and shellfish. Shrimp waste (Meyers and Rutledge, 1971; Saito and Reiger, 1971; Steel, 1971; Ellis, 1978), shrimp protein coagulum and hydrolysate (Simpson *et al.,* 1976; Gagne and Simpson, 1993; Gildberg and Stenberg, 2001),crab wastes (Saito and Reiger, 1971; Spinelli *et al.,* 1974; Kuo *et al*., 1976), crayfish waste(Peterson *et al*., 1966), krill waste(Spinelli, 1978), craw fish waste (Cremades *et al.,* 2001) are also used as carotenoid sources in the formulation of aquaculture feeds.

2.1.3.2. Animal and Poultry Feed

Chitin in shrimp shell is reported to have growth promoting effect in broiler chickens (Zikakis *et al.,* 1982; Nair *et al.,* 1987). Hypolipidemic and hypocholestremic activity of chitin in animals have been reported.(Nagyvary *et al*.,1979; Sugano *et al*., 1980; Hirano *et al.,* 1990). Scientists of Kerala Agricultural University had worked out methods of ensiling prawn waste with rice bran in the proportion 1:1 using tapioca flour or coconut cake as additives (Ramachandran *et al*., 1997). The silage used as cattle feed contained prawn waste, rice bran, tapioca flour and water in the ratio 37:37:10:16. Microbial load and feeding value in cattle were studied. The silage was found to be palatable with high ether extract digestibility. The silage was free from pathogens and fungal growth. It lacked foul odour and hard appendages of the

prawn waste were softened on ensiling. Hence it was also found to be safe for feeding cattle. The hydrolysate of shrimp shell waste can be used as growth stimulating agents in animal feeds (Gildberg and Stenberg, 2001). Ensilation of shrimp waste by *Lactobacillus fermentum* has been reported by Mathew and Nair (2006).

2.1.4. Protein

Shrimp waste is a source of proteins. The carotenoprotein obtained has a high content in essential amino acids, ω-3-fatty acids, and carotenoids mainly astaxanthin. This serves as an excellent nutritional source for patients with malnutrition. The proteins recovered with rich essential amino acids content can find application as flavoring or feed ingredient for animals such as salmon and trout where coloration is important, (Gagne and Simpson, 1993; Simpson *et al.,* 1993; Synowiecki and Al-Khateeb, 2000; Cremades *et al.,* 2001; Armenta and Guerrero-Legarreta, 2009).

2.1.5. Carotenoid Pigments

In addition to the above mentioned conventional uses of shrimp shell waste, new ways of shrimp shell utilization will improve their value as agricultural commodity (Sang *et al.,* 2002). Shrimp waste is one of the important natural sources of carotenoid (Shahidi *et al.,* 1998). Shrimp waste could be the cheapest raw materials for carotenoid recovery (Sachindra and Mahendrakar, 2005). There is a growing interest in the functionality of natural colourants or pigments such as astaxanthin.

Carotenoid pigments are the most important among numerous pigments that are found in nature. They are biologically active compounds with multiple applications in therapy (Pintea *et al*., 2003). To date over 750 natural carotenoids have been identified (Britton, 1995; Britton *et al*., 2004). The carotenoids are broadly classified into “carotenes” or non oxygenated carotenoids and “xanthophylls” oxygen substituted carotenoids (Cardounel *et al.,* 2003). These lipid soluble compounds contribute to the beautiful yellow,

orange, red colours found in microalgae, yeast, salmon, trout, krill, shrimp, cray fish, crustaceans and feathers of some birds (Simpson, 1982).

2.1.5.1. Carotenoid Pigments in Seafoods.

Carotenoids are the most spectacular pigments found in Mollusca, Crustacea and fishes. β-carotene is a very common carotenoid and is a major source of Vitamin A for animals. Lutein is a characteristic pigment of freshwater fishes. Zeaxanthin and lutein were found to be major pigments in freshwater mullets (Matsuno *et al.,* 1975). Tunaxanthin is a characteristic pigment of marine fish. Canthaxanthin has been isolated from a number of aquatic animals. Pigment echinenone has been isolated from brine shrimp (Simpson, 1982). Accumulation of astaxanthin, α-carotene and zeaxanthin has been reported in marine crab (Matsuno *et al.,* 1974). Astaxanthin is the most important marine pigment and is found in diverse groups of sea animals. Astacene is a breakdown product of astaxanthin and is reported to occur in some fish and shellfish. Astacene is formed by oxidation of astaxanthin where the two hydroxyl groups are oxidized to keto groups (Sen, 2005). Astaxanthin and its esters have been found to be the major carotenoids in marine crustaceans (Sahidi *et al.,* 1998). Matsuno and Maoka (1988) reported that astaxnthin is the major carotenoid in meat and shell of marine crab *Paralithodes brevipes.* Khanfari *et al.* (2007) revealed that astaxanthin, astaxanthin monoester, diester and α-carotene were the major pigments in marine shrimp *Penaeus semisulcatus.* Breithaupt (2004) isolated five astaxanthin monoesters, eight astaxanthin diesters and free astaxanthin from shell extract of *Pandalus borealis.* Astaxanthin content of the muscle of five major species of Indian shrimp *viz. Metapenaeus monoceros, M. dobsoni, M .affinis, Feneropenaeus indicus* and *Parapenaeopsis stylifera* are in the range of 0.6-1.4mg/g oil or 0.4-1.4mg/100g of wet muscle (Gopakumar and Nair, 1975). Sachindra *et al.* (2005a) reported that carotenoid content in the wastes of Indian shrimps was found to vary from 35 to 153 μg/g depending on the species, the major pigment being astaxanthin and its esters.

2.1.5.2. Role of Carotenoids in Marine Animals

Carotenoids serve important physiological functions apart from colouration. They function as keys for species identification and as mating signals. Astaxanthin supplementation in fishes provide many physiological benefits such as antioxidant action, vitamin like properties, immune enhancement, hormone like action, increase in vitality thus stimulating breeding, securing maximum survival and growth, resistance to environmental stress, maturation, protection from cataracts, etc. It also provides photoprotection of the external proteins and eggs from UV exposure (Meyers, 1993; Christiansen *et al.,* 1995*;* Torrisen and Christiansen, 1995;Vasallo-Agius *et al.,* 2001; Waagabo *et al.,* 2003; Chien *et al.,* 2003).

2.2. ASTAXANTHIN

Astaxanthin was first chracterised in 1938 from the extract of lobster *Homarus astacus.* Astaxanthin is the most powerful phytonutrient antioxidant. It is regarded as the king of carotenoid family. Astaxanthin has some unique properties that may open up very promising possibilities for neutraceutical, pharmaceutical and cosmetic industries (Murillo, 1992; Jyonouchi *et al*., 1994; Tanaka *et al*., 1995a; Tanaka *et al*., 1995b; Savoure, 1995; Tso and Lam, 1996; Khanfari *et al*., 2007). It belongs to a larger class of phytochemicals known as ‘terepenes’or isoprenoids with its repeated units isoprene (Fig.1) with alternate double and single bonds**.** It is classified as xanthophylls which means ‘yellow leaves’. Xanthophylls are oxygenated carotenoids which include lutein, zeaxanthin, capsanthin, canthaxanthin, astaxanthin, echionine and β-cryptoxanthin (Parada and Anguilera, 2007). Astaxanthin is the most important marine pigment with characteristic red orange colour and is found in a very diverse groups of sea animals including salmon, trout, red sea bream, shrimp, lobster as well as in birds such as flamingo and quail (Torrisen *et al.,* 1989; Guerin *et al*., 2003).

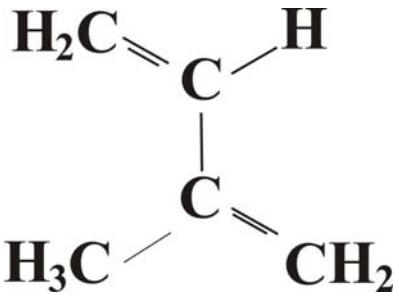


Fig. 1. Basic unit of astaxanthin molecule-isoprene

2.2.1. Astaxanthin in Nature

Astaxanthin is the major pigment in seafood such as salmon, trout, red seabream, shrimp, lobster and fish eggs (Torrisen *et al.,* 1989). In marine environment it is biosynthesized in yeast, microalgae and phytoplankton. In aquatic environments, carotenoids obtained from microalgae are transformed into astaxanthin by zooplankton, which makes it available to higher trophic levels (Andersson *et al.,* 2003*).* Production of astaxanthin in aquatic systems can be affected by changes in nutrient dynamics mediated by phytoplankton community composition. This may have implications for antioxidant protection at higher trophic levels in the food web (Nieuwerburgh, *et al*., 2005).

The yeast *Phaffia rhodozyma* and the algae *Haematococcus pluvialis* provide the most concentrated natural source of astaxanthin. When nutrients become limiting, or when its environments begin to dry in, the algae and the yeast produce massive amounts of astaxanthin. The algae then encyst. Cells with a high concentration of astaxanthin are more resistant to environmental hazards such as light than cells with a low concentration. It is also found in birds like flamingos, quails, and other species (Egeland, 1993; Inborr, 1998). It is a very unstable pigment, particularly free astaxanthin is much sensitive to oxidation. It occurs in many diverse species in nature such as yeast, algae, plants, sponges, coelenterates, polychaetes, molluscs, crustaceans, echinoderms, tunicates, fishes, insects, reptiles, birds and mammals (Karrer and Jucker, 1950; Simpson, 1982).

2.2.1.1. Carotenoproteins and Carotenolipoproteins

Complexes of carotenoids and proteins known as carotenoproteins and carotenolipoproteins dominate in the exoskeleton of crustaceans, coelentrates and many other marine species (Matsuno, 2001). In combination with proteins and lipids carotenoid shows a shift in the absorbance of astaxanthin such that the carotenoprotein gives rise to a wide range of blue, green, yellow, purple and brownish colours of marine life (Tlusty and Hyland, 2005). Astaxanthin gives marine invertebrates their colour by forming complexes with proteins covering the complete visible absorption spectrum and has typical absorption maxima at 472nm in hexane (Britton *et al.,* 1981; Zagalsky *et al.,* 1990; Cianci *et al.,* 2001; Cianci *et al.,* 2002). Form and level of deposition of astaxanthin between tissues and species. It is found either conjugated to protein such as in salmon muscle or lobster exoskeleton or esterified with one or two fatty acids which stabilize the molecule (Torrisen *et al.,* 1989; Lorenz and Cysewski, 2000).

2.2.1.2. Biosynthesis and Bioconversion of Carotenoids

Animals are unable to perform a *de novo* synthesis of carotenoids (Britton, 1995). Bacteria, yeasts, molds and higher plants can form these from acetate by mevalonate pathway (Fig.2). In animals, ingested carotenoid can undergo conversion to another pigment or result in changes such as esterification (Simpson, 1982; Davies, 1985). It is reported that synthesis and accumulation of astaxanthin from β-carotene in *Haematococcus* involves the action of two enzymes, β-carotene ketolase (β-carotene to canthaxanthin) and carotene hydroxylase (canthaxanthin to astaxanthin) (Santos and Mesquita, 1984). Katayama *et al.* (1973) divided fish and shellfish on the basis of bioconversion capabilities as ‘Red carp type’ which can convert lutein, zeaxanthin or intermediates to astaxanthin. But in redcarp type, β-carotene is not an efficient precursor of astaxanthin. Astaxanthin can be stored directly. ‘Sea bream type’ cannot convert β-carotene, lutein or zeaxanthin to astaxanthin, but capable of transferring pigments from the diet to tissue

pigments as free form or esterified and 'Prawn type', β-carotene is converted to astaxanthin and crustaceans belong to this group.

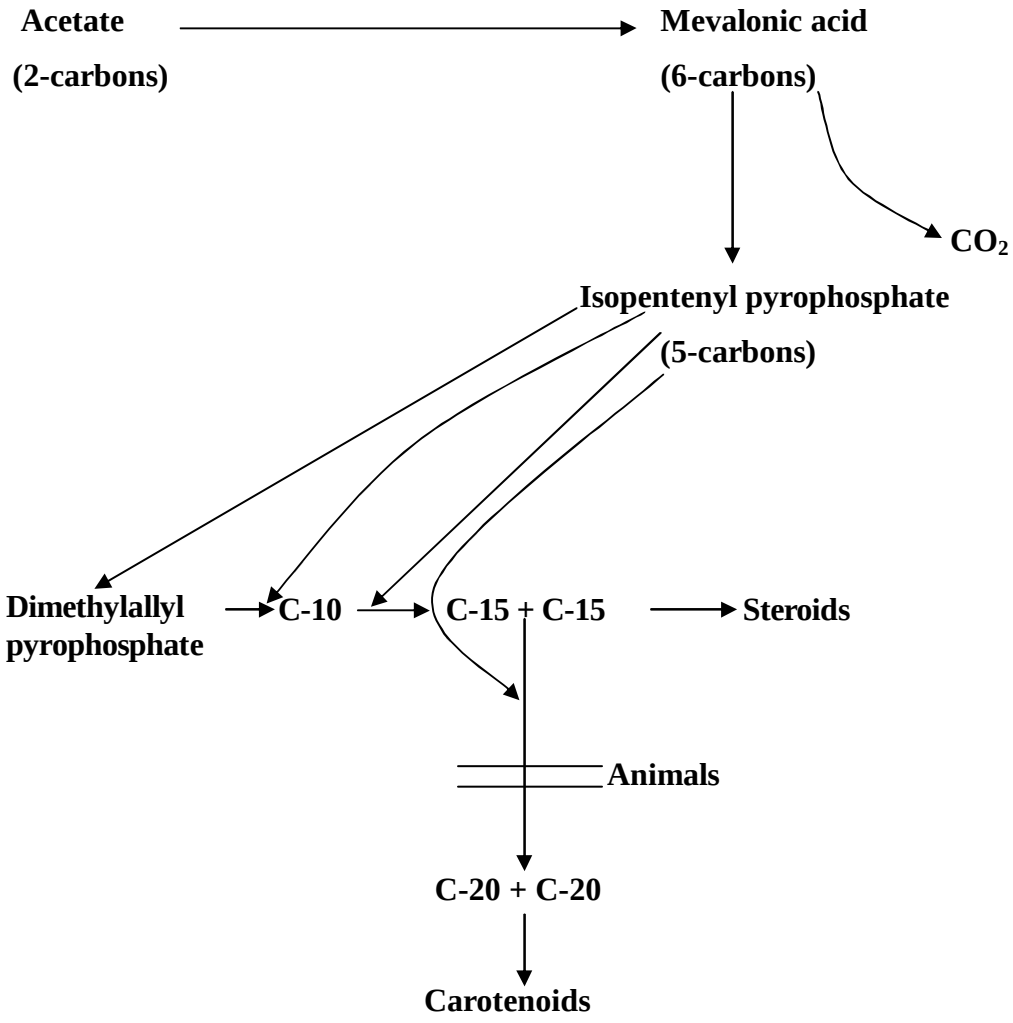


Fig. 2. Biosynthetic formation of carotenoids

2.2.1.3. Potential Sources of Natural Astaxanthin

An ubiquitous green microalga *Haematococcus pluvialis* has been identified as the prime natural source of the pigment astaxanthin for commercial exploitation. It seemed to accumulate the highest levels of astaxanthin in nature more than 40g astaxanthin per kilo of dry mass (40,000 ppm). Some *Haematococcus* strains have been observed to accumulate 70-80g astaxanthin per kilo of dry biomass (Olaizola, 2000). It has the advantage that mass production is possible with population doubling every week.

However much expertise among personnel is required for providing optimum conditions for inducing astaxanthin accumulation in algae, which is its drawback (Droop, 1953; Droop, 1961; Almgren, 1966; Borowitzka *et al.,* 1991, Johnson and An, 1991; Lorenz and Cysewski, 2000). The bacteria *Agrobacterium aurantiacum* can also produce astaxanthin (Yokoyama and Miki, 1995).Another high accumulating organism is phaffia yeast *Xanthophyllomyces dendrorhous.* It has an astaxanthin concentration of 8000 ppm. It has the advantages of fast multiplication and comparatively low price (Gil-Hwan and Eui-Sung, 2003). It also has the problem of being low in nutritional value unlike krill or shrimp. Since it is not ocean based it lacks ocean like traits and it also requires refrigeration once harvested (Parajo *et al.,* 1998).

Arctic shrimp *Pandalus borealis* has an astaxanthin concentration of 1200 ppm. It has the advantage of being nutritionally rich and tasty. It is disadvantageous that only head and shell of this species can be used for astaxanthin extraction. Antartic krill *Euphausia superba* has an astaxanthin concentration of 120 ppm. It reproduces more and faster than other larger ocean creatures. The krill fishing operation is complex, involving high cost and much expertise. Plankton has a **c**oncentration of 60 ppm and salmons have an astaxanthin concentration of 5 ppm. It obtains its colouration from food sources *viz.* algae and crustaceans.

2.2.2. Structure of Astaxanthin

Professor Basil Weedon was the first to map the structure of astaxanthin (Fig.3). It is an oxygenated carotenoid called xanthophyll with a molecular weight of 596.8 Da. Its molecular formula is $C_{40}H_{52}O_4$.

Its chemical name is 3,3'-dihydroxy-β β'carotene-4,4'dione. It has a long, double bonded polyene chain with a 6 membered polar end group. The presence of hydroxyl and keto ending on each ionone ring, explains some unique features such as its ability to be esterified, its uniquely powerful antioxidant activity and a more polar configuration relative to other

carotenoids (Shibata *et al*., 2001; Guerin *et al*., 2003). Like all carotenoids astaxanthin also shares a structural feature termed "polyunsaturation" *ie* they have several "unsaturated" or "double" bonds. The double bonds are arranged in a series alternating with single bonds termed as "conjugated" which means the electrons that make up the double bonds are delocalized or shared evenly over the whole chain. This conjugated structure is responsible for the characteristic yellow, orange, or red colours typical of the carotenoids. The differences in colours depend primarily on the number of double bonds conjugated. Conjugated double bonds are chemically very stable, yet capable of specific chemical reactions. This typical feature explains the uniqueness of astaxanthin. The astaxanthin molecule is similar to that of familiar carotenoid β-carotene, but the small difference in structure confers large differences in the chemical and biological properties of two molecules. With its unique structure and resulting potent antioxidant activity, astaxanthin may be an effective therapeutic modality for variety of conditions, including cardiovascular, immune, anti-inflammatory and neurodegenerative diseases.

2.2.3. Different Chemical Forms of Astaxanthin

Astaxanthin has chemical features that result in the existence of several forms of it which can be classified according to stereoisomers, geometric isomers, and free or esterified forms. All of these forms are found in various natural sources. The predominant stereoisomer of astaxanthin found in krill (*Euphausia superba*, a shrimp-like marine animal) is 3*R*,3'*R* (Bernhard, 1990), and the majority of this is esterified (Foss *et al*., 1987). In wild salmon, the predominant stereoisomer is 3*S*,3'*S*; in salmon flesh the astaxanthin occurs as the free xanthophyll (Bernhard, 1990). The basidiomycete yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) accumulates astaxanthin as its major carotenoid; in this yeast, astaxanthin occurs as the 3*R*,3'*R* stereoisomer and is predominantly esterified. In the green alga *Haematococcus pluvialis*, astaxanthin occurs as the 3*S*,3'*S* stereoisomer (Bernhard 1990). Astaxanthin from *H. pluvialis* occurs primarily as monoesters

(~80%) and diesters (~15%); the predominant fatty acids that make up the esters are C18:1 and C20:0 (Renstrøm and Liaaen-Jensen 1981).

2.2.3.1. Stereoisomers.

Astaxanthin has two chiral (pronounced "ky-ral"), or asymmetric, centers. These are the carbons numbered 3 and 3' (pronounced "three prime") on the two rings in the structure. Chiral asymmetry is analogous to "handedness". A left hand and a right hand are mirror images of each other--they are similar but not identical, and are not superimposable. Similarly, a chiral center can exist in either of two configurations; the same atoms are bonded to the chiral center, but the three-dimensional arrangements are different and not superimposable. Chemists identify chiral centers as being either *R* or *S* (from *rectus* or *sinister*, Latin for "right" or "left"). The two chiral centers in astaxanthin, carbons 3 and 3', can each exist either in the *R* or the *S* form, and thus there are a total of three stereoisomers: 3*S*,3'*S*,(Fig.4) 3*R*, 3'*S*,(Fig.5) or 3*R*,3'*R*(Fig.6). The 3*S*,3'*S* and 3*R*,3'*R* stereoisomers are mirror images of each other and are termed "enantiomers". Each enantiomer has the opposite optical activity of the other, i.e., a solution of a pure enantiomer will rotate plane-polarized light in a direction opposite to that observed for the other enantiomer. The 3*R*,3'*S* form is sometimes termed "*meso*" and is optically inactive because there is a plane of symmetry through the center of the molecule.

2.2.3.2. Geometric Isomers

Carbon-carbon double bonds can have the atoms attached to them arranged in different ways. This arrangement cannot be changed by the atoms twisting or rotating around the bond (since double bonds are not "flexible" in the way single bonds are) without breaking the double bond. If the two largest groups are attached on the same side (looking down the double bond's length) of the double bond, they are termed *Z* (from *zusammen*, German for "together"). If the two groups are on opposite sides of the double bond, they are

termed *E* (from *entgegen*, German for "opposed"**).** Older texts may refer to *Z* as "*cis*" and *E* as "*trans*", however *Z* and *E* are the recommended nomenclature today. Astaxanthin has several double bonds in the linear portion of the molecule, each of which can potentially exist in the *Z* or *E* form. The thermodynamically most stable form of the molecule is all-*E* ("all-*trans*") astaxanthin. This is because in the all-E form, the branching methyl (CH_3) groups on the linear portion of the molecule do not compete for space. In nature, *Z* isomers have been observed at positions 9, 13, and 15, singly or in combination. Thus, several geometric isomers are possible: all-*E*, (9*Z*), (13*Z*), (15*Z*), (9*Z*,13*Z*), (9*Z*,15*Z*), (13*Z*,15*Z*), and (9*Z*,13*Z*,15*Z*) (Bernhard 1990).

2.2.3.3. Free or Esterified.

Astaxanthin has two hydroxyl (OH) groups, one on each terminal ring. These can be "free" (unreacted) hydroxyls, or can react with an acid (such as a fatty acid) to form an ester. If one hydroxyl reacts with a fatty acid, the result is termed a mono-ester (Fig 7). If both hydroxyl groups are reacted with fatty acids, the result is termed a di-ester (Fig 8). Adding a fatty acid to form an ester makes the esterified end of the molecule more hydrophobic. In order of hydrophobicity (difficulty in dissolving in water), we find that di-esters > mono-esters > free.

2.2.4. Synthetic Astaxanthin

Synthetic astaxanthin is produced as the free (unesterified) xanthophyll and as a 1:2:1 mixture of the three stereoisomers: 3*S*,3'*S*; 3*R*,3'*S*; and 3*R*,3'*R*. Meso form or optically inactive form is the major isomer in synthetic astaxanthin. Synthetic astaxanthin is the major form used in salmon feeds.

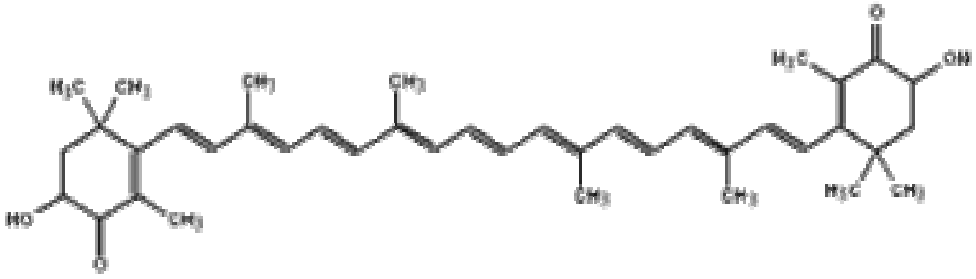


Fig. 3. Structure of astaxanthin molecule

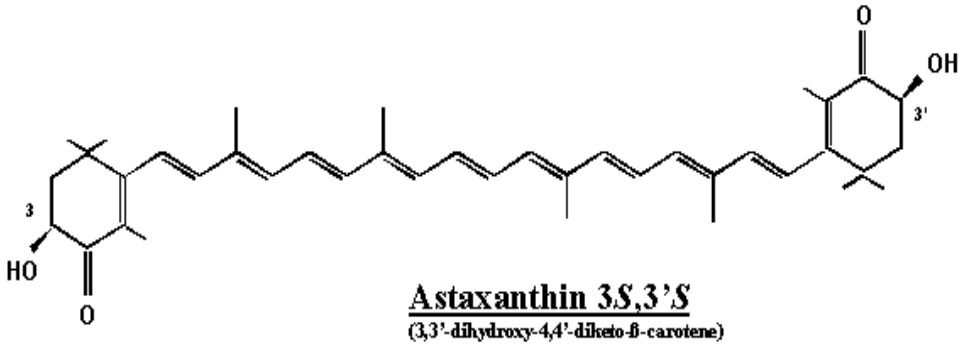


Fig. 4. Structure of astaxanthin stereoisomer 3S,3'S

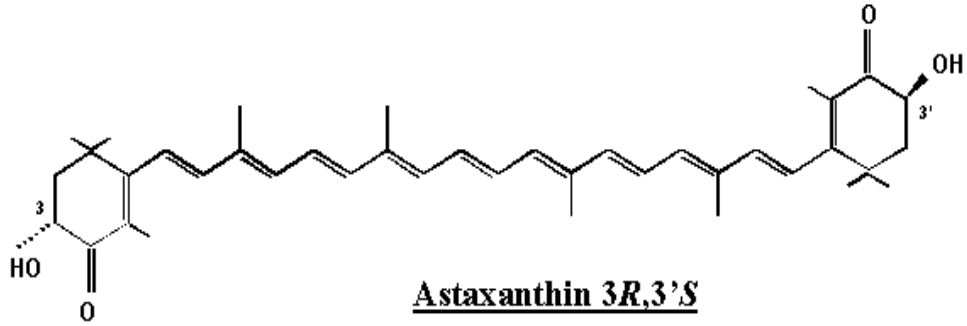


Fig.5. Structure of astaxanthin stereoisomer 3R,3'S

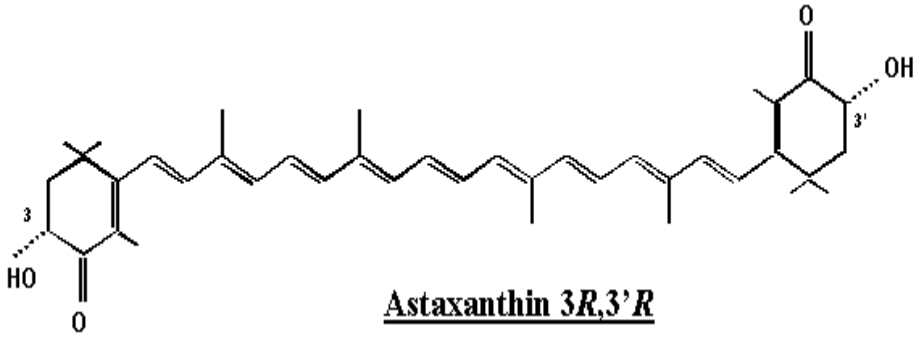


Fig.6. Structure of astaxanthin stereoisomer 3R,3'R

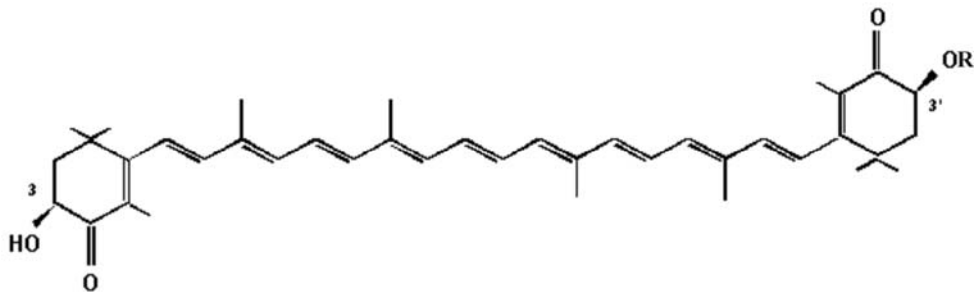


Fig.7. Structure of astaxanthin monoester

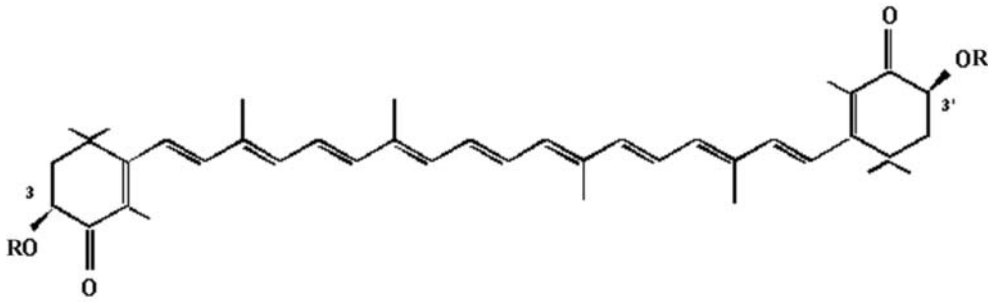


Fig.8. Structure of astaxanthin diester

2.2.4.1. Synthetic Astaxanthin Production Process

Synthetic form is produced from petrochemical sources. The process is complex with a 14 step process and is the longest synthesis sequence used for the production of a single substance. The method for C40 carotenoids is called as 'Double Wittig Condensation'. This involves condensation of a symmetrical C10 dialdehyde as a central C10-element with two equivalents of a C15 phosphonium salt. Sensitive substitution patterns like the oxidation-sensitive -ketol group are the special challenge in the synthesis process (Ernst, 2002).

2.3. COMMERCIAL PRODUCTION OF ASTAXANTHIN

2.3.1. Producers of Natural Astaxanthin

The different brands of natural astxanthin are Ecotone, USA (*P. rhodozyma)* Seeks Funding, Israel (*H.pluvialis),* Bioprawns, Norway (*P. borealis),* AstaReal, Bioastin, Astafactor, BioReal, USA, (*H. pluvialis)*, AstaCarox, Sweden,(*H. pluvialis)*,Parry's natural astaxanthin, India, (*H.*

pluvialis), Jingzhou natural astaxanthin, China,(*H. pluvialis),* Aquasta, UK, (Fermentation of carbohydrates, Dextrose)**,** astaxanthin from algae in Portugal, Krill astaxanthin from Ukraine. Cost of natural astaxanthin as reported in global market is $7000/Kg.

Parry's Nutraceuticals are the leaders in microalgal technology in India. They are the pioneers in cultivation, processing and extraction of microalgae *viz*. *Spirulina platensis*, natural mixed carotenoids from *Dunaliella salina*, Astaxanthin from *Haematoccocus pluvialis*. Parry neutraceuticals has its market in over 40 countries in the world. The different forms of astaxanthin include oil in soft gels, oleoresins, powder which are being marketed in India and also exported as raw material to pharma, food, cosmetic and feed companies to more than 48 countries. Their marketing agent Hallmark Aqua needs, Nellore is marketing a branded product called "Astamark" in coastal areas for the shrimp culture.

2.3.2. Producers of Synthetic Astaxanthin

The industrial producers of synthetic astaxanthin are Hoffmann-La Roche AG and BASF AG under the brand names Carophyll pink and Lucantin pink (Bradford, 1990).

2.4. MAJOR MARKETS OF ASTAXANTHIN

2.4.1. Nutraceuticals

Research efforts to identify functional and bioactive components from many natural sources have been intensified and two new group of products, "Functional foods" and "nutraceuticals" have been introduced into the market. Functional foods enriched with functional components offer medical and physiological benefits to reduce risk of chronic diseases, beyond their basic nutritional function. Nutraceuticals are bioactives isolated or purified from foods to be utilized in the medicinal form (Schmidi, 1993; Defelice, 1995). Natural astaxanthin demand is now emerging in the fast growing multibillion dollar nutraceutical market. Natural astaxnthin fetches a high price of 7000$

per kg in a global market and the synthetic form has a cost of 2000$ per kg. Nutraceutical products available in the market are mostly in the brand names mentioned above. They are available in different concentrations and in combination with other antioxidants. Natural astaxanthin is available as gel capsules, powder and as oleoresins. Some of the commercial products are *viz.* Astavita virgin salmon oil, Neptune Krill oil, AstaZan, AstaFactor sport's formula, FrequenSea, an astaxanthin containing drink, swiss chocolate drink containing astaxanthin.

2.4.2. Personal Care Products

Natural astaxanthin is an active ingredient in many of the cosmetic products, including skin care products, eye care products and body care products. Some of the commercial products are *viz.* Age Antidote Day Cream, Anti Aging Moisturizing complex liquid, Devita's Skin Care treatment products, Derma e astaz/pycnog eye, White Chocolate Body butter with Vitamin C, Hand and Body lotion, Age Defying formulas.

2.4.3. Feed

Largest market for astaxanthin is in aquaculture as a feed ingredient. Both synthetic and natural astaxanthin are used in feeds. It is a FDA approved colourant in animal and fish feeds. Astaxanthin is also considered GRAS (Generally recognised as safe). Synthetic astaxanthin is the major form currently being used in feeds (McCoy, 1999). It is most important in salmon industry as a common feed additive to make up for the lack of a natural source of the pigment (Torrisen *et al.,* 1989). It is commonly used in the aquaculture diets of salmon, trout, seabream (Kalinowski *et al.*, 2005) shrimp (Yamada *et al.,* 1990) and ornamental fishes (Gouveia *et al.,* 2003). Maximum permitted inclusion in feeds is 80mg/Kg which accumulates 4-10mg/Kg in salmons. Level of deposition of astaxanthin differ between tissues and species. Esterified form is the most adaptive form to store pigment without excessive oxidation. Natural astaxanthin is reported to have superior bioefficacy than synthetic.

Natural astaxanthin produces higher pigmentation compared to synthetic astaxanthin provided at the same dietary concentration. Consumers prefer fish fed with natural pigments. Aquagrow enhance for shrimps, Mini wafers colour for loricarids, loaches, crustaceans; Koi and Gold super colour sticks; Astatabin for crustaceans, discus and other fish; Astacolor, Colour enhancing flake feed for discus; Tahitian blend a mix of cryo-preserved marine microalgae for enhancing colour in marine invertebrates are some of the commercial products. Naturose natural astaxanthin produced from *Haematococcus* microalgae is a natural source of astaxanthin being used in fish and poultry feeds. It is incorporated similar to using a premix or added to semi moist feed, as a top coating of pellets with pigment oil mixture or by direct addition to freshly prepared feed. In poultry feed it is used for improving yolk, muscle and skin colouration (Inborr, 1998). Astaxanthin supplementation increases fertility of horses, pigs, and fur animals. Astaxanthin may enhance the effect of antistress agents on farm animals (Hansen *et al.,* 2001).

2.5. NATURAL Vs SYNTHETIC ASTAXANTHIN

The chemical difference between natural and synthetic astaxanthin lies in the stereochemical orientation of molecules in space. Astaxanthin exists in three main enantiometric forms, termed 3*S*,3'*S*, 3*R*, 3'*S* or 3*R*,3'*R*, depending on the spatial orientation of the ydroxyl (OH) groups in chiral carbon number 3. Natural astaxanthin in wild salmon is found in the 3*S*,3'*S* enantiometric form as found in *Haematococcus.* Synthetic astaxanthin from petrochemical sources contains a 1:2:1 mixture of the three stereoisomers a mixture of all the enantiomers of astaxanthin, as a direct result of chemical synthesis, primarily 50 % is the *3R, 3'*S enantiomer (the meso form). Health beneficial effects of 3*S*,3'*S* have been studied and synthetic astaxanthin with a predominance of 3*R*, 3'*S* significant biological effect has not been established. Synthetic astaxanthin is produced as the free (unesterified) xanthophyll and as a: 3*S*,3'*S*; 3*R*,3'*S*; and 3*R*,3'*R*. Meso form or optically inactive form is the major isomer in synthetic astaxanthin.

2.6. PROXIMATE COMPOSITION

Proximate composition of shrimp shell waste has been studied by many authors. It shows variations with respect to the different species studied. Nair *et al.* (2002), reported proximate composition of prawn waste as 75-80 % moisture, 30-35 % ash on dry weight basis, 35-40 % protein, 15-20 % chitin and 3-5 % fat. Sen (2005) had reported the proximate composition of shrimp body peeling waste and shrimp head waste. Proximate composition of *Metapenaeus dobsoni* has been reported by Madhavan and Nair (1974). Spinelli *et al.* (1974) reported proximate composition of red crab waste. Sahidi *et al.* (1992) and Holando and Netto (2006) studied chemical composition of queen crab waste and *Xiphopenaeus kroyeri.* Ramachandran *et al.*(1997) studied the proximate composition of prawn waste used in the preparation of prawn waste rice bran silage as a food for ruminants.

2.7. EXTRACTION OF CAROTENOID PIGMENTS FROM SHRIMP SHELL WASTE

2.7.1. Extraction of Carotenoid Pigments Using Organic Solvents

The carotenoid pigments were extracted from wet tissue of trouts using chloroform/methanol (Saito and Reiger, 1971). Solvents such as acetone can be used for the extraction of carotenoids from crustacean waste (Mandiville *et al.,* 1991; Masatoshi and Junji, 1991; Sachindra *et al*., 2005b). Sahidi *et al*., 1992 extracted carotenoid pigment from snow crab and *Pandalus borealis* waste using chloroform:methanol. Alcohol has been used for extraction of carotenoids from crustacean waste (Kozo, 1997). Kobayashi *et al.* (1997 extracted astaxanthin from *H.pluvialis* cells using 90% acetone (v/v). Barbosa *et al*. (1999) extracted astaxanthin from algal powder using acetone and pooled in petroleum ether and 0.73 % NaCl was added and phase separation was carried out. Yuan and Chen (1997) extracted pigments from *H. lacustris* cells using methanol:dichloromethane (3:1 v/v). Jenson *et al*. (1998) extracted astaxanthin from muscle of rainbow trout using cold acetone.

Sachindra *et al*. (2001) has patented a method for extraction of carotenoids from shrimp waste using solvent mixtures acetone and hexane and isopropyl alcohol and hexane. Charest *et al.* (2001) used alcohol as co-solvent in supercritical CO_2 extraction of astaxanthin from craw fish shells. Felix Valenzuela *et al.*,2001 used ethanol as co-solvent in supercritical CO_2 extraction of astaxanthin from demineralized crab (*Callinectes sapidus*).

Sang *et al*, 2002 used acetone: petroleum ether solution (1:2) for extraction of pigments from shrimp shell waste. De-Dios Naranjo *et al,* (2002) used methanol/chloroform/water and petroleum ether/acetone/ water for extraction of pigment from blue crab wastes. Armenta-Lopez *et al.,* 2002 used organic solvent system to extract out carotenoids from shrimp waste after lactic fermentation.Selective extraction of astaxanthin from crustacean waste has been attempted by supercritical CO_2 extraction technique (Lopez *et al.*, 2004).

Briethaupt, (2004) extracted astaxanthin from *Pandalus borealis* waste using a solvent system methanol: ethylacetate: light petroleum (1:1:1). The phase separation was carried out after addition of saturated NaCl and the extract dried using anhydrous sodium sulphate. Li *et al.* (2005) extracted carotenoids from fish eggs using acetone and the carotenoids in acetone extract was phase separated by using methyl tertiary butyl ether. Holando and Netto (2006) extracted astaxanthin from waste of shrimp *Xiphopenaeus kroyeri* using a solvent mixture of ether: acetone: water (15:75:10). Lopez-Cervantes *et al.* (2006) extracted astaxanthin from shrimp waste using methanol. HPLC analysis of the extract was done using a mixture of water: methanol: dichloromethane: acetonitrile (4.5:28:22:45.5 v/v/v/v).

Nobre *et al.* (2006) extracted carotenoid astaxanthin from *H.pluvialis* using acetone at room temperature. Supercritical fluid extraction of astaxanthin with CO_2 was carried out in *H.pluvialis* algal meal using ethanol as co solvent (Nobre *et al.,* 2006).

Sachindra *et al*., (2006a) studied the extractability of shrimp waste carotenoids using different organic solvents and solvent mixtures to optimize the extraction conditions for maximum yield. The polar solvents used were

acetone, methanol, ethyl methyl ketone and isopropyl alcohol, ethyl acetate and ethanol. The non polar solvents used were petroleum ether and hexane. Solvent mixtures used were acetone and hexane (50:50) and isopropyl alcohol and hexane (50:50).

Khanfari *et al*. (2007) extracted astaxanthin from shrimp processing waste of *Penaeus semisulcatus* using hexane. To recover the pigments extraction was carried out until hexane is colourless. Three groups of solvents *viz.* petroleum ether: acetone: diethylamine (10:4:1), Hexane: Acetone (3:1) and benzene: ethyl acetate were added to shrimp waste after lactic fermentation to recover pigments.

Maoka and Akimoto (2008) extracted carotenoids from carapace of spiny lobster *Panulirus japonicus* using acetone at room temperature. The extract was partitioned between n-hexane and NaCl. The organic layer was dehydrated with anhydrous sodium sulphate and concentrated to dryness.

An efficient and environmentally sustainable extraction method for the enrichment of a high-value pigment, astaxanthin, from a low-value raw material, shrimp waste has been studied by Quan and Turner (2009). Ethanol at elevated temperature and pressure was used as a "green" extraction solvent. The dependence of pressurized liquid extraction (PLE) operating variables (pressure, temperature, extraction time) on the recovered astaxanthin concentration from shrimp waste were studied. The results showed astaxanthin yields of around 24 mg kg^{-1} shrimp waste.

2.7.2. Extraction of Carotenoid Pigments Using Vegetable Oils

Carotenoids are a group of oil soluble pigments. The oil solubilization characteristics of carotenoids have led to studies on recovery of these pigments in oils. Anderson (1975) developed a process for extraction of carotenoids from shrimp processing waste wherein soybean oil was added to the waste, mixed, heated and the oil fraction recovered by centrifugation. Spinelli and

Mahnken (1978) developed a 3-stage counter current extraction process for recovery of astaxanthin containing oil from red crab waste.

Torrison *et al.* (1981) attempted acid ensilaging as a method for stabilization of astaxanthin in shrimp waste during storage prior to oil extraction. Chen and Meyers (1982) used enzymatic hydrolysis of homogenised crawfish waste with a protease and subsequent extraction with soy oil for recovery of carotenoids. Chen and Meyers (1983) studied the effect of acid ensilage treatment of craw fish waste to increase the concentration of astaxanthin in oil recovered after extraction. Acid ensilaging of crawfish waste was found to stabilize the astaxanthin in the waste and also increase the recovery of astaxanthin in soy oil (Chen and Meyers, 1983). Chen and Meyers (1984) developed a power model for the estimation of pigment in different oils, based on absorbance maxima and extinction coefficient of astaxanthin in different oils. Carotenoid in homogenised crawfish waste was recovered by acidifying and heating with soybean oil to recover pigment-enriched oil (Meyers and Chen, 1985). Omara-Alwala *et al.* (1985) reported that the use of propionic acid enhances the recovery of astaxanthin from crawfish waste by 35% on vegetable oil extraction. Yamaguchi *et al.* (1986) adopted supercritical carbon dioxide extraction for separation of oil from Krill, which contained astaxanthin as main pigment. The crude oil extract from shrimp waste silage was found to be more concentrated in astaxanthin than the oil obtained from raw shrimp waste (Inoue *et al.,* 1988). Cod liver oil also has been used to extract pigments from processing discards of snow crab and and shrimp waste (Shahidi and Synowiecki, 1991). No and Meyers (1992) demonstrated that the process of oil extraction of carotenoids from crawfish waste can be integrated with production of chitin and chitosan.

A method has been developed based on silica gel column chromatography for concentration of carotenoids in krill oil (Hara *et al.,* 2001). De Dios-Naranjo *et al*. (2002) studied soybean oil as an extraction system for

pigments from blue crab wastes. Shell waste after lactic fermentation was extracted with soybean oil.

Sachindra and Mahendrakar (2005) studied the extraction yields of shrimp carotenoids from the industrial waste of *Penaeus indicus* in different vegetable oils *viz.* Soy oil, groundnut oil, mustard oil, coconut oil and rice bran oil. The optimized conditions of carotenoid extraction in terms of time of heating, temperature of heating and oil level to waste were studied.

Holanda and Netto (2006) used soy oil for extraction of carotenoids from the shell waste of *Xiphopenaeus kroyeri.* Astaxanthin was exracted from shrimp waste of black tiger species using vegetable oil process. The effect of storage temperature and light on extractability of pigments were studied (Anon, 2006). Handayani *et al.* (2008) studied the extraction of astaxanthin from giant tiger (*Panaeus monodon*) shrimp waste using palm oil to determine the extraction kinetics and thermodynamic parameters. Kang and Sim (2008) extracted astaxanthin from *Haematococcus* culture using common vegetable oils. Astaxanthin containing oils were separated and a high recovery yield of 88 % and above was obtained.

2.7.3. Microbial Extraction of Astaxanthin from Shrimp Shell Waste

Lactic fermentation is a simple and environment friendly method to stabilize crustacean waste and to extract highly unstable carotenoid pigments (Armenta-Lopez, *et al.,* 2002). De-Dios Naranjo *et al.*(2002) carried out lactic fermentation of blue crab waste to reduce protein content in the residue and to stabilize the pigment astaxanthin. Microbial extraction of astaxanthin from shrimp shell waste was carried out by Khanfari *et al.*(2007). Two *Lactobacillus* species *viz. Lactobacillus plantarum, Lactobacillus acidophilus* were used after adding MRS broth as medium containing 1 % lactose sugar and 1 % yeast in presence of 5 % CO_2 at 30^0C.Microbial method was more effective than chemical extraction of astaxanthin from shrimp shell waste of *Penaeus semisulcatus.* No significant difference was noted in the extraction of

astaxanthin using two different species of *Lactobacillus.* Lactose was found to be a better substrate than yeast extract for lactic fermentation (Khanfari *et al.,* 2007). Sachindra *et al.* (2007) studied the recovery of carotenoids from fermented and acid ensiled shrimp waste. Acid ensilaging resulted in the reduction of solvent extraction yield of carotenoids whereas the yield of oil extracted carotenoids was higher in both types of silage. The results indicated that fermentation is a method for stabilization and recovery of carotenoids in the shrimp waste.

Pacheco *et al.*(2009) studied the effect of temperature on chitin and astaxanthin recoveries from shrimp waste of *Litopenaeus sp* using lactic acid bacteria. The fermentations carried out in the 27–36 °C temperature range with lactic acid above 0.319 mmol/g resulted in the highest demineralization and the maximal deproteinizations were attained from 30 to 40 °C. The extraction of free-astaxanthin did not present significant differences between 20 and 35 °C and the proportion of *cis*-stereoisomer forms increased with temperature.

2.8. DEPROTEINISATION OF SHRIMP SHELL WASTE

Deproteinisation of shrimp shell waste helps to stabilize astaxanthin and also increases the yield (Chen and Meyers, 1982; De-Dios Naranjo *et al.,* 2002) Enzyme or alkaline treatments can be used to deproteinise shell waste (Holanda and Netto, 2006).

2.8.1. Enzymatic Hydrolysis of Shell Waste

Recovery of the protein fraction of shrimp waste by enzymatic hydrolysis has been widely studied (Simpson and Haard, 1985; Cano-Lopez *et al.,*1987; Synowiecki and Al-Khateeb, 2000; Gildberg and Stenberg,2001; Mizani *et al.,* 2005; Armenta and Guerrero, 2009). Chen and Meyers (1982; 1983) found that implementation of acid ensiling prior to pigment extraction increased concentration of astaxanthin in oil extract by 40-50 %, and oil recovery by 10 %. Guillou *et al.* (1995) found *Pandalus borealis* waste submitted to acid ensilage resulted in higher yield of astxanthin. Enzymes alcalase or pancreatin

can be used for deproteinisation. Alcalase was used by Synowiecki and Al-Khateeb (2000) for deproteinising *Crangon crangon* shrimp waste and Gildberg and Stenberg (2001) used alcalase for *Pandalus borealis* waste. Protein recovery using alcalase was higher than pancreatin (Quaglia and Orban, 1987; Rebeca *et al.,* 1991; Baeck and Cadwallader, 1995; Shahidi *et al,* 1995; Mizani *et al.,* 2005). Lee *et al.* (1999) investigated efficient extraction conditions of astaxanthin from shrimp wastes for utilizing it as a functional food additive. In order to enhance the stability of pigments, proteolytic enzymes were applied to extract astaxanthin as carotenoprotein. Carotenoprotein was effectively extracted from non-acid ensilaged shrimp wastes by using EDTA medium and a proteolytic enzyme. The reddish top layer and the blackish bottom layer were concentrated and purified and reddish top layer recovery was more effective in terms of yield.Holanda and Netto (2006) used alcalase and pancreatin for deproteinising *Xiphopenaeus, kroyeri* shell waste. Gildberg and Stenberg (2001) used alcalase for producing a high quality chitosan for application in cosmetics from the processing waste of Northern shrimp (*Pandalus borealis*). The shrimp waste proteins were hydrolysed by a commercially available protease (Alcalase) and protein hydrolysate with a high content of essential amino acids were recovered in addition to a concentrate of astaxanthin in the sediment.

Chakrabarthi (2002) and Babu *et al.* (2008) reported that enzyme extraction gave improved extraction of carotenoids and maximum yield over the traditional solvent extraction process and supercritical carbondioxide. Trypsin recovered highest amount of carotenoids compared to papain and pepsin.

Armenta and Guerrero, (2009) analysed amino acid profiles of carotenoproteins extracted from fermented and non-fermented shrimp waste, hydrolysed with a protease and a combination of a protease and a lipase. Protein free astaxanthin derived from shrimp waste finds application in the production of feeds, health products, cosmetics etc.

2.8.2. Alkaline Deproteinization of Shrimp Shell Waste

Alkaline deproteinisation can be carried out using NaOH or KOH. KOH was more efficient than NaOH. Sahidi and Synowiecki (1991) used KOH to deproteinise *Pandalus borealis* shrimp waste. Chang and Tsai (1997) used NaOH to deproteinise pink shrimp *Solenocera melantho* waste. Holanda and Netto (2006) used NaOH and KOH for deproteinising *Xiphopenaeus, kroyeri* shell waste. In addition to crustacean species, solution concentration and temperature are the major factors determining efficiency of deproteinisation (Shahidi and Synowiecki, 1991; Synowiecki and Al-Khateeb, 2000).

Variations in particle size of the waste, temperature, and waste/oil ratio can also lead to differences in extraction (Chen and Meyers, 1982).Extraction yields of carotenoids can vary due to difference in carotenoid content, environmental conditions, species, methods used to extract and quantify carotenoids (Sahidi and Synowiecki, 1991), oxygen, light and intermediary hydroperoxide. Astaxanthin was found to be more stable than other components in the shell waste during extraction (Katayama *et al.*, 1971*)*.Chakrabarti (2002) has reported that the carotenoid yields vary with the raw material. The loss of carotenoids during extraction can occur due to oxidation of carotenoids during handling and processing (Balachandran, 1976; Belitz and Grosch, 1999).

2.9. SEPARATION AND ANALYSIS OF CAROTENOID PIGMENT EXTRACTS

Separation and purification different components in the carotenoid extracts from various sources has been studied by many authors. Thin layer chromatography (TLC) and High performance liquid chromatography (HPLC), liquid chromatography (LC), fast ion bombardment mass spectrometry (FAB-MS) are the methods tried.

The fatty acid profiles of astaxanthin ester of brown shrimp *Crangon vulgaris* had been reported by Snauwaert *et al.* (1973). Renstrom and Liaaen-

Jensen (1981) identified xanthophylls esters by TLC followed by GC detection of fatty acid methyl esters found in shrimp *Pandalus borealis*. Yuan *et al.* (1996; 1997) provided a HPLC method for separation and analysis of astaxanthin esters present in *H. lacustris.* Kobayashi and Sakamoto (1999) isolated astaxanthin esters from *H.pluvialis* extract by TLC. Barbosa *et al.* (1999) identified and quantified major carotenoids in algal powder by TLC. Fractions with astaxanthin monoesters and diesters of Antarctic krill *Euphausia superba* were isolated by C18-HPLC (Takaichi *et al.*, 2003). Pintea *et al.* (2003*)* separated and identified carotenoid pigments in *Calendula officianalis* extract by HPLC. Breithaupt (2004) used HPLC coupled with mass spectrometric analysis to separate different components in *H.pluvialis* extract and *Pandalus borealis* extract.

The fatty acids esterified with astaxanthin of spear shrimp shells *Parapenaeopsis hardwickii* were studied by (Lin *et al.*, 2005). Purification and identification of pigments from the shell extracts of *Penaeus semisulcatus* was studied by Khanfari *et al.*(2007). Astaxanthin, monoester, diester and free astaxanthin were the major components from the carapace extract of spiny lobster *Panulirus japonicus.* Adonixanthin, adonirubin, echinenone, canthaxanthin and pectinolone were also identified in a study based on UV-VIS spectra, H-NMR spectra and HPLC (Maoka and Akimoto, 2008).

2.10. BIOACTIVITY

2.10.1. Factors Affecting Bioactivity

Chirality and stereo differentiation are crucial factors in biological activity because in nature, at a molecular level, asymmetry dominates biological processes, such as enzymatic and most immunological reactions. Chirality is not a prerequisite for bioactivity but in bioactive molecules where one or more chiral centers are present, great differences are usually observed in the activities of the different enantiomers. This is a general phenomenon that

applies to many bioactive substances such as drugs, flavors, fragrances and food additives.

2.10.2. Bioavailability of Carotenoids and Pharmacokinetics

The various steps of digestion, absorption and plasma transport of dietary carotenoids in mammals have been reviewed by Furr and Clark, 1997. After ingestion astaxanthin solubilsed and emulsified with other lipids in presence of bile salts and is absorbed through the enterocytes of the duodenal mucosa and transported to the liver where it binds with a lipoporotein and transferred to chylomicrons for transport through the body to the cells. In the plasma non polar carotenoids like β-carotene, α-carotene, or lycopene are mostly transported by VLDL (very low density lipoproteins) and LDL (low density lipoproteins and the polar carotenoids like zeaxanthin and lutein are more likely to be transported by LDL and HDL (high density lipoproteins). The only study on humans to date that confirms the bioavailability of astaxanthin supplied in a single high dose of 100mg and its transport in the plasma by lipoproteins is by Osterlie *et al*. (2000). The study confirmed the transport of astaxanthin in the plasma by lipoproteins as other carotenoids. In the plasma maximum level of 1.3+/- 0.1 mg/l were reached 6.7+/- 1.2 h after administration, and the plasma astaxanthin elimination half life was 21 +/- 11 h. In the plasma, astaxanthin was present mainly in VLDL containing chylomocrons (36-64 % of total astaxanthin), whereas LDL and HDL contained 29 % and 24 % of total astaxanthin, respectively. Oral bioavailability of astaxanthin can be enhanced by incorporation of lipid based formulations (Odeberg *et al.,* 2003). The major benefits associated with astaxanthin can be listed as follows (Lorenz and Cysewki, 2000; Guerin *et al.,* 2003; Khanfari *et al*., 2007).

- Inhibits lipid peroxidation at the cell level thus protecting the cell membrane and mitochondrial membrane within the cell.
- Crosses the blood- brain barrier, which makes it available to the eye, brain and Central Nervous System to alleviate oxidative stress that

contributes to occular and neurodegenerative diseases such as Glaucoma and Alzheimer's.

- Provides significantly more anti oxidant capacity than other carotenoids and antioxidants such as β-carotene and Vitamin E. Entraps free radicals by adding them to its long, double bonded chain rather than donating an electron.
- Stabilises the cell membrane like a bridge because its polar end group span the cell membrane thus increasing its rigidity and mechanical strength.
- Neutrilises singlet and triplet oxygen (decharges) generated by UVB radiation and other sources.
- Binds to a lipoprotein, serves as an efficient transport vehicle, making it more bioavailable.
- Increases immune system function including heightened production of antibody secreting cells and interleukin -2 and suppression of interleukin gamma.
- Inhibits reactive oxygen species that cause inflammation.
- Enhances the antioxidant actions of Vitamin E and Vitamin C and encourages the release of Vitamin A from the liver when needed.
- Astaxanthin very likely increases cell gap functional communication mechanism, thought to inhibit cancer.

2.10.3. Antioxidant Activity

2.10.3.1. Biological Oxidation

Oxidation is the chemical process by which an atom, molecule or ion robs another of one or more of its electrons. Chemicals exhibiting this tendency for stealing electrons are referred to as oxidizing agents. Perhaps the most familiar oxidizing agent is oxygen itself. When a material is oxidized, its

chemical structure is altered, often irreversibly. In biological systems, such as the human body, a number of powerful oxidising agents can cause damage to cells.

2.10.3.2. Oxidative Stress and Disease

Oxygen is necessary for the metabolic production of energy in our bodies. Mitochondria through the electron transport chain use oxygen to oxidize certain molecules and generate energy in the form of adenosine tri phosphate (ATP). During this process oxygen is reduced to water, producing oxygen derived free radicals or reactive oxygen species (ROS) which play an important role in various diseases. A free radical or reactive oxygen species can be defined as any atom or molecule possessing one or more unpaired electrons (Cuzzocrea *et al*., 2001). Once formed, these highly reactive radicals can start a chain reaction and hence they have significant biological importance. Normal aerobic metabolism produces as its by-products various highly reactive molecules, collectively termed "oxidants". These oxidants include a variety of electron-stealing molecules known as free radicals, as well as the highly reactive singlet form of oxygen (Darley-Usmar and Halliwell 1996). The biologically relevant free radicals derived from oxygen are the superoxide anion($O_2^{\cdot-}$), the per hydroxyl radical (protonated super oxide, HO_2), the hydroxyl radical ($^{\cdot}OH$), and free radical nitric oxide (NO) (Table. 1).

Table. 1. Biologically significant free radicals.

$O_2^{\cdot-}$	Superoxide radical
$^{\cdot}OH$	Hydroxyl radical
$ROO^{\cdot}$	Peroxyl radical
$^1O_2$	Singlet oxygen
$NO^{\cdot}$	Nitric oxide
ONOO	Peroxynitrite
HOCI	Hypochlorous acid

Some of these reactive molecules (e. g., superoxide, hydrogen peroxide, and nitric oxide) are physiologically useful and, in fact, are necessary for life, but can also be harmful if present in excess or in inappropriate situations. ROS become a problem when either a decrease in their removal or their overproduction occurs, resulting in oxidative stress (Athar, 2002). This stress and the resultant damage have been implicated in many diseases and a wealth of preventive drugs and treatments are currently being studied.

Many human diseases and degenerative processes have been linked in some way to the action of free radicals. Free radicals are not necessarily the only cause for these conditions, but may well make the human body more susceptible to other disease-initiating factors, may enhance the progression of diseases, and may inhibit the body's own defenses and repair processes (Cross *et al*. 1987).Phagocytes involved in the immune response against micro-organisms can also generate an excess of free radicals to aid in their defensive degradation of the invader. This stress is partly brought on by environmental parameters, such as physiological stress, air pollution, tobacco smoke, exposure to chemicals, and exposure to ultraviolet (UV) light or other forms of ionizing radiation (Moller *et al*., 1996; Papas 1999). Partly, oxidative stress in animals, including humans, arises as a natural result of the body sustaining itself by aerobic (oxygen-requiring) metabolism (Ames *et al*., 1993; Davies 1995). All of these oxidants can react with various components of a living cell, such as proteins, DNA, or lipids (fats), thus causing damage by changing the chemical structure of these components. Such damage has been linked to a number of pathological conditions including aging (Harman 1981; Ames and Shigenaga 1992; Bianchet *et al.,* 1998), atherogenesis leading to Cardio Vascular Diseases (Steinberg *et al*. 1989; Esterbauer *et al*. 1992; Ames *et al.,* 1993;Tapiero *et al.,* 2004), ischemia-reperfusion injury (Simpson and Lucchesi 1987; Takayama *et al*. 1992), infant retinopathy (Phelps 1987), age-related macular degeneration (Gerster 1991), and carcinogenesis (Moody and Hassan 1982; Marnett 1987; Breimer 1990). Another possible effect of oxygen free radical (OFR), involves their attack on lipids to initiate lipid peroxidation

(Hemnani and Parihar, 1998). The only mechanism which produces malondialdehyde (MDA) in biological systems, is lipid peroxidation. MDA is not the major product of lipid peroxidation, but a typical degradation product. MDA reacts with nitrogenous base of DNA to form DNA adducts. Many observations support the notion that lipid peroxidation plays an important role in carcinogenesis (Vuillaume, 1987).

Oxygen is therefore both necessary and harmful; this sobering conclusion has been referred to as the "paradox of aerobic life" (Davies 1995). The endogenous antioxidants do not completely protect against the sum of oxidative stresses challenging the body, and thus there is net oxidative damage that in the long term contributes to aging and various diseases.

2.10.3.3. Biological Antioxidants

Biological antioxidants are defined as "compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidations" (Palozza and Krinsky, 1992a). The human body has evolved a large array of endogenous antioxidant defenses against oxidative stress including antioxidant enzymes such as superoxide dismutase, catalase, and various peroxidases, as well as the ability to use small molecules with antioxidant activity such as glutathione (Fahey and Sundquist 1991), the hormone melatonin (Reiter *et al*. 1997; Reiter 1998), and uric acid (Yu *et al*. 1998).

There are two broad classes of biological antioxidants *viz.* preventative antioxidants, and radical-scavenging antioxidants Preventative antioxidants, such as catalase and superoxide dismutase, suppress the formation of free radicals. The first line of defense against O_2^- and H_2O_2 mediated injury are antioxidant enzymes: Superoxidedismutase, Glutathione Peroxidase and Catalase. Radical-scavenging antioxidants, such as the flavanoid compounds and vitamin C, serve to "mop up" excess free radicals (Noguchi and Niki, 1999). Vitamin E and the carotenoids are very important biological antioxidants that act in both preventative and radical-scavenging roles.

In addition to the body's endogenous defenses against oxidative stress, diet-derived antioxidants--including ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), and the carotenoids--may be important in protecting against disease and age-related phenomena (Ames *et al*. 1993; Davies 1995; Halliwell 1996). Diet-derived antioxidants may be classified on the basis of their solubility as either lipid-soluble (i. e., soluble in fats), or water-soluble. Lipid-soluble antioxidants include vitamin E and the carotenoids, while vitamin C is a common water-soluble antioxidant.

Superoxide dismutase is an enzyme that can disarm and destroy free radicals, particularly superoxides leading to H_2O_2 production. In mammalian tissues, SOD exists in two main forms, dependent on the metal ion bound to its active site. Cu-Zn-containing SOD is a highly stable enzyme found primarily in the cytosol (Fridovich, 1995). Mn SOD is present in the mitochondrial matrix.

$$O_2 + O_2 + 2H \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide formed by the divalent reduction of oxygen or by the disproportionation of superoxide anions are scavenged by two classes of enzymes the catalases and peroxidases. Catalase has a double function, because it catalyses the decomposition of H_2O_2 to give H_2O and O_2 and also the oxidation of H donors, such as methanol, ethanol, formic acid, phenol with the consumption of one mole of peroxide.

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

$$ROOH + AH_2 \longrightarrow H_2O + ROH + A$$

Peroxidases are more localized in peroxisomes and are widely distributed in human saliva, adrenal medulla, liver, kidney and leucocytes (Vuillaume, 1987). Glutathione peroxidase catalyses the oxidation of GSH to GSSG at the expense of hydrogen peroxides.

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2\ H_2O$$

2.10.3.4. Role of Carotenoids in Preventing Oxidation

All carotenoids share a structural feature termed "polyunsaturation", with several double bonds alternating with single bonds, and the electrons that make up the double bonds in the linear chain are "delocalized", or shared evenly over the whole chain termed as "conjugated". This makes the whole chain relatively electron-rich. Conjugated double bonds are chemically very stable, yet are capable of specific chemical reactions that require their electron-rich yet stable structure. For example, if a carotenoid loses one electron and becomes a carotenoid cation (positively charged ion), the resulting charge of +1 is distributed over the electron-rich chain, a much more stable situation than if the charge were limited to a single location on the compound. This unique structural feature explains the antioxidant potential of such compounds in number of *in vitro and in vivo* studies including animal models and human clinical trials (Seddon *et al.,* 1994; Ames, 1998; Anderson *et al.,* 1999; Biesalski *et al.,* 1999; Cooper *et al.,* 1999; Hennekens, 1999; Lyle *et al.,* 1999).

Carotenoids carry out a protective function against damage by light and oxygen in living organisms. Carotenoids can also act as potent biological antioxidants, in mammalian and human cells mainly by two mechanisms; as quenchers of singlet oxygen and other reactive species, by absorbing the excited energy of singlet oxygen onto the carotenoid chain, leading to the degradation of the carotenoid molecule, but preventing other molecules or tissues from being damaged (Mortensen *et al*, 1997; Boileau *et al.*,1999; Beutner *et al*, 2001). Carotenoids can act also as chain-breaking anti-oxidants by scavenging free radicals thus preventing chain reaction that can lead to the degradation of unsaturated fatty acids, resulting in degradation of lipid-rich membranes within a short time. In photosynthetic cells carotenoids react with other energetically excited molecules (chlorophyll in particular) thus preventing the formation of singlet oxygen, and dissipate excess excitation energy through the xanthophyll cycle.

2.10.3.5. Astaxanthin as an Antioxidant

Astaxanthin's powerful antioxidant activity has been demonstrated in numerous studies showing the detrimental effects of free radicals induced oxidative stress and its potential to target many important health conditions (Papas, 1999; Palozza and Krinsky, 1992b; Naguib, 2000).The relationship between structure and oxygen quenching activity of carotenoids are studied by Hirayama *et al.*(1994) and Shimidzu *et al.*(1996).

Being an oxygenated carotenoid, a xanthophyll, it has greater antioxidant capacity than many of the other carotenoids. With the particular configuration of astaxanthin molecule having both hydroxyl and carbonyl groups at each end, it exhibits greatest antioxidant activity. Peroxyl radical chain breaking ability, incorporation of free radicals into its polyene chains (thereby more effectively eliminating them) and enhancement of Vitamin C as an antioxidant are certain functions of anioxidant. While other carotenoids and antioxidants may perform one or two of these functions, astaxanthin does them all and, in most cases, does them better.

Astaxanthin supplementation to human subjects may decrease *in vivo* lipid peroxidation in healthy men. Astaxanthin's ability to quench singlet oxygen and scavenge free radicals has been demonstrated by a number of *in vitro* studies (Miki, 1991; Kurashige *et al.*,1990; Lim *et al.*, 1992; Oshima *et al.*, 1993; Jorgensen and Skibsted, 1993; Nakagawa *et al.*, 1997; Woodall *et al.*, 1997). Astaxanthin shows very good capability at protecting membranous phospholipids and other lipids, against peroxidation.

Astaxanthin, like vitamin E, is a lipophilic (fat-soluble) antioxidant, and thus might be expected to exert its antioxidant properties in lipid-rich cell membranes and tissues. Feeding studies in rats have shown that in animals deprived of vitamin E, the resistance of lipids (fats) to oxidation was largely restored by feeding the animals with astaxanthin (Kurashige *et al*. 1990; Miki 1991).

In the study by Kurashige *et al*. (1990), rats were fed a vitamin E-deficient diet, with or without astaxanthin supplementation at 1 mg per 100 mg feed, for two to four months; control rats received a vitamin E-sufficient diet. Mitochondria were isolated from liver, and erythrocyte ghosts prepared from blood samples. Mitochondria from vitamin E-deficient rats were preincubated with various concentrations of either astaxanthin or vitamin E, and then exposed to superoxide. An index of lipid peroxidation, the formation of thiobarbituric acid-reactive (TBA-reactive) substances, was measured colorimetrically. The percent inhibition of TBA-reactant formation (as compared to controls with no added antioxidant) was measured. At all concentrations tested, astaxanthin inhibited the formation of TBA-reactants more effectively than did vitamin E, and the IC_{50} concentration was approximately 3 orders of magnitude lower for astaxanthin than for vitamin E. In this system, astaxanthin was a more effective antioxidant than was vitamin E. This dietary administration of astaxanthin, in the absence of vitamin E, partially restored the *in vitro* oxidation resistance of erythrocyte membrane ghosts to the levels found in vitamin E-sufficient rats.

In the study by Miki (1991), rats were fed a vitamin E-deficient diet, with or without astaxanthin supplementation at 1 mg per 100 mg feed, for four weeks; control rats received a vitamin E-sufficient diet. Lipid peroxidation was high in the vitamin E-deficient rats and negligible in control animals. Rats that received the astaxanthin supplementation had peroxidation levels about half that of the vitamin E-deficient rats, again indicating that dietary astaxanthin restored much though not all of the vitamin E-dependent oxidative resistance of the erythrocyte membranes.

Astaxanthin can exert beneficial effects in diabetes with preservation of beta-cell function (Uchiyama *et al.,* 2002). Astaxanthin reduced oxidative stress on the kidneys and prevented renal cell damage and hence can be useful in preventing diabetic nephropathy (Naito *et al.,* 2004). Most of the works on antioxidant activity were employed using all trans isomers. All trans natural

astaxanthin is readily isomerised to cis-trans especially 9-cis and 13-cis compared to all trans isomers *in vivo.*In a study with rat microsome and rabbit erythrocyte ghost membrane lipid peroxidation showed that cis astaxanthin, especially 9-cis exhibited higher antioxidant effect than all trans. The action was in the order 9-cis >13-cis > all trans (Liu and Osawa, 2007). The antioxidant synergism of vitamin E and astaxanthin reduced mlondialdehyde formation in an *in vitro* stimulation of microsomal lipid peroxidation in a feeding study with salmon (Bell *et al.,* 2000).

2.10.3.6. Astaxanthin and Other Carotenoids

Several studies have compared astaxanthin's antioxidant activity with that of other carotenoids. The measurement of antioxidant activity is highly dependent on the experimental system used. Astaxanthin was best among carotenoids at preventing peroxidation of lipids, with up to 10 times higher anti-oxidant efficacy of astaxanthin over beta-carotene, 4 times more effective than lutein in various antioxidant systems. Another study demonstrated a superior capacity of astaxanthin over zeaxanthin, canthaxanthin or beta-carotene at reducing peroxidation of unsaturated fatty acids. Astaxanthin was approximately as effective as canthaxanthin (a xanthophyll structurally similar to astaxanthin), and about 50% more effective than beta-carotene and zeaxanthin, in preventing fatty acid peroxidation in chemical solution (Terao, 1989; Kurashige *et al*, 1990; Miki, 1991; Shimidzu *et al,* 1996) and superior singlet oxygen quenching ability over other carotenoids such as beta-carotene (up to 1.7 to 38times higher, depending on testing conditions) (Di Mascio *et al.,* 1990; Di Mascio *et al.,* 1991; Shimidzu *et al.,* 1996) or lutein and zeaxanthin (Shimidzu *et al.,* 1996).). Lycopene was found to be about a third more effective than astaxanthin. Similar results were found by researchers working with an *in vitro* system of human blood cells treated with different carotenoids and then exposed to singlet oxygen, lycopene was found to be more effective than astaxanthin, which in turn was more effective than beta-carotene (Tinkler *et al.,* 1994).

A second major antioxidant role of carotenoids is in the scavenging of free radicals. An elegant study of carotenoid-radical reactions in chemical solution clearly demonstrated that reactivity rates depend not only on the carotenoid but also on the nature of the radical (Mortensen *et al*. 1997). In a membrane model, astaxanthin was found to be more effective at scavenging peroxyl radicals than was beta-carotene (Palozza and Krinsky, 1992b). Another study using membrane models found similar results, with astaxanthin better at delaying lipid peroxidation than zeaxanthin, canthaxanthin, or beta-carotene (Lim *et al*., 1992). A tissue culture model demonstrated that astaxanthin was superior to beta-carotene or vitamin E in protecting the cells from herbicide-induced oxidative stress (Lawlor and O'Brien, 1995).

Astaxanthin has also been compared to the well-known non-carotenoid antioxidant: alpha-tocopherol (Vitamin E) (Tso and Lam, 1996) and proved, in *in vitro* studies, to have a superior capability to quench singlet oxygen (80 to 550 times higher) and to prevent lipid peroxidation (Kurashige *et al.,* 1990; Miki, 1991). *In vitro* experiments with red blood cells and mitochondria from rats have shown that astaxanthin can be 100 to 500 times more effective at inhibiting lipid peroxidation than vitamin E (Kurashige *et al.,* 1990; Miki, 1991). *In vivo* studies with rats given dietary astaxanthin confirmed its antioxidant abilities. Rengel *et al. (*2000) examined the ability of canthaxanthin and astaxanthin as chain breaking agents in a membraneous vesicle. Astaxanthin showed the strongest antioxidant activity. Oxygen radical absorbance capacity is the highest for natural astaxanthin compared to other natural antioxidants.

One important factor of astaxanthin in humans and other mammals is that (not the case in most aquatic animals) unlike beta-carotene and other carotenoids it has no pro-vitamin A activity. It can therefore not be diverted from its main function as an antioxidant to become part of the pro-vitamin A pool. Hence the risk of hyper-vitaminosis and excessive accumulation of vitamin A is not a concern. Astaxanthin, unlike beta-carotene has the ability to

cross the blood-brain barrier and therefore, directly exert its antioxidant properties in the brain (Tso and Lam, 1996).

The antioxidant properties of astaxanthin are believed to be at the source of most potential benefits of astaxanthin in human health (Dore, 1999) *viz.* Support of the immune system, health of the eye and central nervous system, anti-cancer properties, protection against UV light damage, blood cholesterol regulation and prevention of arteriosclerosis and related ailments, response to bacterial infections, anti-inflammatory response etc.

2.11. Astaxanthin and Photo-protection

Carotenoids have an important role in nature in protecting tissues against UV light mediated photo-oxidation and are often found in tissues directly exposed to sunlight (Noguchi and Niki, 1999; Mc Vean, 1999). Miki (1991) was the first to show that astaxanthin had a stronger protective effect against photo-oxidation than lutein and β-carotene. O'Connor and O'brien (1998) reported that astaxnthin could be more effective than β-carotene and lutein by a factor of upto 200 and 1000 fold respectively. This unique photo protection activity of astaxanthin can be very important for protecting eye and skin from UV induced oxidation damage.

2.11.1. Astaxanthin and Eye Health

Age related macular degeneration and age related cataracts, two main leading causes of visual impairment are related to light induced oxidation. A high dietary intake of carotenoids is associated with a reduced risk for both nuclear cataracts and age related macular degeneration (Jacques, 1999; Lyle *et al*, 1999). Free radicals impair lens crystalline proteins, damage proteolytic enzymes and causes peroxidation of PUFAs in membrane phospholipids leading to death of photoreceptor cells (Gerster, 1991; Jacques, 1999; Snodderly, 1995; Christen *et al.,* 1999; Head, 2001; Snow and Seddon, 1999). Lutein and zeaxanthin accumulate in macula and serve as quenchers of singlet oxygen and absorb blue light (Bone, *et al.,* 1985; Landrum *et al.,* 1999);

Seddon *et al.,* 1994; Lyle *et al.,* 1999). Eventhough astaxanthin has not been isolated in human eye, it is found in the eye and eye parts of a number of animals (Egeland, 1993). A animal study has demonstrated that astaxanthin is capable of crossing blood brain barrier, and like lutein will deposit in the retina of mammals if included in the diet (Tso and Lam, 1996). The retinal photoreceptors of those rats fed astaxanthin were less damaged by UV light injury (Tso and Lam, 1996).

2.11.2. Astaxanthin and Skin Health

Astaxanthin is also related to skin health. Excessive exposure of unprotected skin to sunlight results in sunburn and can also lead to photo-induced oxidation, inflammation, immunosuppression, aging and even carcinogenesis of skin cells. (Fuchs, 1998; Lee *et al*, 2000; Stahl *et al,* 2000). Lee *et al*, (2000) also observed the reduction in UV induced skin erythema by dietary supplementation of 30 mg/day natural carotenoids for 8 weeks, while Stahl *et al,(*2000) showed that α-tocopherol supplementation helped boost the protective effect of β-carotene supplementation. Astaxanthin found in the skin and eggs of salmon and *Haematococcus pluvialis* resting stages is believed to protect against UV light photoxidation (Meyers, 1993; Torrissen *et al,* 1989). *In vitro* protective effect of astaxanthin against UV-induced photooxidation was stronger when compared with β -carotene and lutein (O'Connor and O'Brien, 1998). These findings suggest that astaxanthin has an excellent potential as an oral sun-protectant. Astaxanthin is beneficial in many types of cancers and it's effect on skin cancer is yet to be investigated completely (Fuchs, 1998; Savoure *et al.,* 1995; Black, 1998).

2.12. Astaxanthin and Cellular Health

Antoxidants, and carotenoids in particular, are not only essential to cellular health because they help protect cellular membranes, proteins, DNA, Essential fatty acids, or subcellular components like mitochondria against oxidative damage, but because they also play a role in regulating expression of

genes and in including cell to cell communications (Betram, 1991; Betram, 1999; Allen and Tresini, 2000). Astaxanthin is very good at protecting membranous phospholipids and other lipids against peroxidation (Palozza and Krinsky, 1992a; Naguib, 2000). Astaxanthin's superior role in protecting cellular membranes is believed to derive from its ability to protect both the inner part and external surface of membranes against oxidation. The moieties of its polyene chain and terminal rings rigidify membranes and modify their permeability (Matsushita *et al.*,2000; Goto *et al.,* 2001; Barros *et al.,* 2001).

The number of conjugated double bonds (C=C) is the most effective parameter deciding antioxidant activity, because the activity of carotenoids increased as the number of conjugated double bonds in the carotenoids increased. Second the carbonyl (C=O) and hydroxyl (-OH) groups of carotenoids were important for its activity. In carotenoids with the same number of C=C groups, the quenching abilities depends on the groups in both β-ionone rings. Free astaxanthin with two hydrophilic groups (C=O and -OH) in both β-ionone rings shows decreased oxygen quenching ability in a hydrophobic solvent medium. Esters of astaxanthin with fatty acids have both hydrophilic and hydrophobic esters in β-ionone rings. Esterification of free astaxanthin would compensate for decreased antioxidant activity under hydrophobic conditions and ester part of astaxanthin esters function as a stabilizer to maintain high antioxidant ability between hydrophilic and hydrophobic conditions. The IC_{50} values of astaxanthin esters were lower than those of other carotenoids and, astaxanthin esters consistently inhibited lipid peroxidation by oxygen in both hydrophilic and hydrophobic conditions. This property is more evident in its function as an antioxidant agent in both cytoplasmic and membranous regions of cells (Kobayashi and Sakamoto, 1999).

Astaxanthin is polar and aligns parallel to the lipid component of the cell membrane facilitating its antioxidant and anti inflammatory effects compared to apolar antioxidants like β-carotene, Vitamin C and Vitamin E. This very

well explains the clinical success achieved in case of astaxanthin compared to its counterparts.

2.12.1. Anti-aging and Skin Care.

The mitochondrial theory of aging hypothesized that the cumulative oxidative damage to mitochondria is the main culprit for the senescence of cells, which in turn is responsible for aging (Gershon, 1999). Reactive forms of oxygen can cause cumulative damage to lipids in cell membranes, DNA, and subcellular membranes and structures which is the main cause of ageing process and the development of chronic diseases in older people. ROS such as superoxide, hydroxyl, and peroxyl radicals seem to play an important role in the initiation or promotion of some chronic diseases among older people, such as atherosclerosis, cancer and rheumatoid arthritis. Antioxidants are never demonstrated as agents that can extend life span, but it is believed that antioxidants may help reduce onset of some aging symptoms, oxidative damage in humans or animal models for those diseases (Blumberg and Halpner, 1999).The efficacy of astaxanthin in preventing *in vitro* peroxidation of mitochondria of rat liver cells can be as high as 100 times that of vitamin E. It is found to be 300 times more rejuvenating than Vit A and Vit E (Kurashige *et al*., 1990). Intensive physical activity in older individuals may affect dietary requirements for antioxidants. Eventhough regular exercise is a good way to stay young and healthy, it often increases body's oxygen consumption, especially in muscles and result in increased production of superoxide. Superoxide damages cell membranes and muscle fibres, hence strenuous exercise can cause oxidative damage and injury to muscle tissues. Studies carried out during intense physical activity in animal models and humans recommend the intake of antioxidants such as vitamin E or astaxanthin during exercise (Lignell, 1999). This highlights the unique capacity of astaxanthin in helping to preserve mitochondrial functions and its unique potential in the fight against aging.

Astaxanthin finds much application in anti aging skin care technology. Astaxanthin helps in the improvement of look and feel of hair, skin, nails etc.. It also causes significant reduction in wrinkling, redness and other skin problems (Anon, 2004). Astaxanthin has been proven to reduce the appearance of fine lines and wrinkles showing its unique anti aging and anti wrinkle attributes. It can reduce dark circles and even bags around the sensitive eye area of face. Other natural salmon and algae produced substances such as DMAE (Dimethyl amino ethanol) and astaxanthin can be added to any topical applications, especially antiaging agents.

2.13. Astaxanthin in Detoxification and Liver Function.

The liver is a complex organ where intense catabolism and anabolism take place. Liver functions include active oxidation of lipids to produce energy, detoxification of contaminants, destruction of pathogenic bacteria and virus and dead red blood cells. While performing these functions liver is exposed to significant amounts of free radicals and oxidation byproducts and liver cells are to be protected against oxidative damage. Kurashige *et al.(*1990) demonstrated that astaxanthin was much more effective than vitamin E at protecting mitochondria from rat liver cells against lipid peroxidation. Astaxanthin has also been shown to induce xenobiotic metabolizing enzymes in rat liver, a process which may help prevent carcinogenesis (Gradelet *et al.,* 1996). Gradelet *et al.* (1998) also demonstrated that astaxanthin helped protect rat liver cells against aflatoxins. Segner *et al.*(1989) reported that astaxanthin supplementation helped improve the histology of liver in fish. Astaxanthin protects liver damage in rats induced by CCl_4 by inhibting lipid peroxidation and stimulating the cellular antioxidant system. Astaxanthin blocked Glumate oxaloacetate transferase(GOT), Glutamate pyruvate transaminase (GPT) and Thiobarbituric acid reacting substance (TBARS) levels and also causes an increase in reduced glutathione (GSH) and superoxide dismutase (SOD) activity(Kang *et al.,* 2001).

2.14. Astaxanthin and Antiinflammatory Activity

Inflammation is an immune response that occurs after bodily injury. The inflammation response is often non-specific *ie.* it is identical regardless of the etiology *viz.* harmful organisms, a foreign body, ischemia (deprivation of blood flow), physical trauma, ionizing radiation, electrical energy or extremes of temperature. Inflammation is referred as "double edged sword". A limited amount of inflammation is needed for the healing process to continue and is often a normal part of the body's immune response. Excess inflammation or chronic inflammation can be detrimental to our health. C-reactive protein(CRP) is one of the main culprits behind inflammation. Elevated CRP levels are associated with many infections and diseased conditions (Deodhar, 1989; Kushner, 1990).

Oxidants have been linked to the stimulation of inflammation genes in endothelial cells (Aw, 1999). In inflammation induced clinical conditions such as Crohn's disease, toxic reactive oxygen species are released by phagocytic leucocytes at the site of inflammation (intestinal mucosa and lumen). These, plus increased concentrations of neutrophiles at the site of inflammation, create a pro-oxidative balance that leads to lower levels of antioxidant vitamins and increased levels of markers of oxidative stress and lipid peroxidation (Aghdassi and Allard, 2000).

Reactive oxygen species have been attributed an aggravating role in the inflammation that accompanies asthma (Green, 1995; Casillas *et al.,* 1999; Dworski *et al.,* 1999; Comhair *et al.,* 2000) and exercise induced muscle damage (Dekkers *et al,* 1996). Kurashige *et al,* (1990) identified the anti-inflammatory role of astaxanthin in experimental rats. An inflammation agent *viz.* carrageenan was injected in the paw of experimental rats, fed with astaxanthin, vitamin E or no antioxidant (control). Carrageenan induced swelling of the paw of rats fed with astaxanthin was significantly lower than that of control while Vitamin E did not reduce the swelling of rat paw. This explains the anti-inflmmatory role of astaxanthin.

Dietary astaxanthin was found to help fight symptoms of ulcer disease from *Helicobacter pylori* which causes inflammation of gastric tissues. Astaxanthin supplementation in the diet of mice reduced symptoms of gastric inflammation and was also associated with shifts in inflammation response, which were consistent with the reduced symptoms. Astaxanthin is also reported to have protection against gastric leisions induced by the use of non steroid anti-inflammatory drugs such as naxopen (Kim *et al.,* 2005). Astaxanthin supplementation led to a change in the activity of T-lymphocytes involved in inflammation response, with a shift from a (T helper 1) Th1-response dominated by (interferon) IFN-γ to a Th1/Th2-response dominated by IFN-γ and cytokine (interleukin-4) IL-4 (Bennedsen *et al*., 1999). A dose dependent anti-inflammatory effect by suppression of nitric oxide, prostaglandin E2, and tumour necrosis factor (TNF) has been reported by Ohgami *et al.* (2003). Suppression of T-cell activation makes astaxanthin as effective as commonly used antihistamines and hence may have a role in novel antiasthmatic formulations (Mahmoud *et al.,* 2004).

Astaxanthin influences inflammation also by inhibiting inflammatory mediators known as NF-kappaB and tumour necrosis factor-alpha (TNF-alpha) (Ohgami *et al.,* 2003). In one study with rats uveitis intraocular (eye) infections was induced and injected with varying doses of astaxanthin. Over the disease duration the expression of inflammatory cytokines and chemokines in treated and control animals were studied. Rats injected with astaxanthin showed significant decrease in the number of infectious cells in the anterior chamber of the eye. There was a significantly lower concentration of TNF-alpha and prostaglandin E2 in the aqueous humor of eye. In the early stages uvetis were found to be suppressed by injection of astaxanthin. The number of activated NF-kappaB-positive cells was lower in iris-ciliary bodies of the eye treated with 10 or 100 mg /Kg astaxanthin at 3 h after inoculation with the infection. These results suggest that astaxanthin reduces ocular inflammation in eyes by downregulating proinflammatory factors and by inhibiting the NF-kappaB-dependent signaling pathway (Suzuki *et al.,* 2006). A synthetic form of

astaxanthin, disodium disuccinate astaxanthin also lowered levels of CRP (Lockwood and Gross, 2005). It can also reduce injury to rabbit hearts after researchers occluded the animal's arteries(Lauver *et al.,* 2005). A rat study has reported that astaxanthin accumulated in heart and skeletal muscles and reduced the amount of exercise induced inflammation markers (Aoi *et al.,* 2003).

Krill oil has been found to exert anti-inflammatory effects. Krill oil inhibits production of pro-inflammatory biochemicals (Duetsch, 2007). Krill contains ω-3 PUFAs, potent phospholipids and astaxanthin as its major components. This is also effective against premenstrual syndrome (PMS) and dysmennorhea. It works effectively on both inflammation and pain, 31% reduction in aches and pain has been reported. It improves the emotional and psychological symptoms of patients suffering from PMS by favourably affecting neurotransmitters within the brain. An increase in pregnancy rate, a significant boost in energy, alertness, memory, concentration, stamina and general well being of patients suffering PMS has been reported. Astaxanthin being a major antioxidant in krill oil may be effective in health benefits associated with it (Sampalis *et al.,* 2003).

2.15. Astaxanthin and Cardioprotection

Oxidative damage leads to cardiovascular disease (CVD), one of the major causes of morbidity and mortality in modern society (Ames *et al.,* 1993; Tapiero *et al.,* 2004) Atherosclerosis is the principal cause of CVDs. Initially atheroscelorosis consists of fatty streaks containing "foam" cells, which develop into fibrous plaques. As in other cells cell debris, lipids, cholesterol, and calcium accumulate, plaque deposits are formed which can ultimately block off the flow of blood through the artery and lead to heart attack or stroke. The whole events are triggered by uptake of oxidized LDL by macrophages. Usually LDL in plasma is not oxidized and oxidation of LDL is believed to contribute to the development of atherosclerosis (Goldstein *et al.,* 1979). Hence it might be possible to reduce the risk of atherosclerosis by antioxidant

supplementation (Hennekens, 1999). However, blood levels of HDL (the 'good cholesterol') are indicative of protection against atherosclerosis (Drexel *et al*., 1994).

Epidemiological and clinical data indicate that dietary antioxidants might protect against cardiovascular disease (Frei, 1995). An inverse association between the serum levels of β-carotene and other carotenoids and coronary heart disease has been reported (Kritchevsky, 1999). Supplementation with vitamin E and other carotenoids can decrease the susceptibility of LDL to oxidation (Jilal and Fuller, 1995). Astaxanthin is carried by VLDL, LDL and HDL in human blood. An *in vitro* test and a study with human subjects ingesting daily dosages as low as 3.6 mg astaxanthin per day for two consecutive weeks demonstrated that astaxanthin protects LDL cholesterol against *in vitro* oxidation (Miki *et al,* 1998). This suggests that astaxanthin may help prevent atheroscelorosis and the risk of heart disease by preventing oxidation of LDL cholesterol in the blood (Iwamoto *et al.,* 2000). Finally, astaxanthin could also be beneficial to heart health by reducing inflammation presumably associated with the development of coronary heart disease. C-Reactive proteins and non glycosylated proteins in blood during inflammation were found in trace amounts (Tracy, 1999). Elevated blood levels of CRP are believed to indicate "smoldering" low grade inflammation in arteries of patients without known coronary risk factors (Danesh, 1999; Tracy, 1999; Ridker, 2000).

In an animal model study astaxanthin supplementation led to an increase in the blood levels of HDL (Murillo, 1992), the form of blood cholesterol inversely correlated with coronary heart disease. Thus astaxanthin could benefit heart health by modifying blood levels of LDL and HDL cholesterol. Astaxanthin can also protect the heart directly in mammals. It accumulates in the heart muscle and, following exercise astaxanthin appears to lower the level of exercise induced oxidative damage of lipids and DNA of heart muscle (Aoi *et al*., 2003). In a couple of studies it was shown that astaxanthin reduced

myocardial infarct size following an induced heart attack in Sprague-Dawley rats (Gross and Lockwood, 2004; Gross and Lockwood, 2005; Gross *et al.*, 2006). Astaxanthin is also repored to have a role in reduction of high blood pressure and the delay in the incidence of stroke (Hussein *et al.,* 2005). Disodium disuccinate astaxanthin a water dispersible carotenoid has the ability to reduce myocardial injury in a rabbit model of ischemia/reperfusion (Lauver *et al.,* 2005).

Astaxanthin supplementation may decrease *in vivo* lipid peroxidation indicated by reduced levels of plasma hydroxyl fatty acids. In a study conducted with healthy men to receive daily supplements of astaxanthin 8 mg per day or placebo (microcrystalline cellulose) for 3 months, blood astaxanthin levels increased to 0.032 µ moles per litre and blood levels of 12 and 15 hydroxy fatty acids were also significantly reduced relative to placebo group. Even though levels of fatty acids were lowered no effect on the lag time of combined LDL and VLDL fraction *ex vivo* was noted, Astaxanthin dose higher than 8 mg is required to achieve any impact on lag time (Krappi *et al,* 2007). These results are promising, showing that supplementation of astaxanthin can have a protective role in prevention of heart and vascular disease.

2.16. Astaxanthin and Anticancer Property

Numerous studies have demonstrated the anti-cancer activity of astaxanthin in mammals.Tanaka *et al.* (1994) showed that astaxanthin protected mice from carcinogenesis of the urinary bladder by reducing the incidence of chemically induced bladder carcinoma to 18 % instead of 42 % in the control group receiving no astaxanthin. High carotenoid intake is associated with a decreased risk of various forms of cancer (Muller *et al.,* 2002). In a second study it was found that rats fed a carcinogen but supplemented with astaxanthin had a significantly lower incidence of different types of cancerous growths in their mouths than those rats fed only the carcinogen. Protective effect of astaxanthin against oral cancer was more pronounced than beta-carotene (Tanaka *et al.* (1995a). In a further study Tanaka *et al.* (1995b) found

a significant decrease in the incidence of induced colon cancer in those rats fed astaxanthin versus those administerd only the carcinogen. Chew e*t al.* (1999a) demonstrated that astaxanthin was effective in fighting mammary cancer by showing that astaxanthin could reduce growth of induced mammary tumours by more than 50 %. Astaxanthin could inhibit the proliferation of human breast cancer MCF-7 cell line *in vitro* and the action of carotenoids may be worked through different pathways (Li *et al.,* 2002). They further showed that anti-cancer activity of astaxanthin was higher than that of beta-carotene and canthaxanthin. In a study with Balb/c mice astaxanthin was found to reduce Meth-A induced tumor cell growth evidenced by lower tumour size compared to control (Jyonouchi *et al.*, 2000). Astaxanthin and other carotenoids inhibited the invasion of rat ascites hepatoma cell AH109A when co cultured with rat mesentry derived mesothelial cells (Kozuki *et al.,* 2000*).*

Anderson (2001) showed that astaxanthin was able to inhibit the enzyme 5-alpha-reuductase responsible for prostate growth and proposed astaxanthin supplementation as a method to fight benign prostate hyperplasia and prostate cancer. Jyonouchi *et al.*(2000) reviewed mechanisms explaining anticancer activity of astaxanthin,which included preventing oxygen-mediated cytotoxicity or genotoxicity, inducing xenobiotic-metobiolizing enzymes in rat liver as suggested by Gradelet *et al.*(1996) and stimulating immune response mechanisms such as T-cells that fight tumor growth. Jewell and O' Brien, (1999) demonstrated that astaxanthin was also able to induce xenobiotic metabiolizing enzymes in the lung and kidney. Bertram,(1991; 1999) also suggested that carotenoids role in cell communications at gap-junctions played a role in slowing the growth of cancer cells. He hypothesized that carotenoids affect DNA regulating RNA responsible for gap junction communication. Carotenoids enhance intercellular gap junction communication that play an important role in cell growth control and carcinogenesis (Zhang *et al,* 1991). It has been shown that astaxanthin supplementations in rats inhibits the stress induced suppression of tumor fighting natural killer cells.

2.17. Astaxanthin and Immunomodulating Properties

Immune response cells are particularly sensitive to oxidiative cells and damage of their membranes by free radicals because they rely heavily on cell to cell communications *via* receptors in the cell membrane.The phagocytic action of some of these cells releases free radicals which can rapidly damage these cells if they are not neutralized by antioxidants (Hughes, 1999). Positive effects of astaxanthin on immune response mechanisms in aquatic invertebrates have been demonstrated (Kawakami *et al.,* 1998). For salmon astaxanthin is considered a vitamin that is essential for the proper development and survival of juveniles (Christiansen *et al.,* 1995). With mammals astaxanthin has been shown to significantly influence immune function in a number of *in vitro* and *in vivo* assays using animal models. Studies on the immunological significance of the astaxanthin; effects on the antitumour effector activity of natural killer cells suggested that astaxanthin improves antitumour immune responses by inhibiting lipid peroxidation (Kurihara *et al.,* 2002).

Astaxanthin enhances *in vitro* antibody production by mouse spleen cells stimulated with sheep red blood cells done by use of mitogen responses to spleen cells, thymocyte proliferation, interleukin 2 production and antibody production in response to SRBC (Jyonouchi *et al.,* 1991) partly by excerting actions on T-cells especially T-helper cells (Jyonouchi *et al.,* 1993). Astaxanthin can also partially restore decreased humoral immune reponses in old mice (Jyonouchi *et al.,* 1994). Studies on human blood cells *in vitro* demonstrated that astaxanthin can enhance immunoglobulin production in reponse to T-dependent stimuli ((Jyonouchi *et al.,* 1995a).These immuno modulating properties are not related to pro vitamin A activity,because astaxanthin,unlike β-carotene does not have such activity in mammals (Jyonouchi *et al.,* 1991). Astaxanthin has also been found effective in preventing development of symptoms in autoimmune-prone mice (Tomita, *et al.,* 1993). Astaxanthin was found to suppress IFN-gamma production by cloned murine Th 1 cells and increased the number of antibody secreting cells

in primed and unprimed spleen cells. In the culture of Th2 clone astaxanthin enhanced the number of antibody secreting cells (Jyonouchi *et al.,* 1996). In a study with *in vivo* modulatory activity of beta-carotene, canthaxanthin and astaxanthin, mice fed with astaxanthin and beta-carotene had enhanced phytohemagglutin induced lymphoblastogenesis compared to unfed ones.Astaxanthin also enhanced lymphocyte cytotoxic activity (Chew *et al.,* 1999b). It has also been suggested that astaxanthin's immunomodulating functions may be related to its antitumour activity (Jyonouchi *et al.,* 2000). The immununomodulating capacity of astaxanthin is superior to that of β-carotene and canthaxanthin. (Jyonouchi *et al.,* 1995b; Jyonouchi *et al.,* 1996;). In an *in vitro* cell culture experiment with different carotenoids, astaxanthin caused significant stimulatory effect on cell proliferative response of spleen cells and thymocyte in Balb/c mice. Cytokine inducing activity was highest for astaxanthin *(*Okai and Okai, 1996).

2.18. Astaxanthin and Neuro Degenerative Dieases

The nervous systems including the brain, spinal cord and peripheral nerves is rich in both unsaturated fats(which are prone to oxidation) and iron, which has shown prooxidative properties (Halliwell, 1992). The high levels of poly unsaturated lipids, intense metabolic, aerobic activity, rich irrigation with blood vessels and elevated iron levels found in tissues of the nervous systems, like the brain, spinal cord and peripheral nerves, make these tissues particularly susceptible to oxidative damage (Halliwell, 1992; Dawson and Dawson, 1996; Fachinetti *et al.,* 1998). There is substantial evidence that oxidative stress is a causative or ancillary factor in the pathogenesis of major neuro degenerative dieases. This include Parkinson's disease (Fahn and Cohen, 1992; Borlongan *et al.,* 1996; Ebadi *et al.*,1996) Alzheimer's disease (Behl, 1999; Markesbery and Carney, 1999) and Amyotrophic lateral Sclerosis(ALS, "Lou Gehrig's diease") (Simonian and Coyle, 1996; Ferrante *et al.,* 1997; Hall *et al.,* 1998) as well as stroke, trauma and seizures. It has been suggested that Alzheimer's diease may be linked to diet, with reduced risk associated with diets high in

antioxidants (Grant, 1997; Pratico, 2002). A number of *in vitro* studies have shown that dietary antioxidants, vitamins and carotenoids can protect nervous tissues from damage by oxidative stress (Cotter *et al.,* 1995; Tagami *et al.,* 1998; Mitchell *et al.,* 1999).

A number of *in vivo* and clinical studies have provided evidence that dietary supplementation with lipid soluble antioxidants can help fight neurological diseases. In a Dutch study it was found that the risk for Parkinson's disease was lower for subjects who had higher dietary intake of antioxidants (De Rijk *et al.,* 1997). In another study it was found that persons suffering from Parkinson's disease had consumed less of the small molecule antioxidants betacarotene and vitamin C than did non sufferes of the disease, implying that dietary antioxidants do play a protective role (Hellenbrand *et al.,* 1996). About 20% of familiar Amyotropic Lateral Sclerosis (ALS) cases are associated with a mutuation in the gene for copper/zinc superoxide dismutase, an important antioxidant enzyme and *in vitro* experiments demonstrated that expression of the mutant enzymes in neuronal cells caused cell death, which could be prevented by antioxidant small molecules such as glutathione and vitamin E (Ghadge *et al.,* 1997). Rats fed natural astaxanthin demonstrated that astaxanthin is able to cross the blood brain barrier in mammals and can extend its antioxidant benefits beyond that barrier (Tso and Lam, 1996). Astaxanthin which has a stronger antioxidant activity than betacarotene or vitamin E is therefore an excellent candidate for testing in Alzheimer's disease and other neurological diseases. A mice study showed neuroprotective effect of high astaxanthin doses (Hussein *et al.,* 2005). Mice were subjected to transient cerebral ischemia and those pretreated with astaxanthin performed better, probably due to the antioxidant activity of astaxanthin on ischemia-induced free radicals, which resulted in the reduced impairment of spatial memory in the mice. A protective action of shrimp carotenoids against the ethanol effects on cortical spreading depression was noted in rats. This protective effect could be related to the antioxidant properties of shrimp head carotenoid (Bezerra *et al.,* 2005).

3. MATERIALS AND METHODS

3.1. PREPARATION OF RAW MATERIAL

Shell waste from the deep sea shrimp *Aristeus alcocki* (Plates 1 and 2) was collected from the processing plants RF Exports Pvt. Ltd, Chandiroor and Caps Seafoods Pvt. Ltd, Vypeen. The waste was transported to the laboratory in an insulated box in iced condition. *Aristeus alcocki* is processed as headless (HL) (Plate 3a) and peeled undeveined (PUD) (Plate 3b), the major product styles marketed under the trade name "red ring". The product styles generate waste as cephalothorax, abdominal shell and tail portion. Adhering meat from the cephalothorax was removed and the waste was washed under running water and dried under shade. They were packed in polyethylene bags and stored at -20^{0}C until used. Another lot of wet waste after removing the adhering meat was packed in polythene covers was and stored at -20^{0}C. Both wet and dried wastes were homogenized in a laboratory mixer prior to extraction of astaxanthin and estimation of different components in the shell.

3.2. STANDARDISATION OF THE EXTRACTION OF ASTAXANTHIN FROM SHRIMP SHELL WASTE.

Extraction of astaxanthin from shrimp shell waste was standardized based on the methods of using different organic solvents and vegetable oils. Ground wet and dried shrimp shell waste was extracted using four different organic solvents and three different vegetable oils with and without deproteinization (Fig.9). Waste was subjected to deproteinisation using alkali and enzyme. Quantification of astaxanthin was done spectrophotometrically using the equation of Kelley and Harmon (1972). Extraction method that gave maximum yield was selected for further studies.

3.2.1. Extraction using Ether: Acetone: Water (15:75:10, v/v/v)

A known weight of sample (1g) was extracted with ether: acetone: water (15:75:10,v/v/v) according to the method of Chen and Meyers (1982) and the extract was filtered and evaporated to dryness in a hot air oven at 60^0C. The residue was dissolved in hexane and astaxanthin was quantified by measuring absorbance at 470 nm using the equation of Kelley and Harmon (1972) as explained in 3.3.

3.2.2. Extraction using Acetone

A known weight of sample (1g) was extracted three times with 10ml each of acetone according to the method of Barbosa *et al.,* 1999 and the extract was collected in a separating funnel, added 12.5ml of petroleum ether (BP 40-60^0C) and 9.4ml of 0.73 % NaCl solution. Mixed and let stand. Collected the epiphase. To the lower phase added an equal volume of water, mix and allowed to separate and the epiphase was collected and evaporated to dryness in a hot air oven at 60^0C. The residue was dissolved in hexane and astaxanthin was quantified by measuring absorbance at 470 nm and using the equation of Kelley and Harmon, (1972).

3.2.3. Extraction using Hexane:Isopropanol (3:2, v/v)

A known weight of sample (1g) was extracted three times with 10ml each of hexane:isopropanol (3:2, v/v) according to the method of Sachindra *et al.,* 2006 and the pooled extract was washed with equal volume of 1 % NaCl solution, allowed to separate in a separating funnel and the epiphase was collected and dehydrated with anhydrous sodium sulphate, evaporated to dryness in a hot air oven at 60^0C. The residue was dissolved in hexane and astaxanthin was quantified by measuring absorbance at 470 nm using the equation of Kelley and Harmon (1972).

Plate. 1. Species studied – *Aristeus alcocki*

Plate. 2. Shrimp shell waste of *Aristeus alcocki* used for the extraction of astaxanthin

Plate. 3a. Headless (HL) – Red ring

Plate. 3b. Red ring – Peeled undeveined (PUD)

3.2.4. Extraction using 90 % Acetone

A known weight of sample (1g) was extracted with 10ml of 90% acetone (v/v) according to the method of Kobayashi *et al.,* 1997 for 5 min, 10ml of the solvent was again added and the sample was soaked for 1 h and the extract was filtered and evaporated to dryness in a hot air oven at 60^0C. The residue was dissolved in hexane and astaxanthin was quantified by measuring absorbance at 470 nm using the equation of Kelley and Harmon (1972).

3.2.5. Extraction using Vegetable Oils

A known weight of sample (1g) was extracted with vegetable oils *viz* coconut oil, soybean oil and sunflower oil according to the method of Sachindra and Mahendrakar (2005). The ratio of oil : waste used was 2:1 for wet sample and 4:1 for dried samples. An antioxidant BHT was added @ 0.05% and heated at 70^0C for 150 min, centrifuged and the pigmented oil was recovered. Astaxanthin was quantified by measuring absorbance at 485nm.

3.2.6. Preparation of Extract for Bioactivity Studies

Astaxanthin extract for feeding studies and other assays were prepared based on the method of Barbosa *et al.,* 1999. About three kilogram of the wet shrimp shell waste without deproteinisation was used for bulk extraction of pigment using acetone. The extract was concentrated using vacuum rota evaporator at 60^0C. The concentrated extract was quantified, flushed with N_2 and kept in amber coloured bottles at 4^0C. Required volume was pipetted, evaporated off the solvent and dissolved in suitable solvents and used for various assays.

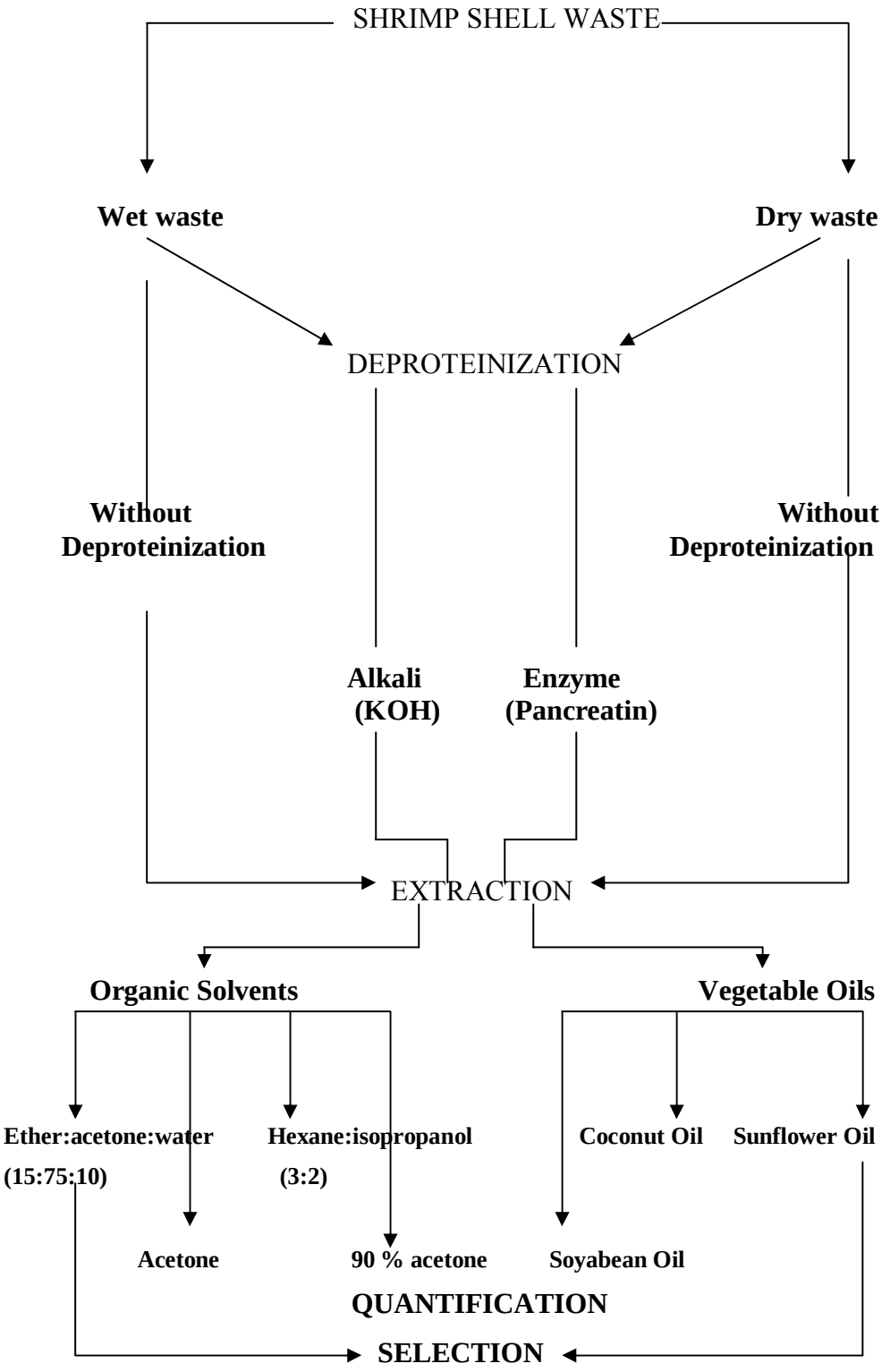


Fig. 9. Flow chart showing standardization of extraction of astaxanthin

3.3. QUANTIFICATION OF ASTAXANTHIN

Extracted astaxanthin was redissolved in hexane and quantified by measuring absorbance at 470 nm and using the equation of Kelley and Hormon (1972).

$$AST(\mu g / g) = \frac{A \times D \times 10^6}{100 \times G \times d \times E_{1cm}^{1\%}}$$

AST = Astaxanthin concentration in µg/g

A = Absorbance

D = Volume of extract in hexane

10^6 = Dilution multiple

G = Weight of sample in g

d = Cuvette width

E = Extinction co-efficient, 2100

For samples extracted in oil, quantification was done by measuring absorbance at 485nm and extinction co-efficient used was 2155.

3.4. DETERMINATION OF PROXIMATE COMPOSITION OF SHRIMP SHELL WASTE

Moisture, total lipids, ash and total nitrogen were determined according to AOAC (1990). Chitin was determined according to Spinelli *et al.* (1974), by extracting samples with 2% NaOH (w/v) followed by demineralization with 5 % HCl. The total protein content was calculated by substracting the nitrogen content of the chitin (6.89 %) (Synowiecki and Al-Khateeb, 2000) from the total nitrogen content, then using a conversion factor of 6.25. These analyses were done in triplicate for both wet and dried samples and average values for each has been recorded. All the chemicals used in the various tests were of either AR grade or GR grade.

3.4.1. Moisture Content

Moisture content of products was determined by the method of AOAC (1990).

Principle

Determination of moisture in the sample is done by drying the sample at elevated temperatures. Percentage of water is calculated from the difference in weight of sample before and after drying.

Apparatus

Analytical balance, hot air oven, petridish (with out lid), desiccator.

Procedure

About 5gm of sample was accurately weighed in a clean dry petridish using electric balance and was dried to a constant weight at a temperature of 105°C in a hot air oven. The dried material was cooled in a dessicator. The moisture content was calculated as the percentage loss of weight of the sample upon drying.

$$\% \text{ Moisture content} = \frac{(W_2 - W_3)g}{(W_2 - W_1)g} \times 100$$

Where,

W_1 = Initial weight of dry petridish.

W_2 = Weight of petridish + sample before drying.

W_3 = Weight of petridish + sample after drying.

$(W_2 - W_1)$ g = Weight of sample in grams.

$(W_2 - W_3)$ g = Weight of water in the sample in grams.

3.4.2. Protein Content

Protein content of the samples was estimated by the Microkjeldahl's method (AOAC, 1990).

Principle

The sample is digested with concentrated H_2SO_4 in presence of suitable catalysts like $CuSO_4$ / Hg/ HgO, so that protein N_2 is converted to ammonium sulphate. The ammonium sulphate formed is distilled with alkali and the ammonia evolved is absorbed in boric acid containing suitable indicator such as Tashirho's indicator. The ammonia absorbed is then titrated against N/70 H_2SO_4. From the titre value the percentage of total nitrogen is calculated. The total protein content of the waste was calculated by subtracting the nitrogen content of chitin (6.89 %) (Synowiecki and Al-Khateeb, 2000) from the total nitrogen content, then using a conversion factor of 6.25.

True protein % in shrimp shell waste = (Total nitrogen – Chitin nitrogen) x 6.25

Reagents

- Concentrated H_2SO_4(N_2 free)
- Digestion mixture ($CuSO_4$: K_2 SO_4 = 1 : 8)
- 40 % NaOH
- Tashirho's indicator – Dissolve 100mg of methyl red and 25mg of methylene blue in 100 ml ethyl alcohol.
- 2 % boric acid solution.
- *N/70* H_2SO_4 - Concentrated H_2SO_4 is approximately 36*N*. Prepare an approximately 0.1 *N* H_2SO_4 by diluting 2.8 ml of concentrated H_2SO_4 to one litre with distilled water. This solution is the then standardized against 0.1 *N* Na_2CO_3 solution using methyl orange as indicator. The standardized sulphuric acid is further diluted to obtain *N/70* H_2SO_4.

Procedure

One gram of sample was accurately weighed and transferred to a Kjeldahl's flask. A pinch of digestion mixture ($CuSO_4$: K_2 SO_4 = 1 : 8) and 10ml of concentrated H_2SO_4 were added and digested by heating at a

temperature of 300°C for 12h over a heating mantle. About 25ml of distilled water was then carefully poured into the flask along the side. The flask was swirled to dissipate off the heat evolved. When the solution attained room temperature, it was quantitatively transferred to a 50ml standard flask with distilled water washings. The solution was then made up to 50ml using distilled water and mixed thoroughly. Five ml of this solution was subjected to distillation using a distillation unit ('Kjelplus' make). 10ml 10N NaOH solution was added to the sample solution for distillation. The vapours were collected in 5ml of 2% boric acid that was previously mixed with 2 drops of Tashirho's indicator. The boric acid was titrated against standard N/70 H_2SO_4 to the pink end point.

$$\text{Total nitrogen},\% = \frac{V \times 14 \times 100 \times 50}{1000 \times 70 \times 5 \times W}$$

Where,

V= volume of N/70H_2SO_4

W= weight of sample.

3.4.3. Ash Content

Ash content of fresh fish was estimated by the method of AOAC (1990).

Principle

A known weight of the sample is ignited at a temperature of 550-600^{0}C to burn off completely the organic materials. The residue obtained is called ash which contains different mineral elements.

Apparatus

Clean dry silica crucible and muffle furnace.

Procedure

About 3g of dried sample was weighed accurately in a silica crucible. It was then ignited in a muffle furnace at a temperature of 550°C until sample

was free of carbon. It was then allowed to cool in a dessicator and weighed. The difference in weight was expressed as a percentage of sample weight to denote total ash.

$$\% \text{ Ash content} = \frac{(W_2 - W_3)g}{(W_2 - W_1)g} \times 100$$

Where,

W_1 = Initial weight of dry petridish.

W_2 = Weight of petridish + sample before ignition.

W_3 = Weight of petridish + ash after ignition.

$(W_2 - W_1)$ g = Weight of sample in grams.

$(W_2 - W_3)$ g = Weight of ash in the sample in grams.

3.4.4. Fat Content

The Soxhlet method of fat content estimation was followed (AOAC, 1990).

Principle

Fat content of moisture free sample is determined by extraction with suitable solvent. Petroleum ether can be used as solvent. Extraction is done by using soxhlet apparatus.

Reagents

- Petroleum ether (BP 60-80°C)

Apparatus

Soxhlet extractor is the laboratory apparatus used for the extraction of a lipid from the sample. Moisture free sample containing lipid is placed inside a "thimble" made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent petroleum ether. The Soxhlet is then equipped with a

condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the lipid will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over 16-20 h. During each cycle, a portion of the lipid dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, yielding the extracted lipid. The non-soluble portion of the extracted solid remains in the thimble, and is discarded.

Procedure

Two gram of moisture free sample was taken in an extraction thimble. The electric heating unit was adjusted so that the solvent, petroleum ether (60°C-80°C), siphons over 5-6 times per hour. The extraction was carried out for 16-20 h. The solvent was then transferred to a pre weighed beaker and evaporated off on a boiling water bath, then cooled to room temperature in a dessicator and weighed. The difference in weight was expressed as percentage of sample weight.

$$\% \text{ Fat content} = \frac{(W_2 - W_1)g}{W} \times 100$$

Where,

W = Weight of sample in grams.

W_1 = Weight of beaker.

W_2 = Weight of beaker + fat.

$(W_2 - W_1)$ g = Weight of fat in the sample in grams

3.4.5. Chitin Estimation

Chitin content in the raw material was estimated by the method of Spinelli *et al. (*1974).

Principle

A known weight of the sample is deproteinised by heating with alkali (NaOH) and the residue is demineralised using an acid HCl to obtain the residue as chitin. The weight of residue is expressed as a percentage of sample weight.

Procedure

One gram dry sample was ground to about 32mm mesh and digested in 100 ml of 2 % NaOH at 100^0C for one hour. The digest was filtered on a medium tared sintered glass crucible to refilter the residue. The digested residue was transferred to a beaker, treated for 12h at room temperature with 100ml of 5 % HCl and refiltered on the sintered glass crucible. The residue was washed with hot distilled water until there was a negative test for chloride. The crucible was dried at 110^0C for 16 h and weighed.

$$\% \text{ Chitin} = \frac{(W_2 - W_1)g}{W} \times 100$$

Where,

W = Weight of sample in grams.

W_1 = Weight of crucible.

W_2 = Weight of crucible + residue.

$(W_2 - W_1)$ g = Weight of residue in grams

3.5. DETERMINATION OF DIFFERENT COMPONENTS IN SHRIMP SHELL WASTE EXTRACT BY THIN LAYER CHROMATOGRAPHY

Analysis of different components in the shrimp shell extract was done using thin layer chromatography of the extract based on the method of Kobayashi and Sakamoto (1999).

3.5.1. Thin Layer Chromatography

Separation of free and esterified astaxanthin present in the shrimp shell extract was done using thin layer chromatography based on the method of Kobayashi and Sakamoto (1999). TLC was carried out in 0.25mm silica gel G coated plates and developed in a solvent system consisting of acetone/n-hexane (3:7 v/v) in the dark to prevent photoreduction of carotenoids.

A known volume of extract was taken in a round bottom flask. The petroleum ether was evaporated under nitrogen. The residue was dissolved in 2 ml of acetone. Spotted the extract on the TLC plate. The bands obtained were identified using standard astaxanthin and internationally accepted Rf values for astaxanthin monoester and astaxanthin diester. The different fractions *viz.* astaxanthin, astaxanthin monoester, astaxanthin diester were quantified by scraping out the respective bands in TLC plate. The astaxanthin present in the scraped out sample was redissolved into acetone and quantified as described earlier (3.3).

3.6. ANALYSIS OF FATTY ACIDS IN ASTAXANTHIN MONOESTER AND ASTAXANTHIN DIESTER BY GAS LIQUID CHROMATOGRAPHY

3.6.1. Saponification

The astaxanthin monoester and diester fractions separated in TLC were redissolved in acetone and the extract was taken in a round bottom flask. Acetone was evaporated under nitrogen. Added 10 ml of methanol and 1 ml of 40 % NaOH. Saponified for 30 min. Unsaponifiable matter was removed with petroleum ether. Aqueous layer was acidified with 1 ml of concentrated HCl. FFA was extracted with 5 ml of petroleum ether (BP 40^0C-60^0C) and dried under anhydrous sodium sulphate.

3.6.2. Gas Chromatography

The free fatty acids obtained from above were quantitatively converted to fatty acid methyl esters (FAME) using Boron triflouride-methanol reagent (Metcalfe *et al.*, 1966) as described below.

A known volume of the free fatty acid extract in petroleum ether was taken in a round bottom flask and evaporated completely under nitrogen and 5 ml of 0.5 N methanolic NaOH was added. This mixture was refluxed under a water condenser for 5-10 min under a nitrogen atmosphere. 6 ml of boron trifluoride –methanol (BF_3-CH_3OH) solution was added using automatic pipette through condenser and continued boiling for 2 min. Heat was removed, and 6 ml of saturated NaCl solution was added to the contents of the flask. The stoppered flask was shaken vigourously for 15 min while the solution was turbid. The aqueous phase was transferred to a 250 ml separator and extracted with two 30 ml portions of the petroleum ether (B.P. 60-80^{0}C). The combined extract was washed with three 20 ml portions of water, dried over anhydrous Na_2SO_4, filtered and evaporated free of solvent under a stream of nitrogen on a steam bath.

Methyl esters of fatty acids thus obtained were separated by gas liquid chromatography equipped with Elite 225 (Perkin Elmer) capillary column and a flame ionization detector in the presence of hydrogen and air. The carrier gas was nitrogen and the flow rate was 0.5ml/min.

Other GLC conditions were: injector temperature – 250^{0}C; temperature programme- 110^{0}C-4 min 2.7^{0}C/min-240^{0}C-5min; Detector at 275^{0}C. The fatty acid measurement of peak areas and data processing were carried out by electronic integrator. Individual fatty acids were expressed as per cent.

3.7. DEPROTEINISATION OF SHRIMP SHELL WASTE

3.7.1. Deproteinization using Alkali

Alkali deproteinization of shell waste is carried out according to the method of Sahidi and Synowiecki (1991). 10 g of wet waste was diluted in 100 ml of 1 % KOH and heated at a temperature of 90^0C for 2 h with intermittant stirring. Cooled and centrifuged at 12000 g for 15 min at 4^0C. Dry the residue at 60^0C for 6 h and determine the final weight. The total nitrogen of the supernatant was determined and protein calculated using the Microkjeldahl's method (AOAC, 1990).

The percentage of deproteinisation was calculated using the formula

$$\% \text{ Deproteinization} = \frac{\text{Protein in supernatant}}{\text{Protein in waste}} \text{ x } 100$$

3.7.2. Deproteinization using Enzyme

Enzymatic deproteinization of shrimp waste was carried out using enzyme pancraetin according to the modified method of Holanda and Netto (2006). To establish the conditions of hydrolysis, the E/S ratio was varied and temperature was maintained at 40^0C at pH 8.5. An E/S ratio of 1:50 was selected. The hydrolysis was carried out in a glass bottle for 48 h maintained at 40^0C and pH was monitored. The pH was maintained at 8.5 using 1N NaOH. The reaction is stopped by heating at 90^0C for 5 min. The insoluble fraction was separated by centrifugation at 16000 g for 15 min at 4^0C. The nitrogen content of the supernatant was determined by Microkjeldahl's method (AOAC, 1990) and the protein content was calculated. The percentage deproteinisation was calculated as in the previous case (3.7.1).

3.8. ASSAY OF *IN VITRO* ANTIOXIDANT ACTIVITY

3.8.1. Superoxide Anion Scavenging Activity

Reagents

- $FeSO_4(NH_4)_2SO_4$. 6 H_2O : 0.16 Mm
- Nitroblue tetrazolium(NBT) : 50µM
- Riboflavin : 2µM
- NaCN : 3 µg in 6mM EDTA
- KH_2PO_4-Na_2HPO_4 buffer, pH 7.8 : 67mM

Superoxide anion (O_2^-) generated from the photo reduction of riboflavin was detected by nitroblue tetrazolium (NBT) reduction method of McCord and Fridovich (1969). The reaction mixture contained 6mM ethylene diamine tetra acetic acid (EDTA) containing 3µg NaCN; 2µM riboflavin; 50µM NBT; buffer (67mM KH_2PO_4-Na_2HPO_4, pH 7.8) and various concentrations of astaxanthin extracts in a final volume of 3ml. The tubes were illuminated under incandescent lamp for 15 min. The optical density (OD) at 530 nm was measured before and after illumination. The inhibition of superoxide radical was determined by comparing the absorbance values of the control with that of treatments. Quercetin was used as standard.

$$\text{Percentage inhibition} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} x100$$

3.8.2. Inhibition of Lipid Peroxidation

The lipid peroxidation induced by Fe^{2+} - ascorbate system in beef liver homogenate was estimated by thiobarbituric acid reaction method of Ohkawa *et al.* (1979).

3.8.2.1. Preparation of Liver Homogenate

Fresh beef liver was procured from slaughter house in ice box. It was washed in distilled water. One gram of liver tissue was homogenized in 0.1 M Tris-HCl buffer pH 7.0 and centrifuged at 5000 rpm for 15 min at 4^0C. The supernatant was collected and used for the assay.

Principle

The tissue malondialdehyde was allowed to react with TBA. The MDA-TBA adduct formed during the reaction in the acidic medium was extracted to the organic layer and the absorbance was measured at 532nm.

Reagents

- Tris-HCl buffer pH 7.0 : 0.1 M
- Tris-HCl buffer pH 7.0 : 20 mM
- KCl : 30 mM
- $FeSO_4(NH_4)_2SO_4$. 6 H_2O : 0.16 mM
- Ascorbic acid : 0.06 mM
- Sodium dodecyl sulphate(SDS) : 8.1 %
- Thiobarbituric acid : 0.8 %
- Acetic acid : 20 %
- n-butanol pyridine mixture : 15:1 v/v

Procedure

The reaction mixture contained 0.1 ml of beef liver homogenate (25 % w/v) in Tris-HCl buffer (20mM, pH 7.0); 30 mM KCl; 0.16 mM $FeSO_4(NH_4)_2SO_4$. 6 H_2O; 0.06 mM ascorbate and various concentrations of astaxanthin extracts in a final volume of 0.5 ml. The reaction mixture was incubated at 37^0C for Ih. After the incubation period, 0.4 ml was removed and treated with 0.2 ml of sodium dodecyl sulphate (SDS) (8.1 %); 1.5 ml of 0.8 % thiobarbituric acid and 1.5 ml of 20 % acetic acid (pH 3.5). The total volume was made up to 4 ml with distilled water and the kept in a water bath at 95-100^0C for 1h. After cooling 1 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1, v/v) were added to the reaction mixture, shaken vigourously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and absorbance at 532 nm was measured. Inhibition of lipid

peroxidation was determined by comparing the OD of treatment with that of control. Catechin was used as the standard.

3.8.3. Hydroxyl Radical Scavenging Activity

Hydroxyl free radicals degrades 2-deoxy ribose to form thiobarbituric acid reactive substances (TBARS) (Elizebeth and Rao, 1990).

Principle

Hydroxyl radical scavenging activity was determined by studying the competition between deoxyribose and the astaxanthin extract for the hydroxyl radical generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction). Hydroxyl radical formed by Fenton's reaction is as follows.

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}.$$

Reagents

- Deoxyribose : 2.8 mM
- Ferric chloride : 0.1 mM
- KH_2PO_4-KOH buffer (pH 7.4) : 20 mM
- EDTA : 0.1 mM
- H_2O_2 : 1.0 mM
- Ascorbic acid : 0.1 mM
- TBA-TCA-HCl reagent : 15 % w/v TCA & 0.375 % w/v TBA in 0.25 N HCl

Procedure

The reaction mixture contained 2.8 mM deoxyribose; 0.1 mM $FeCl_3$; 20 mM KH_2PO_4-KOH buffer (pH 7.4); 0.1 mM EDTA; 1.0 mM H_2O_2 ; 0.1 mM ascorbic acid and various concentrations of astaxanthin extract in a final volume of 1 ml. The TBARS formed was estimated by reaction with TBA to form pink coloured complex (Ohkawa *et al.*,1979). The hydroxyl radical

scavenging activity was determined by comparing absorbance of control with that of treatments. Catechin was used as standard.

3.9. ANIMALS

Male Albino rats of Wistar strain one month old weighing 80-120 g and male Balb/c mice 4-5 weeks old weighing 20-25 g purchased from Small Animal Breeding Centre, Kerala Agricultural University, Mannuthy, Thrissur were used for the study. The animals were housed under hygienic conditions in polypropylene cages under 12 hour light and dark cycle. All procedures involving animal care and experiments were in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and with the approval of Institutional Animal Ethics Committee (IAEC).

3.10. CHEMICALS

Isopropanol, n-hexane, n-butanol, acetone, potassium chloride, potassium hydroxide, absolute ethanol, petroleum ether (B.P.40-60^{o}C), petroleum ether (B.P.60-80^{o}C), Pancreatin, Ferrous ammonium sulphate, DMSO, ethylene glycol monoethyl ether, were purchased from Merck, India Ltd, Mumbai. Soybean oil, coconut oil and sunflower oil were of food grade. Thiourea, 2,4 dinitrophenyl hydrazine, ascorbic acid (for analytical work) 5,5'-dithio bis nitrobenzoic acid (DTNB), glutathione (GSH), glucose, sodium pyrophosphate, sodium chloride, sodium citrate, citric acid, Tris buffer, Tris-hydrochloride buffer, SDS, sucrose, phenazine methosulphate, nitroblue tetrazolium, hydrogen peroxide, deoxyribose, reduced nicotinamide adenine dinucleotide (tetrasodium) (NADH), reduced nicotinamide adenine dinucleotide phosphate (tetrasodium) (NADPH), oxidised glutathione (GSSG), thiobarbituric acid, TCA, bovine serum albumin (BSA), NADP, carrageenan, adenosine dinucleotide phosphate (ADP), ATP sodium salt were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai. Alpha naphthyl acetate, alloxan, Pararosaniline were purchased from Loba Chemie Pvt. Ltd., Mumbai. Tissue

culture media RPMI-1640, Foetal Bovine serum (FBS) and trypan blue were purchased from Himedia Laboratories, Mumbai. Phytohaemagglutinin (PHA) and PBS were obtained from Genei, Bangalore. Astaxanthin standard, isoproterenol, Queretin and catechin were purchased from Sigma chemicals, USA. All other reagents were of Analytical Reagent quality.

3.11. ASSAY OF ANTIINFLAMMATORY ACTIVITY

3.11.1. Animals

Antiinflammatory activity of astaxanthin was studied using male Balb/c mice. Male Balb/c mice weighing 20-25 g body weight, purchased from Small Animal Breeding Centre, Kerala Agricultural University (KAU), Thrissur, were used for the study.

3.11.2. Carrageenan Induced Paw Edema

Carrageenan induced paw edema was used for determining the acute anti-inflammatory activity of astaxanthin. Animals were divided into four groups containing six animals in each group. In all the groups, inflammation was produced by injecting 0.025 ml of a 1% freshly prepared carrageenan solution in the right hind paw of the mice. One group with carrageenan alone served as positive control. The second and third groups were administered with astaxanthin extract at a concentration of 0.5 mg/Kg and 1.0 mg/Kg body weight intraperitoneally one hour prior to carrageenan injection. The fourth group was administered with a standard reference drug Diclofenac 10 mg/Kg intraperitoneally. The paw thickness was measured using vernier calipers before and 3 h after carrageenan injection (Ajith and Janardhanan, 2001)

Increase in paw thickness was calculated using the formula $P_t–P_o$, where P_t is the thickness of paw at time 't' (i.e. 3h after carrageenan injection) and P_o is the paw thickness at '0' time. Percentage inhibition was calculated using the formula (1-T/C) x 100 where C is the increase in paw thickness of the control and T is that of the treatments.

3.12. CARDIOPROTECTIVE ACTIVITY

3.12.1. Animals

Cardioprotective effect of astaxanthin was examined in isoproterenol induced myocardial infarction in rats (Rona *et al*, 1959). Male albino Wistar strain rats weighing 80-120 g body weight, purchased from Small Animal Breeding Centre, Kerala Agricultural University (KAU), Thrissur, were used for the study.

3.12.2. Experimental Design

The animals were housed in groups of four in polypropylene cages with a 12:12 light/dark cycle. Sufficient number of control groups and test groups were maintained so that at least 6 animals were available for each assay. The animals in the groups I & II were fed on normal diet purchased from College of Veterinary and Animal Sciences, KAU, Thrissur. The animals of the groups III & IV were fed on normal diet containing astaxanthin @ 10 mg/Kg feed. They were provided with food and water *ad libitum*. The experimental duration was 45 days.

3.12.2.1. Preparation of Test Diet

For preparing test diet, required volume of quantified extract containing astaxanthin was pipetted into a beaker and solvent evaporated off over a water bath at 60^0C, mixed uniformly with normal feed and stored in air tight containers at 4^0C .

3.12.2.2. Isoproterenol Induced Myocardial Infarction

At the end of feeding study myocardial infarction was produced in animals of groups II (fed on normal diet) and in group IV (fed on normal diet containing astaxanthin @ 10 mg/kg feed) by subcutaneous injection of isoproterenol (6mg in physiological saline per 100g body weight) twice at an interval of 24h. Simultaneously, the animals of groups I and III were injected with physiological saline alone. Animals surviving the second injection were

sacrificed at 36h after first injection. Blood was collected in ice cold containers and the serum was separated for the determination of diagnostic marker enzymes.

3.12.3. Preparation of Tissue Homogenates

At the end of the experiment period, the rats in each group were fasted overnight, stunned by a blow at the back of the neck, killed by decapitation and blood and heart were collected. The heart was dissected out and rinsed thoroughly in ice-cold saline to remove the blood and kept in ice-cold containers. The tissues were gently blotted between the folds of a filter paper. Small amounts were weighed and homogenized in a suitable solvent or buffer to carry out the biochemical analyses. The supernatants were used for the estimation of superoxide dismutase(SOD), catalase(CAT), glutathione peroxidase(GPx), glutathione reductase(GR), reduced glutathione(GSH), glutathione S-transferase (GST), ascorbic acid, malondialdehyde(MDA). The residue containing the membrane bound enzyme was used for the assay of Na^+ K^+ ATPase.

3.12.4. Preparation of Serum

Blood was collected and serum was separated from blood cells by centrifugation at 2000 rpm for 30 min. Serum was collected and used for the assay of cardiac enzymes *viz.* lactate dehydrogenase(LDH), serum glutamate oxaloacetate transaminase(SGOT), serum glutamate pyruvate transaminase(SGPT), creatine kinase(CK), creatine kinase MB(CK-MB).

3.12.5. Analysis of Cardiac Marker Enzymes

Activities of serum enzymes such as lactate dehydrogenase (LDH), Creatine phosphokinase (CPK), Glutamate oxaloacetate transferase (GOT), Glutamate pyruvate transferase (GPT) and Creatine kinase-MB (CK-MB) were determined using commercial diagnostic kit supplied by AGAPPE Diagnostics, Mumbai, India. The principle of the estimations carried out is as given below

3.12.5.1. Lactate Dehydrogenase (LDH, EC 1.1.1.27)

Principle

LDH catalyses the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH acivity in serum is proportional to the increase in absorbance due to reduction of NAD

$$\text{Pyruvate} + \text{NADH} + H^+ \underset{\text{LDH}}{\rightleftarrows} \text{L- lactate} + NAD^+$$

Reagents

- Reagent 1 (R_1)

Tris buffer (pH 7.4)	50 mmol/l
Pyruvate	1.2 mmol /l
EDTA	5 mmol/l
Reagent 2 (R_2)	NADH0.15 mmol/l

- Working reagent — R_1:R_2 – 4:1

Procedure

Wavelength	340 nm
No. of readings	3
Interval	60 Sec
Blank	distilled water

Sample 40 µl serum + 2 ml working reagent.

Calculation

LDH activity in U/L = ▲OD/min × 16030

3.12.5.2. Creatine Phosphokinase (CPK, EC 2.7.3.2)

Principle

Kinetic determination of CPK is based on the following reactions

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow[\text{CK}]{} \text{Creatinine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xrightarrow[\text{HK}]{} \text{ADP} + \text{G}-6-\text{P}$$

$$\text{G-6-P} + \text{NADP} + \text{G-6-P-DH} \longrightarrow 6\ \text{PG} + \text{NADPH} + \text{H}^{+}$$

Reagents

- Reagent 1 (R_1)

Imidazole (pH 6.7)	125 mmol/l
D-Glucose	25 mmol /l
N-Acetyl-L-cysteine	25 mmol/l
Magnesium acetate	12.5 mmol/l
NADP	2.52 mmol/l
EDTA	2.02 mmol/l
Hexokinase	≥ 6800 U/l

- Reagent 2 (R_2)

Creatine phosphate	250 mmol/l
ADP	15.2 mmol/l
AMP	25 mmol/l
Diadenosine pentaphosphate	103 µmol/l
G-6-PDH	≥ 8800 U/l

- Working reagent R_1:R_2 – 4:1

Procedure

Wavelength	340 nm
No. of readings	3
Interval	60 Sec
Blank	distilled water

Sample 50 µl serum + 2 ml working reagent.

Calculation

CPK activity in U/L = ▲OD/min × 6508

3.12.5.3. Glutamate Oxaloacetate Transferase (GOT, EC 2.6.1.1)

Principle

Kinetic determination of SGOT is based on the following reactions

$$L-\text{aspartate} + 2\ \text{oxyglutarate} \xrightarrow[\text{GOT}]{} L-\text{glutamate} + \text{oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H \xrightarrow[\text{MDH}]{} L-\text{malate} + NAD^{+}$$

(MDH –malate dehydrogenase)

Reagents

- Reagent 1 (R_1)

Tris buffer (pH 7.5)	88 mmol/l
L-Aspartate	260 mmol /l
LDH	≥1500 U/l
MDH	≥900 U/l

- Reagent 2 (R_2)

α – Ketoglutarate	12 mmol
NADH	0.24 mmol/l

- Working reagent R_1:R_2 – 4:1

Procedure

Wavelength	340 nm
No. of readings	3
Interval	60 Sec
Blank	distilled water

Sample 200 µl serum + 2 ml working reagent.

Calculation

SGOT activity in U/L = ▲OD/min × 1745

3.12.5.4. Glutamate Pyruvate Transferase (GPT, EC 2.6.1.2)

Principle

Kinetic determination of SGPT is based on the following reactions

$$L-\text{Alanine} + 2-\text{oxoglutarate} \xrightarrow[\text{GPT}]{} L-\text{glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow[\text{LDH}]{} L-\text{lactate} + NAD^{+}$$

Reagents

- Reagent 1 (R_1)

Tris buffer (pH 7.5)	110 mmol/l
L-Alanine	600 mmol /l
LDH	≥1500 U/l

- Reagent 2 (R_2)

α – Ketoglutarate	16 mmol
NADH	0.24 mmol/l

- Working reagent R_1:R_2 – 4:1

Procedure

Wavelength	340 nm
No. of readings	3
Interval	60 Sec
Blank	distilled water

Sample 200 µl serum + 2 ml working reagent.

Calculation

SGPT activity in U/L = ▲OD/min × 1745

3.12.5.5. CK-MB

Principle

Procedure involves measurement of CK- activity in the presence of antibody to CK-M monomer. This antibody completely inhibits the activity of CK-MM and half the activity of CK-MB & CK-BB. Then CK method is used to determine CK-B activity.

Reagents

- Reagent 1 (R_1)

Imidazole (pH 6.7)	125 mmol/l
D-Glucose	25 mmol /l
N-Acetyl-L-cysteine	25 mmol/l
Magnesium acetate	12.5 mmol/l
NADP	2.4 mmol/l
EDTA	2.02 mmol/l
Hexokinase	≥ 6800 U/l

- Reagent 2 (R_2)

Creatine phosphate	250 mmol/l
ADP	15.2 mmol/l
AMP	25 mmol/l
Diadenosine pentaphosphate	103 µmol/l
G-6-PDH	≥ 8800 U/l

- Working reagent R_1:R_2 – 4:1

Procedure

Wavelength	340 nm
No. of readings	3

Interval	60 Sec
Blank	distilled water

Sample 80 µl serum + 2 ml working reagent.

Calculation

CK-MB activity in U/L = ▲OD/min × 8254

3.12.6. Assay of Na^+-K^+ ATPase Activity(EC 3.6.1.37)

Na^+-K^+ ATPase is a membrane bound enzyme involved in the transport of various cations and steroidal glycoside digoxin. Na^+-K^+ ATPase was assayed by the method of Blostein (1968).

Principle

Na^+-K^+ ATPase present in the tissue acts on ATP present in the reaction mixture releasing inorganic phosphorus which is estimated as the activity of enzyme.

Reagents

Sucrose	:	0.25 M
Tris-HCl buffer	:	1 mM (pH 7.4) containing 1mM EDTA
Tris-HCl buffer	:	1 mM (pH 7.4) containing 10mM EDTA
Tris-HCl buffer	:	2 mM (pH 7.4)
NaCl	:	0.25 M
$MgSO_4$	:	0.00138 M
KCl	:	0.0125 M
Na-ATP	:	0.00125 M
Tris-HCl buffer	:	0.0625 M (pH 7.4) containing 3.12×10^{-4} M EDTA

3.12.6.1. Preparation of Membrane from Heart Tissue

A known weight of the heart tissue was homogenized under ice cold conditions in 0.25 M sucrose and centrifuged at 3500 rpm for 10 min at 4^0C. The residue containing the cell debris was washed with cold normal saline twice by centrifugation at 3500 rpm for 10 min at 4^0C. The cells were then lysed with distilled water and cell membranes washed twice with 1 mM Tris-HCl buffer (pH 7.4) containing 1mM EDTA at 4^0C followed by washing with 1 mM Tris-HCl buffer (pH 7.4) containing 10mM EDTA and finally thrice with 2mM Tris-HCl buffer (pH 7.4). The heart membrane was finally suspended in 0.0625 M Tris-HCl buffer (pH 7.4). The membrane bound enzyme was released by freezing the suspension and thawing it and repeating the process. This suspension was used for the assay of the enzyme (Dhanya *et al.,* 2003).

Procedure

Two sets of tubes, one test and the other control containing 0.4 ml of a mixture of NaCl, $MgSO_4$ and KCl, 0.4 ml of Na-ATP, 0.16 ml of 0.0625 M Tris-HCl buffer (pH 7.4) containing 3.12 x 10^{-4} M EDTA in a total volume of 0.96 ml, were pre incubated at 37^0C for 5 min and 0.2 ml of enzyme preparation were added to the test. The tubes were incubated at 37^0C for 1 h and were transferred to powdered ice and ice cold 12 % TCA was added to both followed by 0.2 ml of enzyme preparation to the control. This final incubation mixture was used for the estimation of inorganic phosphorus.

3.12.6.2. Estimation of Phosphorus

Phosphorus was estimated by the method of Fiske and SubbaRow (1925).

Principle

The protein free sample was treated with an acid molybdate solution, which formed phosphomolybdic acid from any phosphate present. The phosphomolybdic acid was reduced by the addition of 1,2,4-

aminonaphtholsulfonic acid (ANSA) reagent, to produce a blue colour whose intensity was proportional to the amount of phosphate present.

Reagents

H_2SO_4	:	5 N
Ammonium molybdate	:	2.5 %
ANSA reagent	:	Mix 0.2 g ANSA, 1.2 g sodium bisulphite, 1.2 g sodium sulphite, ground well and dissolved 50 mg ANSA reagent in 2ml distilled water.
Standard	:	KH_2PO_4, 136 mg in 100 ml distilled water, diluted 10 ml of this to 100 ml and used as working standard

Procedure

Pippeted 1 ml of above incubation mixture from control and tests. Added 1 ml of 5 N H_2SO_4. Added 1 ml of 2.5 % ammonium molybdate, 0.1 ml of freshly prepared ANSA (aminonaphthosulphonic acid) reagent. Made up the volume to 10 ml using distilled water and read the OD at 660 nm within 10 min. Blank was prepared by mixing 1 ml distilled water, 1 ml 5 N H_2SO_4, 1ml 2.5 % ammonium molybdate, 0.1 ml ANSA and 6.9 ml distilled water. As standard took 0.5 ml working standard and proceeded as for blank.

3.12.7. Assay of Antioxidant Enzymes

3.12.7.1 .Glutathione Peroxidase (GPx, EC 1.11.1.9)

Glutathione peroxidase was estimated by the method of Rotruck *et al.* (1973).

Principle

$$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$$

Reagents

- Tris buffer : 0.4 M, pH 7.0
- Sodium azide solution : 10 mM
- Glutathione solution (GSH) : 2 mM
- Trichloroacetic acid (TCA) : 10%
- Ethylene diamine tetraacetic acid (EDTA) : 0.4 mM
- Hydrogen peroxide (H_2O_2) : 0.2 mM

Procedure

100 mg of tissue was homogenized in a 2ml of Tris buffer and centrifuged at 5000 rpm for 10 min. To 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1 ml sodium azide and 0.5 ml tissue homogenate were added and mixed well. To this mixture 0.2 ml of GSH followed by 0.1 ml H_2O_2 solution were added. The contents were mixed well and incubated at 37^{o}C for 10 min. along with a control containing all reagents except tissue homogenate. After 10 min. the reaction was arrested by the addition of 0.5ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH by the method of Patterson and Lazarrow (1955).

3.12.7.2. Glutathione-S-transferase (GST, EC 2.5.1.18)

Glutathione-S-transferase was estimated by the method of Habig *et al.* (1974)

Principle

GST catalyse the conjugation of reduced glutathione *via* the sulfhydryl group, to 1-chloro 2, 4 dinitrobenzene. The conjugate can absorb light in the UV range which is measured spectrophotometrically.

Reagents

- Phosphate buffer : 0.3 M (pH 6.5)
- 1-chloro 2,4 dinitrobenzene : 30 mM
- Glutathione solution : 30 mM

100mg tissue is homogenized with 2 ml of 0.3 M phosphate buffer centrifuged at 5000 rpm for 10 min. Pippeted 1.0 ml of phosphate buffer to a test tube, added 0.1 ml of 1-chloro 2,4 dinitrobenzene and 0.1 ml of enzyme solution. To this mixture was added 1.3 ml of double distilled water and incubated at 37^0C for 5 min. Added 0.1 ml of 30 mM GSH and change in absorbance was measured at 340 nm for 3 min at one minute interval against a blank containing all reagents except the enzyme solution. Instead of enzyme solution 0.1 ml of phosphate buffer was used.

3.12.7.3. Glutathione Reductase (GR, EC 1.6.4.2)

Glutathione reductase was estimated by the method of Beutler and Bernes (1996).

Principle

$$GS–SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+.$$

Reagents

- Phosphate buffer : 0.12 M (pH 7.2)
- EDTA : 155 mM
- GSSG : 6.3 mM
- NADPH : 9.6 mM in 1% sodium bicarbonate

Procedure

100 mg tissue was homogenized in 2 ml of 0.12 M phosphate buffer centrifuged at 5000 rpm for 10 min. 2.6 ml buffer, 0.1 ml EDTA and 0.1 ml GSSG act as reagent mixture to which was added 0.1 ml of the homogenized

sample and kept for 5 min. Then 0.05 ml of NADPH was added, mixed thoroughly and read at 340 nm for 5 minutes against buffer blank.

3.12.7.4. Catalase (CAT, EC 1.11.1.6)

Catalase level in tissues was determined using the method of Machley and Chance (1954).

Principle

Catalase catalyses the decomposition of H_2O_2. In the UV range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in extinction at 230 nm.

$$2\ H_2O_2 \longrightarrow 2H_2O + O_2$$

Reagents

- Phosphate buffer : 0.12 M, pH 7.2
- H_2O_2 Phosphate buffer : 0.16 ml H_2O_2 diluted to 100 ml with buffer

Procedure

100 mg of tissue was homogenized in 2 ml phosphate buffer at 1-4^{o}C and centrifuged at 5000 rpm for 10 min. Pipetted 3 ml H_2O_2-phosphate buffer into the experimental cuvette and 40 µl of the enzyme solution was added. Change in OD was measured at 230 nm at 0 sec, 30 sec and 60 sec, respectively against control cuvette containing enzyme solution and H_2O_2 free phosphate buffer. Specific activity is expressed in terms of units / mg protein, where one unit has been defined as the velocity constant per second.

3.12.7.5. Superoxide Dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase in tissues was determined using the method of Kakkar *et al.* (1984).

Reagents

- Sodium pyrophosphate buffer : 0.052 M (pH 8.3)
- Tris-HCl buffer : 0.0025 M (pH 7.4)
- Sucrose : 0.25 M
- Phenazine methosulphate : 186 µM
- Nitroblue tetrazolium : 300 µM
- NADH : 780 µM
- Ammonium Sulphate solution : 90 %
- Glacial acetic acid
- n-butanol

Procedure

100 mg tissue was homogenized in 2 ml 0.25 M sucrose and differentially centrifuged at 10,000 rpm under cold condition to get the cytosol fraction. Protein fraction was precipitated from the supernatant with 90% ammonium sulphate and dialysed overnight with 0.0025 M Tris-HCl buffer, pH 7.4. The supernatant was used as the enzyme source. The assay mixture contained 1.2 ml sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, 0.3 ml nitroblue tetrazolium, appropriately diluted enzyme preparation 0.2 ml and water in a total volume of 3 ml. The reaction was started by the addition of 0.2 ml 780 µM NADH. After incubation at 30^{o}C for 90 seconds, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and upper butanol layer was taken out. Colour intensity of chromogen in the butanol was measured at 560 nm against n-butanol blank. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the OD at 560 nm of chromogen production by 50 % in one minute under the assay condition and expressed as specific

activity in milliunits / mg protein. Protein estimation was carried out on the same enzyme source by using protein estimation kits based on Bradford method.

3.12.8. Estimation of Enzyme Protein

Protein content of the tissue homogenates were assayed using protein estimation kit of Genei based on method of Bradford (1976). This is a rapid and accurate method for estimation of protein concentration.

Principle

The assay relies on the binding of the dye coomassie blue G250 to protein. The quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is achieved by measuring the absorbance of the solution at 595 nm or 625 nm.

Reagents

- 10 % TCA
- N NaOH
- Standard protein BSA
- Bradford reagent

Procedure

Pipetted out 0.1 ml of extract and added 0.9 ml water and 1 ml 10% TCA. Centrifuged at 5000 rpm for 10 min. Removed the supernatant completely and dissolved the precipitate in 1 ml 0.1 N NaOH. 100µl of protein was used for estimation. 2ml of Bradford's reagent was added, mixed and kept for 10 min. The optical density was measured at 595/620 nm using UV spectrophotometer (UV-VIS JASCO 530) against distilled water as blank. Working standard was 1mg/ml BSA.

3.12.9. Assay of Antioxidants

3.12.9.1. Ascorbic Acid

Ascorbic acid level in tissues was determined by using the method of Roe (1954).

Principle

Ascorbic acid is oxidized to dehydroascorbic acid which couples with 2,4 dinitrophenyl hydrazine to yield an ozazone which gives red colour with strong sulphuric acid which is measured photometrically.

Reagents

- Trichloroacetic acid (TCA) : 6 %
- Thiourea reagent : 50 % in alcohol
- 2,4 dinitrophenyl hydrazine : 2 % in 9N H_2SO_4
- Conc. H_2SO_4 : 85 %
- Ascorbic acid standard : 1 mg /ml of 6% TCA
- Activated charcoal

Procedure

Immediately after sacrificing the animals a 100 mg of tissue was homogenized in 5 ml ice cold 6 % TCA in a pre-chilled mortar. The extract was taken in a stoppered test tube and shaken well with added activated charcoal and allowed to stand for 15 min. The clear supernatant was filtered through No. 41 Whatman filter paper. To 4 ml of supernatant, added a drop of Thiourea reagent and 1 ml of 2 % 2, 4 dinitrophenyl hydrazine in 9 N H_2SO_4 and incubated for 3 h at 37^{o}C in a water bath. At the end of the incubation, placed the test tubes in an ice bath and added carefully 4 ml of 85 % H_2SO_4. Kept for 30 min.in a refrigerator. Centrifuged and OD measured at 540 nm in a spectrophotometer against a blank containing all reagents and 4 ml of 6 % TCA instead of extract. The values are expressed in mg/100g tissue.

3.12.9.2. Glutathione (GSH)

Glutathione level in tissue was determined using the method of Patterson and Lazarrow (1955).

Reagents

- Phosphate buffer : 0.5M, pH 7.5
- Alloxan : 0.1 M
- NaOH : 0.5 N
- NaOH : 1 N
- Standard GSH : 3 mg dissolved in 5 % 25 ml metaphophoric acid

Procedure

100 mg of tissue was homogenized in 600 µl phosphate buffer and centrifuged at 5000 rpm for 10 min. To the supernatant added 600 µl alloxan, 600 µl phosphate buffer and 600 µl NaOH (0.5 N) was incubated at 25^0C for 6 min. The reaction was stopped by the addition of 600 µl 1N NaOH. Absorbance was noted at 305 nm in a quartz cuvette of 1 cm length path in a spectrophotometer. A control tube was maintained with phosphate buffer instead of extract. The values were expressed in mg / 100 g tissue.

3.12.10. Estimation of Lipid Peroxidation Products

3.12.10.1. Malondialdehyde (MDA)

Malondialdehyde was estimated by the method of Beuge and Aust (1978).

Principle

The tissue malondialdehyde was allowed to react with TBA. MDA-TBA adduct was formed during the reaction in the acidic medium and the absorbance was measured at 532nm

Reagents

- Tris-HCl buffer : 0.1M, pH 7.5
- TCA-TBA-HCl reagent : 15% w/v TCA and 0.375% w/v 2- thiobarbituric acid in 0.25 N HCl.

Procedure

100 mg tissue was homogenized with 2 ml Tris-HCl buffer. 2 ml of TCA-TBA-HCl reagent was added to 1 ml of the tissue homogenate and mixed thoroughly. The contents were heated in a boiling water bath for 15 minutes. After cooling, the flocculent precipitate was removed by centrifugation at 1000 x g for 10 minutes. The absorbance of the pink coloured supernatant was read at 535 nm against a blank that does not contain the sample. The concentration of malondialdehyde was calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

3.13. IMMUNOSTIMULATORY EFFECT

3.13.1. Experimental Design

The animals (male Balb/c mice) were housed in groups of two in polypropylene cages with a 12:12 light/dark cycle. Sufficient number of control groups and test groups were maintained so that at least 6 animals were available for each assay. The animals in the control group (Group I) were fed on normal diet purchased from College of Veterinary and Animal Sciences, KAU, Thrissur. The animals of the test group (Group II) were fed on normal diet containing astaxanthin @ 10 mg/Kg feed. They were provided with food and water *ad libitum*. The experimental duration was 45 days. At the end of experimental period animals were sacrificed and spleen, bone marrow cells and blood were collected for the following assays.

The immunostimulatory action was determined by assaying the Splenic T-lympocyte mitogen response, bone marrow cell proliferation assay, plaque formation cell assay, circulating antibody titre and α-naphthyl esterase activity.

3.13.2. Splenic T-lympocyte Mitogen Response

At the end of the feeding study, the animals were sacrificed; spleen removed asceptically and made into single cell suspension. The cells from both the control and test animals were cultured (10^6 cells/ml) in the presence and absence of mitogen phytohaemagglutinin (PHA) (5µg/ml) in RPMI-1640 medium containing 10% FBS (final volume 1 ml) and antibiotics in a humidified atmosphere of 5 % CO_2 at 37^0C. After 48 h, 50µl MTT dye [3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added and further incubated for 4h. MTT solution was dissolved in RPMI-1640 medium at 4 mg/ml. It was passed through 0.45µ filter, to remove any amount of insoluble residue. After 4 h the liquid contents of the well were discarded and added 100 µl MTT lysis buffer (2g SDS dissolved in 5ml dimethyl formamide and 5 ml distilled water) to each well to solubilize the formazan crystals to produce a solution suitable for measurement of absorbance. After adding MTT lysis buffer the plates were incubated further for 2h in dark. The results were reported as absorbance measured at wavelength 570nm (Singh *et al.*, 2007).

3.13.3. Bone Marrow Cell Proliferation Assay

Bone marrow cell proliferation assay was carried out by following the method of Kumar *et al.* (1999). Total bone marrow cells were collected from control and test animals and made into single cell suspension in RPMI-1640 as described above. The cells (10^6 cells/ml) were cultured in the presence and absence of mitogen PHA (5µg/ml) in RPMI-1640 medium containing 10% FBS (final volume 1 ml) and antibiotics in a humidified atmosphere of 5 % CO_2 at 37^0C. After 48 h, 50µl MTT dye [3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added and further incubated for 4h. MTT solution was dissolved in RPMI-1640 medium at 4 mg/ml. It was passed through 0.45µ filter, to remove any amount of insoluble residue. After 4 h the liquid contents of the well were discarded and added 100 µl MTT lysis buffer (2g SDS dissolved in 5ml dimethyl formamide and 5 ml distilled water) to each

well to solubilize the formazan crystals to produce a solution suitable for measurement of absorbance. After adding MTT lysis buffer the plates were incubated further for 2h in dark. The results were reported as absorbance measured at wavelength 570nm.

3.13.4. Plaque Formation Cell Assay

3.13.4.1. Preparation of SRBC Suspension

Collected 10 ml of sheep blood to 10 ml of Alsever's solution. Mixed gently and kept at 4^0C till used. Removed the supernatant and washed with physiological saline. Resusupended in saline and centrifuged at 700g for 5 min. Noted the volume of packed cells and made in to desired suspension in normal saline.

3.13.4.2. Preparation of Alsever's Solution

Glucose	:	2.05 g
Sodium citrate	:	0.80 g
Citric acid	:	0.055 g
Sodium chloride	:	0.42 g

Dissolved in 100 ml triple distilled water and autoclaved for 10 min at 10lbs.

Procedure

Modified slide technique of Jern's Plaque assay was adopted for plaque formation cell assay (Mehrotra, 1992). At the end of experimental period the control and test animals were immunized with 1 ml of 5 % SRBC intraperitoneally. The spleen was collected from the sacrificed animals on the 5^{th} and 10^{th} day following immunization. A single cell suspension of the spleen cells was prepared in PBS ($8x10^6$ cells/ml). To 0.5 ml of 0.5% agarose prepared in PBS, 50 µl of 10% SRBC and 50 µl of spleen cell suspension were added, mixed well and poured over a glass slide. The slides were allowed to solidify and then incubated with fresh human serum as a source of complement

for 1 h at 37^0C. The plaques formed were counted using a colony counter and represented as plaque forming cells (PFCs /million spleen cells).

3.13.5. Circulating Antibody Titre

Blood was collected from the immunized animals on the 3rd and 5th day following immunization. The blood was allowed to clot and serum was separated by centrifugation. Two-fold serial dilution of the serum samples were made in physiological saline and mixed (1:1) with 1% SRBC in physiological saline. Agglutination was noted after incubation at room temperature for 3 h (Nelson and Davey, 1992).

3.13.6. Alpha-naphthyl Acetate Esterase Activity in Bone Marrow Cells

Naphthyl acetate esterase activity in the bone marrow is an indicator of maturation of stem cells to monocytes-macrophages. Total bone marrow cells from control and test animals were made in to a single cell suspension. A smear was prepared, dried and stained according to the method of Bancroft and Cook (1994).

Reagents

Phosphate buffer : pH 6.8; 946 mg Na_2HPO_4 in 100ml distilled water 9.07 mg KH_2PO_4 in 100 ml distilled water. Mix 87 ml of Na_2HPO_4 with 13 ml of KH_2PO_4 solution.

Solution A : Pararosaniline 1g/20 ml distilled water + 5 ml conc.HCl. while gently warming, filter and store in dark at 4^0C

Solution B : 4 % solution of NaNO2 (should be prepared one day before use)

Solution C : Alpha naphthyl acetate 50 mg/2.5 ml ethyleneglycol monoethyl ether

Haematoxylin : Dissolved 2.5 g haematoxylin and 90 g potash alum in 500 ml distilled water. Add 20 mg NaI or KI and 50 ml ethanol. Stored at room temperature.

Fixative solution	: Formaldehyde	25ml
	Acetone	45 ml
	Distilled water	30 ml
	Na_2HPO_4	20 mg
	KH_2PO_4	100 mg
	pH	6.6
NH_4OH	: 0.3 ml in 100 ml distilled water.	

Procedure

Separated out the bone marrow cells and made in to a single cell suspension. Centrifuged and removed supernatant. Made a thick smear on a glass slide. Fixed it using a fixative solution for 1-2 min at 4-10^{0}C. To prepare reaction mix; prepared solution I by mixing 44.5 ml phosphate buffer and 2.5 ml solution C, kept for 45 min. Prepared solution II in the 44th minute; mixed 1.2 ml of solution A and 1.2 ml of solution B, kept for one minute. Mixed solutions I and II. Poured that solution mixture to a petridish in which the fixed smear was put and kept for 15 min. Washed it with distilled water and counter stained with haematoxylin for one minute. If excessively stained placed the slide in 0.3 % ammonium hydroxide. Allowed to dry and observed under the microscope. A total of 4000 cells were counted in triplicates and number of cells positive for esterase were counted.

3.14. ANTI-TUMOUR ACTIVITY

3.14.1. Tumour Cell Line and their Maintenance

Dalton's Lymphoma ascites (DLA) tumour cell lines were provided by Amala Cancer Research Institute, Thrissur. The tumour cell line was maintained by serial intraperitoneal (i.p.) transplantation in mice. Full grown tumour cell lines were aspirated from the mouse peritoneum, washed thrice with PBS and suspended in PBS. About 1x10^6 cells were injected intraperitonealy into a new healthy mouse.

3.14.2. Assay of Direct Cytotoxic Activity *In Vitro*

The *in vitro* cytotoxicity of astaxanthin was assayed using DLA cell lines. The ascitic tumour cells (1 x 10^6 cells) were suspended in 0.5 ml of PBS containing 7.35 µg, 14.7 µg, 29.4 µg of astaxanthin and were incubated at 37^0C for 8 h in moist air containing 5% CO_2. The viability of cells at 2, 4, 6 & 8 h were assayed by Trypan-blue exclusion method (Talwar, 1974).

3.14.3. Assay of Antitumour Activity *In Vivo*

Antitumour activity of the extracts was determined using ascites and solid tumour models.

3.14.3.1. Ascite Tumour Model

Male Balb/c mice weighing 20 $\pm$ 5 g were used for the study. The animals were grouped into four groups consisting of six mice in each group. DLA cells (1 x 10^6 cells/animal) in PBS were transplanted as intraperitoneal injections to each mouse in all groups. The group 1 administered with DLA alone was taken as positive control. Astaxanthin was given as intraperitoneal injections on the 2^{nd} & 4^{th} day after tumour transplantation to group 2, group 3 and group 4 at concentrations 0.5 mg astaxanthin/Kg body weight, 1.0 mg/Kg and 5mg/Kg respectively. The animals were fed on normal diet and water *ad libitum*. Mortality rate will be observed as percent increase in life span (ILS). It is calculated using the formula (Ahluwalia *et al.*, 1984).

$$\% \text{ ILS} = (1\text{-}T/C) \times 100$$

Where,

T = mean survival time of the astaxanthin treated group

C = mean survival time of the control

The details of treatment given to each group is shown below.

Group 1 (Control group) : 1 x 10^6 cells of DLA

Group 2 (Test group) : 1 x 10^6 cells of DLA (0.5 mg /kg astaxanthin)

Group 3 (Test group) : 1 x 10^6 cells of DLA (1.0 mg /kg astaxanthin)

Group 4 (Test group) : 1 x 10^6 cells of DLA (5 mg /kg astaxanthin)

3.14.3.2. Solid Tumour Model

Male Balb/c mice weighing 20 $\pm$ 5 g were used for the study. The animals were grouped into four groups consisting of six mice in each group. Viable DLA cells (1 x 10^6 cells in 0.1 ml/animal) in PBS were transplanted subcutaneously into the right hind limb of each mouse in all groups. The group 1 administered with DLA alone was taken as positive control. Astaxanthin was given as intraperitoneal injections after 24 h of tumour transplantation to group 2, group 3 and group 4 at concentrations 0.5 mg astaxanthin/Kg body weight, 1.0 mg/Kg and 5mg/Kg respectively and continued for four consecutive days. The development of tumour in each group was measured using vernier calipers twice a week for five weeks and tumour volume was calculated using the formula

$V = (4/3)\ \pi\ a^2\ b/2$ where a is the minor diameter and b is the major diameter (Ma *et al.,* 1991).

The animals were fed on normal diet and water *ad libitum*. At the end of fifth week the animals were sacrificed under diethyl ether anaesthesia and the tumours extirpated and weighed.

The percent inhibition was calculated by the formula (1-B/A) x 100 where A is the average tumour weight of the control group and B is that of the treated group (Chihara *et al.,* 1970).

3.14.4. Assay of Anticancer Activity *In Vitro*

3.14.4.1. Cell Lines Used

HeLa cell lines were procured from National Centre for Cell Science (NCCS), Pune, India

Designation	:	**HeLa**
Tissue	:	Adenocarcinoma, cervix
Growth properties	:	Adherent
Organism	:	Homosapiens
Morphology	:	Epithelial
Metastatic site	:	Cervix

3.14.4.2. Maintenance of Cell Lines

All the cells were routinely maintained in a complete medium which contains DMEM, 10% FBS and antibiotics. The cells were incubated at 37^0C and 5% CO_2 atmosphere. At first, healthy cells were seeded into T-25 cm^2 tissue culture flasks and allowed to become 80% confluent. The cells were then trypsinised and seeded into 96 and 12-well plates for *in vitro* MTT assays. The cells were grown in monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM), (Himedia) containing 10% foetal bovine serum (Himedia) and 1 % antibiotics in a humidified atmosphere of 5% carbon dioxide at 37^0C.

3.14.4.3. Sub Culturing

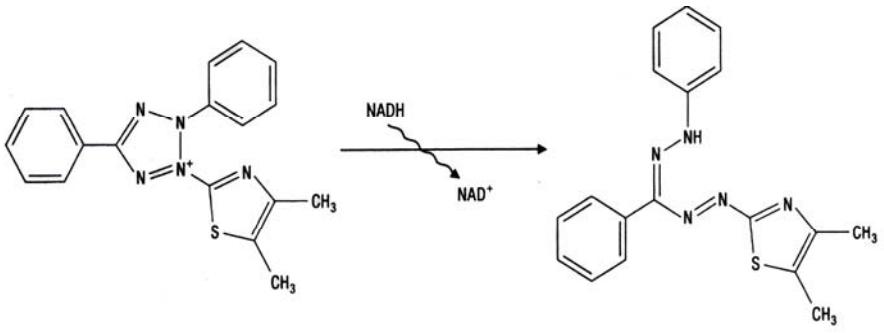
When cells were grown to confluency the medium was removed and washed once with PBS. 0.25% Trypsin -EDTA solution was added, and incubated for 1-2 min. at 37^0C. Fresh medium (with serum) was added and cells were dispensed gently by a pipette. A known number of cells were dispensed into new flasks or microtitre plates for further experiments.

3.14.4.4. MTT Cell Proliferation Assay

This assay was done using revised method of Mossmann (1983). The assay is based on the metabolic activities of the viable cells. In this assay a tetrasolium salt MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was used (Fig. 10). MTT is cleaved by succinate dehydrogenase which is present in metabolically active cells, into water insoluble dark blue formazan crystals. These water insoluble formazan crystals can be solubilised using isopropanol or other organic solvents. After it is solubilised, the formazan formed can easily and rapidly be quantified in a microplate reader.

Reagents

- Dulbecco's Modified Eagle's Medium (DMEM) (Himedia, Mumbai)
- 10% FBS (Himedia, Mumbai)
- Streptomycin 100 µg/ml (PAA Laboratories, GmH, Austria)
- Pencillin 100 units/ml (PAA Laboratories, GmH, Austria)
- Amphotericin 2.5 µg/ml (PAA Laboratories, GmH, Austria)
- 0.25% Trypsin – EDTA in phosphate buffered saline (PBS) (PAA Laboratories, GmH, Austria)
- MTT (Sigma Chemicals, USA)



MTT **FORMAZAN**

($C_{18}H_{16}BrN_5S$) **(**3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl -2H-tetrazolium bromide)

Fig. 10. Molecular structure of MTT and its corresponding reaction product

HeLa cells were seeded into a 96-well microtitre plate (5000 cells/well) and incubated for 24 h. The astaxanthin extract was added in different concentrations (5, 7.5, 10, 12.5, 15, 17.5, 20 μg/ml). The 96-well microtitre plates contained 100 μl cell suspensions with the seven concentrations, each with six replicates. Three wells contained cells in astaxanthin free medium to determine the control cell survival and percentage of live cells after culture. The outermost wells were avoided to reduce the drying effect, instead they were filled with PBS for maintenance of humidity. The diluted concentrations were made in the medium. The cells were incubated for 24 h at 37^0C. Then the medium was removed and fresh medium was added along with 20 μl MTT (5 mg/ml) to each well. The plates were incubated for 2 h. The yellowish MTT was reduced to dark coloured formazan by the viable cells. The formazan crystals formed were solubilised with MTT lysis buffer (20% SDS in 50% formamide). The plates were kept for 2 h incubation at 37^0C. The colour developed was quantitated with a microplate reader (BIORAD systems, USA) (Measuring wave length: 570 nm, Reference wave length 630 nm).

The cells survival (CS) is expressed as percentage.

CS = (OD drug exposed well / Mean OD control wells) X 100 $\pm$ SD

LC_{50} (Lethal Concentration for killing 50% of cells seeded) value indicates the effectiveness of the astaxanthin concentration used, the lesser the value, the more effective the pigment is.

3.15. STATISTICAL ANALYSIS

Results are expressed as mean ± SD. The statistical analysis of all the experiments were done using the statistical package SPSS.

4. RESULTS

4.1. PROXIMATE COMPOSITION OF SHRIMP SHELL WASTE

The proximate composition of fresh and dried shrimp shell waste are presented in Table 2. The major components in shrimp shell waste are protein, ash and chitin.

Table 2. Proximate composition of shrimp shell waste of *Aristeus alcocki*, Ramadan, 1938 in fresh and dried conditions, per cent by weight

Samples / Components	Fresh (Mean ± SD)	Dried (Mean ± SD)
Moisture	70.74 ± 0.56	14.57 ± 0.16
Ash	10.36 ± 1.13	29.40 ± 0.69
Chitin	9.80 ± 0.53	23.80 ± 0.80
Lipid	1.03 ± 0.11	2.06 ± 0.02
Protein	5.40 ± 0.28	29.71 ± 0.85

n = 3

4.2. DEPROTEINISATION OF SHRIMP SHELL WASTE

Deproteinisation of shell waste using alkali (KOH) and enzyme (Pancreatin) was carried out. Alkali deproteinsation was more efficient than enzyme deproteinisation. Per cent deproteinisation using alkali for wet and dry waste were 77.68 and 51.53 respectively (Table 3) per cent deproteinisation obtained with enzyme pancreatin for wet and dry waste were 66.61 and 35.34 respectively for wet and dry waste (Table 3) .

Table 3. Extent of deproteinisation of *Aristeus alcockii* shrimp waste using enzyme and alkali, percentage of total protein

Treatment / Sample	Alkali (KOH) (Mean ± SD)	Enzyme (Pancreatin) (Mean ± SD)
Wet	77.68 ± 0.31	66.61 ± 0.21
Dry	51.53 ± 0.25	35.34 ± 0.04

n = 3

4.3. EXTRACTION OF CAROTENOIDS FROM SHRIMP SHELL WASTE

Analysis of variance (Table 4) for extraction yields of carotenoids in different extraction media with different samples showed that the yield of carotenoids differed significantly between extraction solvents, between wet and dry samples and between deproteinised and non deproteinised samples. The mean extraction yields of carotenoids from shrimp shell waste with and without deproteinisation in dry and wet condition are presented in Table 5. The highest carotenoid yield of 87.14 ± 4.55 µg/g (Plate. 5) was obtained with wet non deproteinised waste (Plate. 4) using acetone (Fig. 11). The carotenoid yield from dry samples were significantly lower than those of wet samples. Similarly the carotenoid yields from deproteinised samples were significantly lower than those of non deproteinised samples.

Oil extraction of carotenoids was carried out using coconut oil, soybean and sunflower oil. The carotenoid yield was significantly lower when oil was used as the extraction medium. Of the three oils, coconut oil gave the highest yield (23.05 ± 2.09 µg/g) (Plate. 6).

Another important observation was that for any dry sample (deproteinised or non deproteinised), 90 % acetone was found to be the best solvent for carotenoid extraction (Fig. 12).

Table 4. ANOVA for extraction yields of carotenoids for different samples and different extraction media

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Samples	35309.32	5	7061.86	6035.25*
Extraction medium	8054.48	6	1342.41	1147.26*
Interaction	13206.78	30	440.23	376.22*
Error	147.43	126	1.170	
Total	**56718.01**	**167**		

*Significant at 5 % level Critical difference: 0.284

Table 5. Extraction yields of carotenoids using different solvents and vegetable oils

Extraction Media / Raw material	Ether:acetone: water (µg/g)	Acetone (µg/g)	Hexane: Isopropanol (µg/g)	90% acetone (µg/g)	Coconut oil (µg/g)	Soybean oil (µg/g)	Sunflower oil (µg/g)
Dry (D)	$21.25 \pm 0.86^{a,g}$	$15.74 \pm 0.83^{b,g}$	$20.34 \pm 0.35^{c,g}$	$22.79 \pm 1.33^{d,g}$	$3.32 \pm 0.23^{e,g}$	$1.361 \pm 0.15^{f,g}$	$1.48 \pm 0.20^{f,g}$
EDD	$0.47 \pm 0.86^{a,h}$	$1.15 \pm 0.19^{a,h}$	$0.29 \pm 0.05^{a,h}$	$1.17 \pm 0.13^{a,h}$	ND	$0.65 \pm 0.30^{a,c,g}$	$0.43 \pm 0.04^{c,h}$
ADD	$0.36 \pm 0.04^{a,h}$	$0.74 \pm 0.08^{a,h}$	$0.19 \pm 0.06^{a,h}$	$0.75 \pm 1.00^{a,h}$	$0.22 \pm 0.03^{a,h}$	$0.31 \pm 0.02^{a,h}$	$0.37 \pm 0.07^{a,h}$
Wet (W)	$40.22 \pm 1.15^{a,i}$	$87.14 \pm 4.55^{b,i}$	$70.27 \pm 2.58^{c,i}$	$41.46 \pm 2.51^{d,i}$	$23.05 \pm 2.09^{e,i}$	$20.27 \pm 0.88^{f,i}$	$18.06 \pm 0.82^{g,i}$
EDW	$10.56 \pm 0.23^{a,j}$	$21.43 \pm 1.33^{b,j}$	$12.04 \pm 0.99^{b,j}$	$1.10 \pm 0.18^{c,h}$	$5.07 \pm 0.17^{d,j}$	$3.79 \pm 0.39^{e,j}$	$4.01 \pm 0.61^{f,j}$
ADW	$9.70 \pm 0.75^{a,k}$	$17.38 \pm 0.59^{b,k}$	$9.84 \pm 0.72^{b,k}$	$1.36 \pm 0.31^{c,h}$	$4.51 \pm 0.36^{d,j}$	$2.88 \pm 0.13^{d,k}$	$2.40 \pm 0.21^{e,k}$

Values are mean ± SD of four different estimations. Values having the same superscript in same column and same row are not significantly different at 5 % level. First superscript represents the extraction medium and second supersript represent the sample.

D - Dry Non deproteinised sample

W - Wet Non Deproteinised sample

EDD - Enzyme Deproteinised Dry Sample

EDW - Enzyme Deproteinised Wet Sample

ADD - Alkali Deproteinised Dry Sample

ADW - Alkali Deproteinised Wet Sample

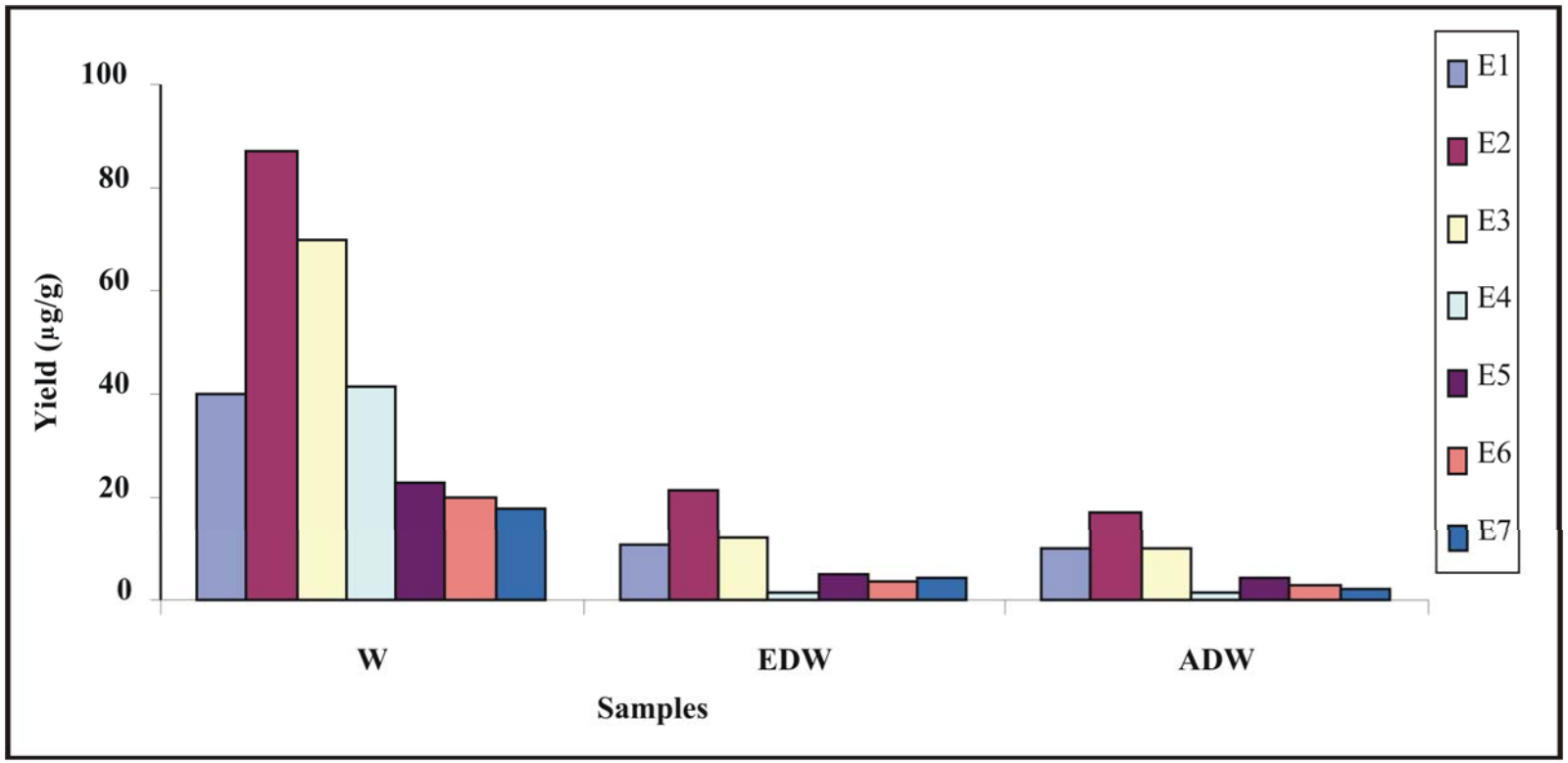


Fig. 11. Extraction yields of wet samples in different extraction media

E1	=	Ether:Acetone:Water (15:75:10 v/v/v)	E2	=	Acetone
E3	=	Hexane:Isopropanol (3:2 v/v)	E4	=	90 % Acetone
E5	=	Cononut Oil	E6	=	Soybean Oil
E7	=	Sunflower Oil			

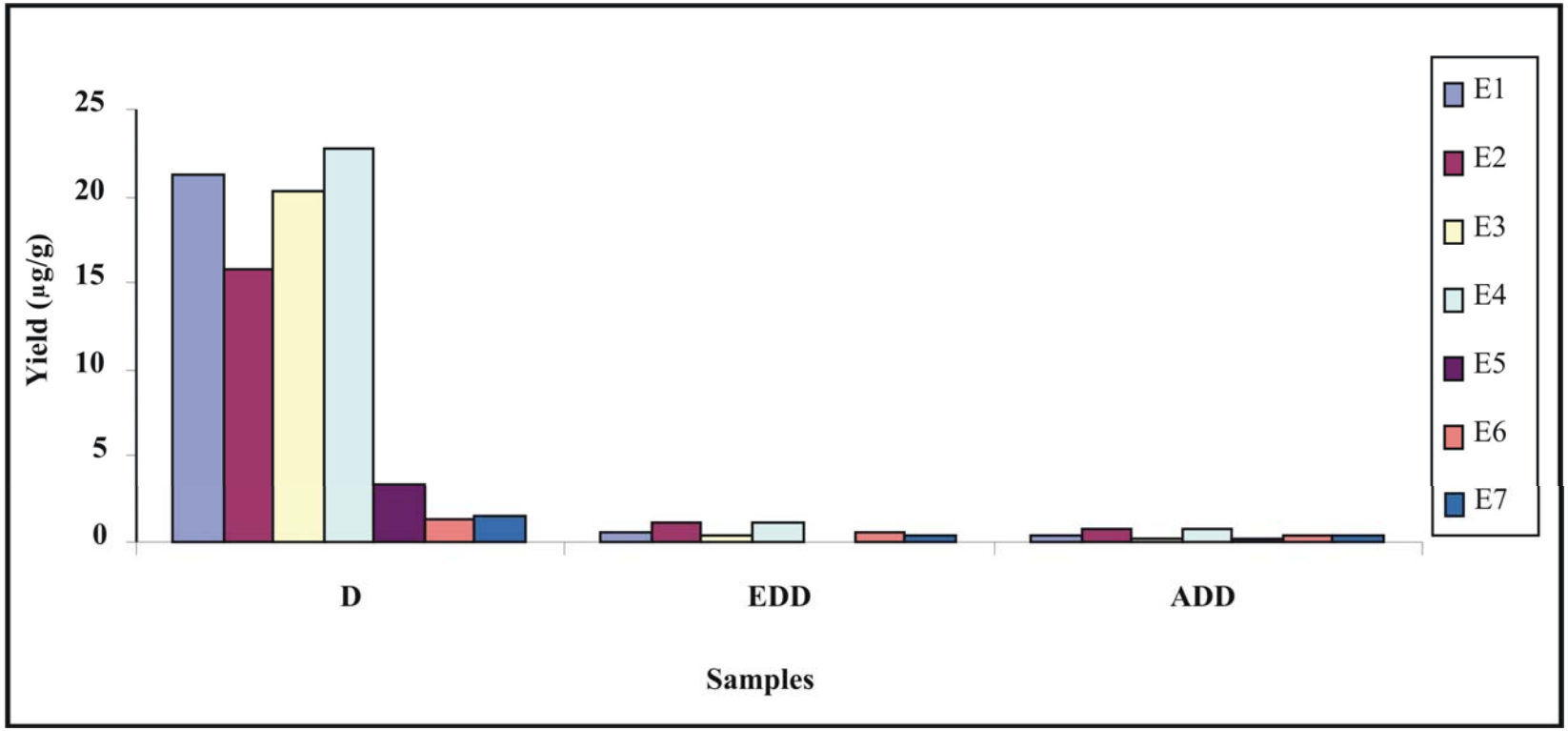


Fig. 12. Extraction yields of dry samples in different extraction media

E1	=	Ether:Acetone:Water (15:75:10 v/v/v)	E2	=	Acetone
E3	=	Hexane:Isopropanol (3:2 v/v)	E4	=	90 % Acetone
E5	=	Cononut Oil	E6	=	Soybean Oil
E7	=	Sunflower Oil			

Plate. 4. Shrimp shell waste of *Aristeus alcocki* before and after extraction of astaxanthin.

Plate.5. Astaxanthin in petroleum ether

Plate.6. Astaxanthin in cococnut oil

4.4. DETERMINATION OF DIFFERENT COMPONENTS IN SHRIMP SHELL WASTE EXTRACT BY THIN LAYER CHROMATOGRAPHY

Thin layer chromatographic separation of carotenoid extracts from *Aristeus alcocki* yielded three distinct bands (Plate. 7). The R_f values for the three bands were respectively 0.33, 0.60, 0.78 (Table 6), which corresponded to astaxanthin, astaxanthin monoester, astaxanthin diester as reported by Kobayashi and Sakamoto (1999). The astaxanthin band was further confirmed by using standard astaxanthin (Sigma Aldrich, USA). Spectrophotometric quantification of three bands showed that the extract contained astaxanthin: astaxanthin monoester: astaxanthin diester in the ratio 1:1:2.

Table 6. R_f value of different carotenoids in the extract from *Aristeus alcocki*, (mean of three determinations)

Carotenoid	R_f value
Astaxanthin diester	0.78
Astaxanthin monoester	0.60
Astaxanthin	0.33

4.5. FATTY ACID COMPOSITION IN ASTAXANTHIN MONOESTER AND ASTAXANTHIN DIESTER

The fatty acid composition in astaxanthin monoester and astaxanthin diester are given in Tables 7 and 8, respectively. Astaxanthin monoester contained 49.29 % saturated fatty acids, 30.43 % monounsaturated fatty acids and 20.28 % PUFAs. Astaxanthin diester contained 41.94 % saturated fatty acids, 29.91 % monounsaturated fatty acids and 29.85 % PUFAs. In the case of monoester, the main fatty acids esterified with astaxanthin were palmitic acid (18.38 %) and oleic acid (14.40 %). The main PUFAs present in the monoester were EPA (4.83%) and DHA (6.58 %). In the case of diester, the main fatty acids esterified with astaxanthin were palmitic acid (20.47 %), oleic acid (18.19 %) and PUFAs: EPA (8.79%) and DHA (11.36%).

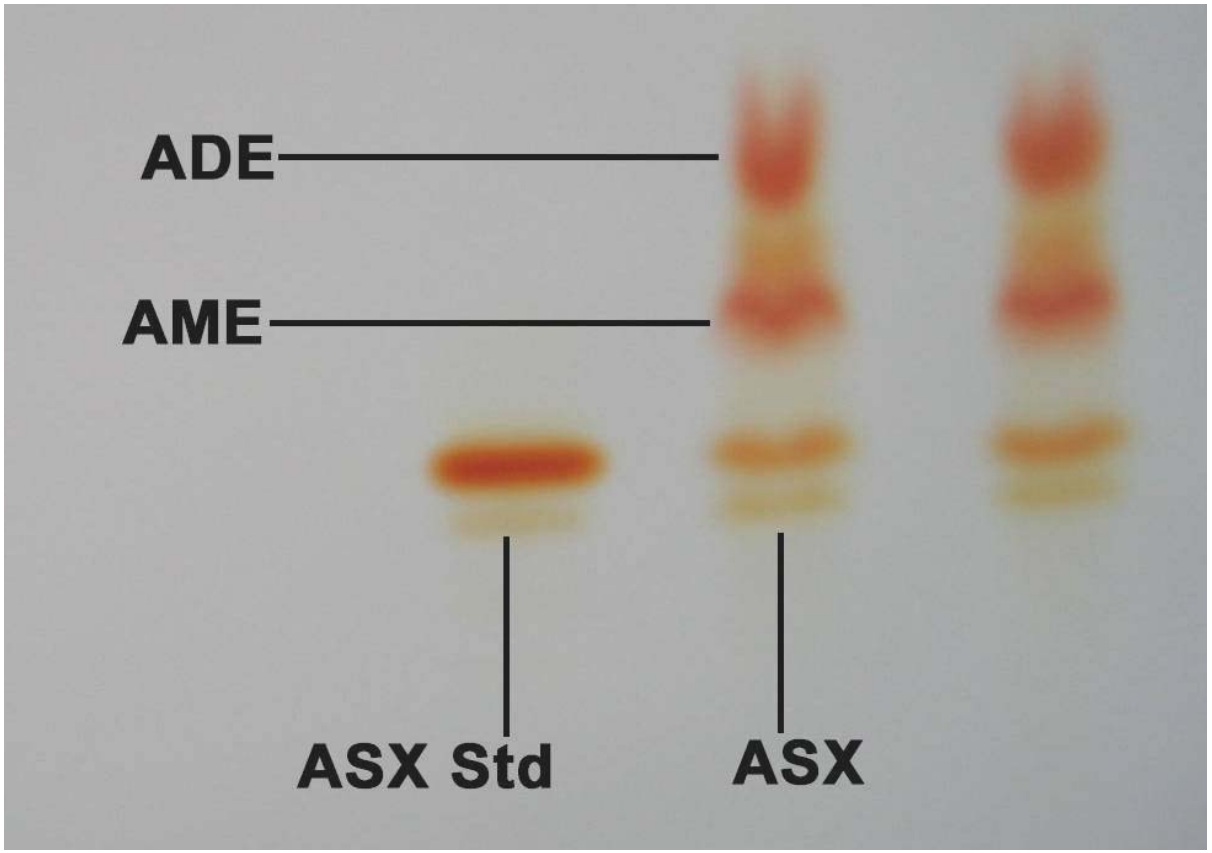


Plate. 7. Thin layer chromatography plate showing astaxanthin, astaxanthin monoester and astaxanthin diester in the carotenoid extract from *Aristeus alcocki* shell waste.

ASX STD – Astaxanthin Standard
AME – Astaxanthin Monoester
ASX – Astaxanthin in the Extract
AME – Astaxanthin Diester

Table 7. Fatty acid composition of astaxanthin monoester, as per cent of fatty acid

Sl.No.	Component	Area %
1	C12	3.002
2	C14	4.161
3	C16	18.382
4	C16:1	2.930
5	C17	0.910
6	C18	9.281
7	C18:1	14.400
8	C18:2	1.236
9	C18:3	1.867
10	C20:1	8.468
11	C20:4	2.844
12	C20:3 & C21	2.922
13	C20:5	4.832
14	C22	7.388
15	C22:1	1.758
16	C22:6	6.577
17	C24	6.165
18	C24:1	2.878

Table 8. Fatty acid composition of astaxanthin diester, as per cent of fatty acid

Sl.No.	Component	Area %
1	C14	2.121
2	C16	20.472
3	C16:1	4.249
4	C17	0.947
5	C18	8.800
6	C18:1	18.197
7	C18:2	2.232
8	C18:3	1.116
9	C20:1	6.333
10	C20:4	3.988
11	C20:3 & C21	1.690
12	C20:5	8.797
13	C22	3.201
14	C22:2	0.665
15	C22:6	11.360
16	C24	4.708
17	C24:1	1.125

4.6. *IN VITRO* ANTIOXIDANT ACTIVITY

4.6.1. Superoxide Anion Scavenging Activity

Astaxanthin extracted from shrimp shell waste showed significant hydroxyl scavenging activity. IC_{50} (50 % inhibiting concentration) value for the shrimp shell extract is 27.91 ± 0.54 ng/ml (Table 9). Astaxanthin possessed

significantly higher activity than quercetin. The results indicate that astaxanthin possessed significant antioxidant activity.

4.6.2. Inhibition of Lipid Peroxidation

Astaxanthin extracted from shrimp shell waste showed significant lipid peroxidation inhibition activity. IC_{50} value for the shrimp shell extract is estimated as 26.54 ± 0.42 ng/ml.(Table 9).

4.6.3. Hydroxyl Radical Scavenging Activity

Astaxanthin extracted from shrimp shell waste showed significant hydroxyl scavenging activity. The shrimp shell extract showed an IC_{50} value of 56.43 ± 1.06 ng/ml (Table 9). Astaxanthin possessed significantly higher activity than catechin. The results indicate that astaxanthin possessed significant antioxidant activity.

Table 9. *In vitro* antioxidant activity of astaxanthin from shrimp shell waste (*Aristeus alcocki)*, IC_{50}

Activity	Astaxanthin (Mean ± SD)	Quercetin (Mean ± SD)	Catechin (Mean ± SD)
Superoxide radical scavenging activity	27.91 ± 0.54 ng/ml	41.21 ± 0.76 µg/ml	-
Inhibition of lipid peroxidation	26.54 ± 0.42 ng/ml	-	432 ±10.2 µg/ml
Hydroxyl radical scavenging activity	56.43 ± 1.06 ng/ml	-	842 ±16 µg/ml

n = 6

4.7. ANTIINFLAMMATORY ACTIVITY

Analysis of variance for increase in paw thickness of Balb/c mice with different treatments (Table 10) showed that the astaxanthin extract from shrimp shell waste significantly reduced carageenan induced paw edema. The reduction in edema was noted in a dose dependent manner. Astaxanthin concentrations at 0.5 mg/kg body weight and 1.0 mg/kg body weight inhibited

the inflammation by 47.83 and 67.11 percent (Table 11). The inhibition of inflammation at 1.0mg/kg body weight was greater than the standard reference drug diclofenac (Fig.13).

Table 10. ANOVA for increase in paw thickness of Balb/c mice in carrageenan induced paw edema

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	164.05	3	54.68	1579.71*
Error	0.69	20	0.035	
Total	**164.74**	**23**		

*Significant at 5 % level Critical difference: 0.12

Table 11. Effect of astaxanthin extract on carageenan induced paw edema

Paw thickness / Treatment	Initial paw thickness (mm)	Paw thickness on 3 h (mm)	Increase in paw thickness (mm)	% inhibition
Control	15.00 ± 0.09	25.37 ± 0.31	10.37 ± 0.13[a]	-
Standard (Diclofenac)	15.66 ± 0.08	20.54 ± 0.24	4.88 ± 0.26[b]	52.94
0.5mg astaxanthin/kg body weight	15.55 ± 0.11	20.96 ± 0.29	5.41 ± 0.18[c]	47.83
1.0mg astaxanthin /kg body weight	15.76 ± 0.09	19.17 ± 0.14	3.41 ± 0.16[d]	67.11

Values expressed as mean ± S.D, n=6 animals, Values having the same superscript in same column are not significantly different at 5 % level.

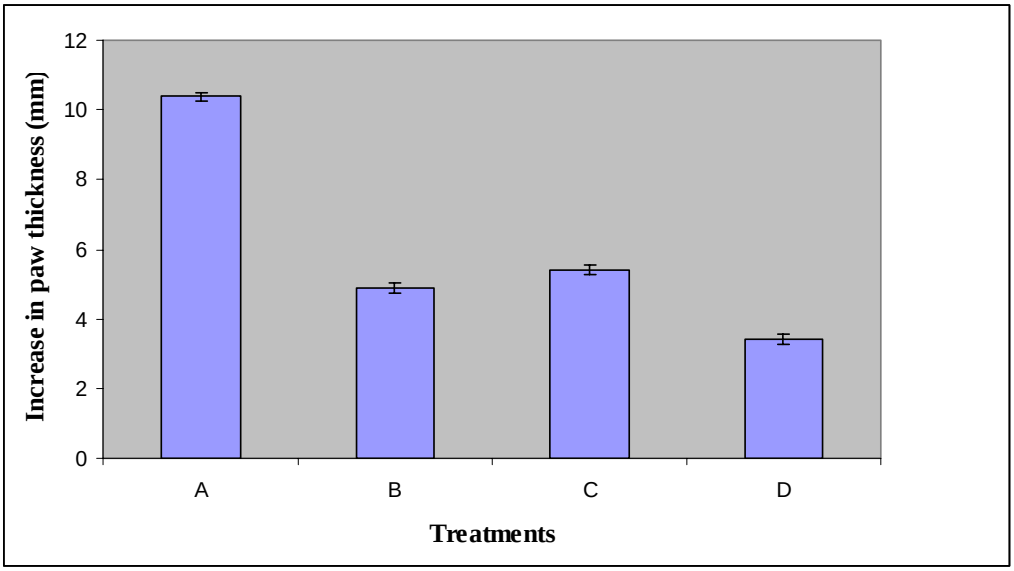


Fig. 13. Effect of astaxanthin extract from *Aristeus alcocki* on carageenan induced paw edema in mice

A = Control

B = Standard (Diclofenac)

C = 0.5 mg/kg body weight astaxanthin

D = 1.0 mg/kg body weight astaxanthin

4.8. CARDIOPROTECTIVE ACTIVITY

4.8.1. Analysis of Cardiac Marker Enzymes

Analysis of variance for the activities of cardiac enzymes in normal and experimental groups showed that there is significant difference in the activities of the enzyme among the different groups (Tables 12, 13, 14, 15 and 16). In the study a significant rise in the activities of cardiac enzymes (LDH, CPK, GPT, GOT and CK-MB) was observed in the serum of Group II isoproterenol-administered rats compared to that of Group I control rats (Table 17). Feeding astaxanthin @ 10mg/kg body weight along with normal diet to the experimental rats of Group IV prior to isoproterenol administration decreased

the activities of these marker enzymes as compared to Group II isoproterenol-injected rats fed on normal diet alone. Activities of enzymes did not show any significant variation between Group I and Group III.

Table 12. ANOVA for comparison of LDH activities in serum of normal and experimental rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	2398661	3	799553.7	3257.54*
Error	4908.93	20	245.4467	
Total	**2403570**	**23**		

*Significant at 5 % level Critical difference: 9.04

Table 13. ANOVA for comparison of CPK activities in serum of normal and experimental rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	4373870	3	1457957	6859.91*
Error	4250.653	20	212.5326	
Total	**4378120**	**23**		

*Significant at 5 % level Critical difference : 8.41

Table 14. ANOVA for comparison of GPT activities in serum of normal and experimental rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	13748	3	4582.66	24.60*
Error	3725.73	20	186.29	
Total	**17473.73**	**23**		

*Significant at 5 % level Critical difference 7.88

Table 15. ANOVA for comparison of GOT activities in serum of normal and experimental rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	17133.51	3	5711.17	20.54*
Error	5559.67	20	277.98	
Total	**22693.18**	**23**		

*Significant at 5 % level Critical difference: 9.62

Table 16. ANOVA for comparison of CK-MB activities in serum of normal and experimental rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	209835.6	3	69945.18	235.92*
Error	5929.334	20	296.4667	
Total	**215764.9**	**23**		

*Significant at 5 % level Critical difference: 9.94

Table 17. Activities of lactate dehydrogenase, creatine kinase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase and creatine kinase MB in serum of normal and experimental rats

Groups / Enzyme	Group I	Group II	Group III	Group IV
	Activity (Units / litre) (Mean ± SD)			
LDH	442.3 ± 13.31	1205.25±15.16#	434.43 ± 16.87	795.36 ± 17.03*
CPK	426.3 ± 8.97	1459.0± 22.57#	431.06 ± 5.93	941.87 ± 14.99*
GPT	25.24 ± 8.01	84.69 ± 22.35#	27.10 ± 7.77	42.87 ± 11.00*
GOT	84.26 ± 8.24	148.14 ± 30.0#	81.42± 8.01	108.0 ± 10.02*
CKMB	112.82±10.0	336.45±17.4#	108.56±25.11	222.16±12.47*

n = 6

Group I and III, normal controls, rats fed on normal diet and normal diet containing astaxanthin @ 10mg/kg, respectively, for a period of 45 days; Group II and IV, myocardial infarction was induced by isoproterenol administration after 45 days of feeding with normal diet and normal diet containing astaxanthin @ 10mg/kg

*Significant at 5% level as compared to Group II

Significant at 5% level as compared to Group I

4.8.2. Assay of Na^+-K^+ ATPase Activity (EC 3.6.1.37)

Analysis of variance for activities of Na^+-K^+ ATPase in heart tissues of normal and experimental groups of rats (Table 18) showed that there is significant difference in the activities of the enzyme among the different groups. The reduction in activity in Group II was significant compared to Group IV. Mean activities of Na^+-K^+ ATPase in the heart tissues of normal and experimental rats are presented in Table 19. A significant reduction in the activities of the enzyme was noted with isoproterenol administration when compared to control. Prior administration with astaxanthin helped to maintain the activities of these enzymes to normal levels in Group IV compared to Group II. Activities of enzymes did not show any significant variation between Group I and Group III.

Table 18. ANOVA for comparison of Na^+-K^+ ATPase activities in heart tissues of normal and experimental groups of rats.

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	25.47	3	8.49	50.44*
Error	3.36	20	0.16	
Total	**28.84**	**23**		

*Significant at 5 % level Critical difference: 0.23

Table 19. Activities of Na^+-K^+ ATPase in heart tissues of normal and experimental groups of rats.

Groups / Enzyme	Group I (Mean±SD)	Group II (Mean±SD)	Group III (Mean±SD)	Group IV (Mean±SD)
Na^+-K^+ ATPase (µg of P liberated h^{-1} mg^{-1} protein)	5.99 ±0.47	3.814 ± 0.29#	6.15 ± 0.55	4.3 ± 0.13*

n = 6

Group I and III, normal controls, rats fed on normal diet and normal diet containing astaxanthin @ 10mg/kg, respectively, for a period of 45 days; Group II and IV, myocardial infarction was induced by isoproterenol administration after 45 days of feeding with normal diet and normal diet containing astaxanthin @ 10mg/kg

*Significant at 5% level as compared to Group II

Significant at 5% level as compared to Group I

4.8.3. Assay of Antioxidant Enzymes

Analysis of variance (Table 20, 21, 22, 23 and 24.) for the antioxidant enzymes showed that there is significant difference in the activities of the enzyme among the different groups. The activities of these enzymes were significantly lower in the heart tissues of Group II rats as compared to Group I normal control rats. Activities of glutathione dependent antioxidative enzymes GPx, GST, GR and antiperoxidative enzymes CAT and SOD are presented in Table 25. Astaxanthin supplementation @ 10mg/kg body weight helped to maintain the activities of these enzymes to normal levels in Group IV compared to group II.Activities of enzymes did not show any significant variation between Group I and Group III.

Table 20. ANOVA for comparison of GPx activities in the heart tissues of normal and experimental groups of rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	19.32	3	6.44	50.92*
Error	2.53	20	0.13	
Total	**21.85**	**23**		

*Significant at 5 % level Critical difference: 0.205

Table 21. ANOVA for comparison of GST activities in the heart tissues of normal and experimental groups of rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	2775964	3	925321.4	44.28*
Error	417908.5	20	20895.43	
Total	**3193873**	**23**		

*Significant at 5 % level Critical difference 83.45

Table 22. ANOVA for comparison of GR activities in the heart tissues of normal and experimental groups of rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	16.97	3	5.65	76.22*
Error	1.18	20	0.07	
Total	**18.15**	**23**		

*Significant at 5 % level Critical difference 0.15

Table 23. ANOVA for comparison of CAT activities in the heart tissues of normal and experimental groups of rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	80.40	3	26.80	108.52*
Error	4.94	20	0.24	
Total	**85.34**	**23**		

*Significant at 5 % level Critical difference: 0.28

Table 24. ANOVA for comparison of SOD activities in the heart tissues of normal and experimental groups of rats

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	15.56	3	5.18	107.13*
Error	0.97	20	0.04	
Total	**16.53**	**23**		

*Significant at 5 % level Critical difference: 0.12

Table 25. Activities of glutathione peroxidase (GPx), glutathione-S-Transferase (GST), glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD) in the heart tissues of normal and experimental groups of rats.

Group / Enzyme	Group I (Mean ± SD)	Group II (Mean ± SD)	Group III (Mean ± SD)	Group IV (Mean ± SD)
GPx (µg of GSH oxidized min^{-1} mg^{-1} protein)	6.28 ± 0.41	4.14 ± 0.22#	6.36 ± 0.36	5.38 ± 0.40*
GST (µ mol 1-chloro-2,4-dinitrobenzene conjugate formed min^{-1} mg^{-1} protein)	1580 ± 176	776 ± 91#	1565 ± 179	1070 ± 111*
GR (m mols of NADPH min^{-1} mg^{-1} protein)	7.46 ± 0.18	5.34 ± 0.42#	7.58 ± 0.16	6.96 ± 0.13*
CAT (n mol H_2O_2 decomposed min^{-1} mg^{-1} protein)	8.28 ± 0.63	3.76 ± 0.27#	8.10 ± 0.56	7.40 ± 0.45*
SOD (milliunits mg^{-1} protein)	3.48 ± 0.28	1.48 ± 0.14#	3.41 ± 0.23	3.01 ± 0.19*

n = 6

Group I and III, normal controls, rats fed on normal diet and normal diet containing astaxanthin @ 10mg/kg, respectively, for a period of 45 days; Group II and IV, myocardial infarction was induced by isoproterenol administration after 45 days of feeding with normal diet and normal diet containing astaxanthin @ 10mg/kg

*Significant at 5% level as compared to Group II

Significant at 5% level as compared to Group I

4.8.4. Assay of Antioxidants

Analysis of variance of (Table 26.) ascorbic acid levels in different groups showed that there is significant difference in the levels of ascorbic acid among the different groups. A significant reduction in the level of ascorbic acid in the heart tissues of Group II animals as compared to the Group I normal control was noticed. Similar results were observed with the levels of GSH (Table 27) in normal and experimental groups of rats. Mean levels of reduced glutathione and ascorbic acid in the heart tissue of experimental and control rats are presented in Table 28. A significant reduction was seen in the level of ascorbic acid in the heart tissues of Group II animals as compared to the Group I normal control. Prior administration of astaxanthin @ 10mg/kg body weight maintained the levels of GSH and ascorbic acid to near normal values in Group IV compared to Group II. Levels of ascorbic acid and GSH did not show any significant variation between Group I and Group III.

Table 26. ANOVA for ascorbic acid levels in the heart tissues of normal and experimental groups of rats.

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	219.21	3	73.07	11.13*
Error	131.20	20	6.56	
Total	**350.41**	**23**		

*Significant at 5 % level Critical difference: 1.47

Table 27. ANOVA for GSH levels in the heart tissues of normal and experimental groups of rats.

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	7540.17	3	2513.39	1229.19*
Error	40.89	20	2.04	
Total	**7581.06**	**23**		

*Significant at 5 % level Critical difference: 0.82

Table 28. Levels of ascorbic acid and glutathione in the heart tissues of normal and experimental groups of rats.

Group / Antioxidant	Group I (Mean±SD)	Group II (Mean±SD)	Group III (Mean±SD)	Group IV (Mean±SD)
Ascorbic acid (mg/100g tissue)	125.55 ± 3.51	118.91 ±1.11#	126.33 ± 3.17	121.49 ± 1.59*
Glutathione (mg/100g tissue)	220.89 ± 2.04	180.78 ± 0.70#	221.58 ± 1.67	192.90 ± 0.84*

n = 6

Group I and III, normal controls, rats fed on normal diet and normal diet containing astaxanthin @ 10mg/kg, respectively, for a period of 45 days; Group II and IV, myocardial infarction was induced by isoproterenol administration after 45 days of feeding with normal diet and normal diet containing astaxanthin @ 10mg/kg

*Significant at 5% level as compared to Group II

Significant at 5% level as compared to Group I

4.8.5. Estimation of Lipid Peroxidation Products

Analysis of variance (Table 29) for the levels of malondialdehyde in the heart tissues of normal and experimental groups of rats showed that there is significant difference in the levels of MDA among the different groups. A significant rise in the level of lipid peroxides in Group II animals as compared to Group I was noticed. Mean levels of lipid peroxidation product MDA in the heart tissue of normal and experimental rats are presented in Table 30. The rats fed on astaxanthin containing diet *ie*. Group IV showed a reduction in the levels of MDA as compared to Group II. Levels of MDA did not show any significant variation between Group I and Group III.

Table 29. ANOVA for MDA levels in the heart tissues of normal and experimental groups of rats.

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	15.56	3	5.18	107.13*
Error	0.97	20	0.048	
Total	**16.53**	**23**		

*Significant at 5 % level Critical difference: 0.03

Table 30. Levels of malondialdehyde in the heart tissues of normal and experimental groups of rats.

Group / Parameter	Group I	Group II	Group III	Group IV
Malondialdehyde (nmol/100g tissue)	0.92 ± 0.06	1.87 ± 0.08#	0.91 ± 0.04	1.60 ± 0.05*

n = 6

Group I and III, normal controls, rats fed on normal diet and normal diet containing astaxanthin @ 10mg/kg, respectively, for a period of 45 days; Group II and IV, myocardial infarction was induced by isoproterenol administration after 45 days of feeding with normal diet and normal diet containing astaxanthin @ 10mg/kg

*Significant at 5% level as compared to Group II

Significant at 5% level as compared to Group I

4.9. IMMUNOSTIMULATORY EFFECT

4.9.1. Splenic T-lympocyte Mitogen Response

Student's t test for the proliferation of spleen cells in the absence and presence of mitogen showed that in the absence of mitogen, there was no significant difference in the OD between the control and the test group (Table 31). The mean observations in the present study are shown in the Table 33. The effect of feeding astaxanthin @ 10mg/kg feed for a period of 45 days showed a two fold increase in the OD when the spleen cells of mice are stimulated with PHA, when compared to the control group receiving no astaxanthin.

Table. 31. t test for the proliferation of spleen cells in the absence and presence of mitogen

Source of variation	Degrees of freedom	t-critical	t-value
Spleen cells without mitogen	18	2.10	0.72
Spleen cells PHA (5µg/ml)	18	2.10	8.18*

4.9.2. Bone Marrow Cell Proliferation Assay

The results of student's t test also showed that there was a significant increase in the proliferation of bone-marrow cells of rats fed on astaxanthin @ 10mg/kg feed compared to the control (Table 32). Bone marrow cells of astaxanthin fed group showed a four times increase in proliferation as indicated by the results (Table 33). Rate of proliferation is not affected by mitogen treatment as the bone marrow cells did not have any mature T-cells.

Table. 32. t test for the proliferation of bone marrow cells in the absence and presence of mitogen

Source of variation	Degrees of freedom	t-critical	t-value
Bone marrow cells without mitogen	18	2.10	5.84*
Bone marrow cells PHA (5µg/ml)	18	2.10	11.69*

Table 33. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on splenic T-lymphocyte mitogen response and bone marrow cell proliferation assay

Type of cells / Treatment	Spleen cells(MeanOD ± SD)		Bone marrow cells (MeanOD ± SD)	
	No mitogen	PHA (5µg/ml)	No mitogen	PHA (5µg/ml)
Control	0.131 ± 0.04	0.23 ± 0.11	0.14 ± 0.01	0.15 ± 0.01
Treatment	0.141 ± 0.02	0.56 ± 0.08*	0.58 ±0.25*	0.63 ± 0.12*

* P<0.05, n =10

4.9.3. Plaque Formation Cell Assay

Student's t test for the number of plaque forming cells showed that there was a significant increase in the number of plaque forming cells in mice fed on astaxanthin @ 10mg/kg feed (Table 34). The plaque forming cells were maximum on the 5th day and it was more than double that of the control (Table 35).

Table. 34. t test for the number of plaque forming cells on 5th and 10th day

Source of variation	Degrees of freedom	t-critical	t-value
5th day	10	2.22	39.95*
10th day	10	2.22	26.22*

Table 35. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on plaque forming cells

No.of PFCs / Treatment	No of PFCs / million spleen cells on (Mean ± SD)	
	5th day	10th day
Control	177 ± 9	157 ± 5
Test	440 ± 138*	358 ± 18*

* P<0.05, n = 6

4.9.4. Circulating Antibody Titre

There was a 16 times increase in the circulating antibody titre in the serum of test animals (Table 36) on 5th day following immunisation.

Table 36. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on circulating antibody titre

Treatment	Circulating antibody titre (Mean ± SD)	
	3rd day	5th day
Control	16	16
Test	64	256

n = 6

4.9.5. Alpha-naphthyl Acetate Esterase Activity in Bone Marrow Cells

Results on the effect of feeding astaxanthin to mice on esterase activity is presented in Table 37. The group fed on astaxanthin showed an increase in the number of cells with esterase activity. The percentage increase in the cells positive to esterase was 28 % compared to control.

Table 37. Effect of feeding astaxanthin extracted from shrimp shell waste of *Aristeus alcocki* on esterase activity in bone marrow cells

No. of cells / Treatment	Cells with positive staining in total of 4000 cells (Mean ± SD)	% increase
Control	616	-
Test	788	28

n = 3

4.10. ANTITUMOUR ACTIVITY

4.10.1. Direct Cytotoxicity

Results of the present study indicate that astaxanthin extract shows direct cytotoxic activity against DLA. The results show that in a dose dependent manner astaxanthin show *in vitro* cytotoxicity. The % viability of the DLA cells were found to be 14.58% at 2 h at a concentration of 7.35µg astaxanthin/ml and at higher concentration of 15µg astaxanthin/ml the % viability was reduced to 4.34 % (Table 38). At higher concentrations no viable cells were found. The same effect was noted with lower concentrations of astaxanthin for a duration of more than 2 h.

Table 38. Antitumour activity of astaxanthin extract from shell waste of *Aristeus alcocki* against DLA *in vitro*

Viability / Treatments	% Viability		
	0 h	**2 h**	**4 h**
Control	100	100	100
7.35 µg astaxanthin/ml	100	14.58 %	0
15 µg astaxanthin/ml	100	4.34 %	0
30 µg astaxanthin/ml	100	0	0

4.10.2. Ascite Tumour

Antitumour activity was exhibited in a dose dependent manner. Analysis of variance (Table 39) for increase in life span of Balb/c mice showed that astaxanthin extract from shrimp shell waste showed significant antitumour activity against DLA induced ascite tumour. Astaxanthin increased the life span from 15 days to 25 days. This accounted for 67 % increase in the life span in DLA bearing mice (Table 40). This significant increase in life span was noted at a dose of 5 mg astaxanthin/kg body weight. At astaxanthin concentrations of 0.5 mg/kg and 1.0 mg/kg also a proportionate increase in life span was noted.

Table 39. ANOVA for increase in life span of Balb/c mice in the DLA induced ascites tumour

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	319.70	3	106.56	69.29*
Error	30.75	20	1.53	
Total	350.45	**23**		

*Significant at 5 % level Critical difference: 0.71

Table 40. Antitumour activity of astaxanthin extract of *Aristeus alcocki* on ascites tumour in Balb/c mice

Groups	Treatment	Life span in days* (Mean ± SD)	% increase in life span
Group I	Control	15.10 ± 0.75^{a}	-
Group II	0.5 mg astaxanthin /kg body weight	19.20 ± 1.94^{b}	27.2
Group III	1.0 mg astaxanthin /kg body weight	21.45 ± 1.11^{c}	42.1
Group IV	5 mg astaxanthin /kg body weight	25.20 ± 0.75^{d}	66.9

n = 6

*Mean life span of mice dying within 30 days of transplantation of tumour cells. Values in the same column with non-identical superscripts are significantly different, $P < 0.05$.

4.10.3. Solid Tumour

Analysis of variance (Table 41 and Table 42) for decrease in tumour volume and tumour growth showed that astaxanthin extract from shrimp shell waste possesed significant antitumour activity against DLA induced solid

tumour. Reduction in tumour development was exhibited in a dose dependent manner. The extract at a concentration of 0.5mg/kg body weight prevented the tumour development by 49.21 % with respect to control and the extract at a higher concentration of 5mg/kg body weight prevented the tumour development by 82.55 % with respect to control (Table 43).

Table 41. ANOVA for decrease in tumour volume of Balb/c mice in the DLA induced solid tumour

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	104.07	3	34.69	246.56*
Error	2.81	20	0.14	
Total	**106.88**	**23**		

Significant at 5 % level Critical difference: 0.21

Table 42. ANOVA for decrease in tumour weight of Balb/c mice in the DLA induced solid tumour

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between groups	157.08	3	52.36	455.46*
Error	2.30	20	0.11	
Total	**159.38**	**23**		

Significant at 5 % level Critical difference: 0.19

Table 43. Antitumour activity of astaxanthin extract of *Aristeus alcocki* on solid tumour in Balb/c mice

Tumour Measurements / Groups	Treatment	Volume on 5th week (cm^3)	% decrease in tumour volume	Weight of tumour (g)	% decrease in tumour weight
Group I	Control	6.85 ± 1.1^{a}	-	8.25 ± 0.98^{a}	-
Group II	0.5mg astaxanthin /kg body weight	3.42 ± 0.53^{b}	50.10	4.19 ± 0.25^{b}	49.21
Group III	1.0mg astaxanthin /kg body weight	2.46 ± 0.57^{c}	64.08	2.73 ± 0.18^{c}	66.90
Group IV	5 mg astaxanthin /kg body weight	1.25 ± 0.32^{d}	81.75	1.44 ± 0.21^{d}	82.55

Values are mean ± SD of six values. Values in the same column with non-identical superscripts are significantly different, $P < 0.05$

4.11. ANTI- CANCER PROPERTY

To quantify the effects of the astaxanthin on cell growth, cell viability was first assayed by reduction of MTT at 24 h after the addition of control medium or various concentrations of the astaxanthin. HeLa cells exhibited a clear dose dependent decrease in cell viability (Plate.8) upon treatment with astaxanthin (Table 44). As the dose increased to a maximum (20 µg/ml), the percentage viability decreased to 9.63%. Astaxanthin exhibited a strong cytotoxicity on HeLa cells and the LC_{50} value was found to be 9.379 µg/ml (Fig. 14).

Table. 44. Anticancer property of astaxanthin extract of *Aristeus alcocki* on cervical cancer cell line HeLa, % cell survival

Concentration of astaxanthin (µg/ml)	Cell survival (%)
0	100
5.0	75.56 ± 9.74
7.5	57.04 ± 5.20
10.0	54.07 ± 6.54
12.5	50.37 ± 2.30
15.0	41.48 ± 7.26
17.5	23.85 ± 3.79
20.0	9.63 ± 3.35

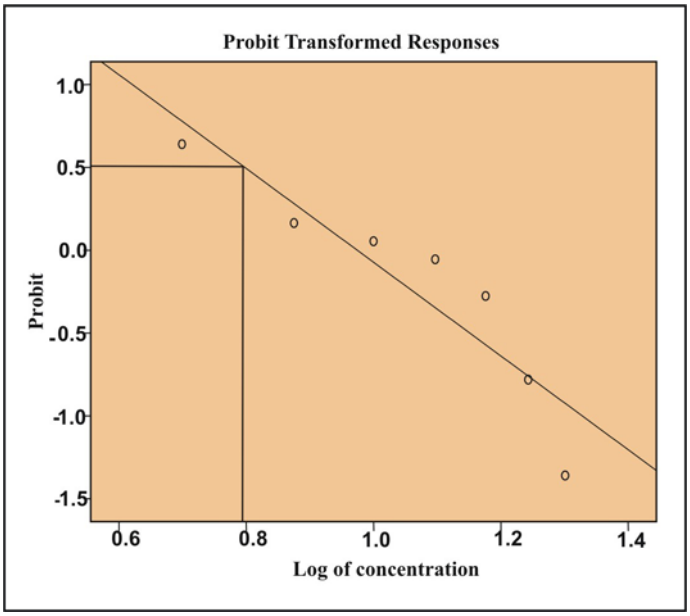


Fig. 14. Bioassay analysis

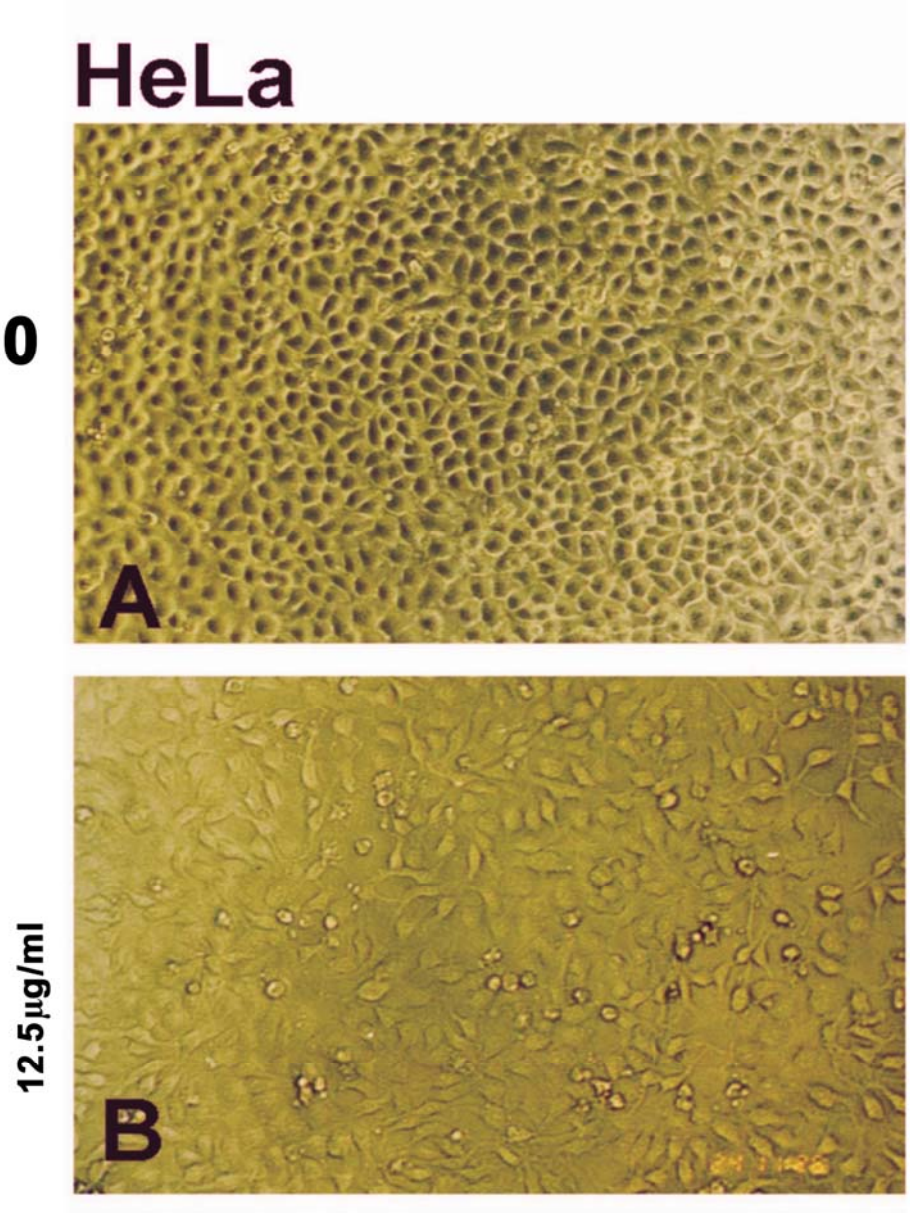


Plate. 8. Effects of different concentrations of astaxanthin on cervical cancer cell HeLa after 24 h

A - (Control) Untreated HeLa cells

B - HeLa cells treated with 12.5 µg/ml Astaxanthin

5. DISCUSSION

5.1. PROXIMATE COMPOSITION OF SHRIMP SHELL WASTE

Proximate composition of shrimp shell waste vary with species and many other factors. Nair *et al.* (2002), reported proximate composition of prawn waste as 75-80 % moisture, 30-35 % ash (dry basis), 35-40 % protein (dry basis), 15-20 % chitin (dry basis) and 3-5 % fat (dry basis). The values observed in the present study also correlates with the above values and also with the values reported by Madhavan and Nair (1975).

5.2. EXTRACTION OF CAROTENOIDS FROM SHRIMP SHELL WASTE

The raw material used for the present study was shrimp shell wastes of deep sea shrimp *Aristeus alcocki* commonly called as Scarlet shrimp or Arabian red shrimp. To ensure maximum yield of carotenoid pigments this deep sea species was selected. Sachindra *et al.* (2006b) reported that the yield of waste (head and carapace) from processing of the deep sea shrimps *Aristeus alcocki* and *Solonocera indica* ranged from 62.6-65.6%. This deep sea shrimp *Aristeus alcocki* is marketed in seafood industry under the trade name "Red Ring" because of the deep pinkish hue of the product. The major product styles are peeled and undeveined (PUD) and headless (HL). It is a demersal marine species which dwells at a depth of 270-1086 m. Bright colours and colour luminescence are adaptations for camouflage of deep sea fishes. Deep sea species are generally red or dark coloured that hide them against background by reflecting very little background light at depths (Kapoor and Khanna, 2004;Gupta and Gupta,2006). Carotenoid pigments are a minor but important component of shell waste. In marine crustaceans astaxanthin and its esters are the major carotenoid pigments present. (Sahidi *et al.*, 1992). Common sources of natural astaxanthin are the green algae *Haematococcus pluvialis* , the red yeast, *Phaffia rhodozyma* , as well as crustacean byproducts(Higuera-Ciapara *et al.*, 2006).

The carotenoid astaxanthin was quantified in the shrimp shell extract of *Aristeus alcocki* in the present study at 470 nm in hexane. Cianci *et al.* 2001 has reported that astaxanthin has typical absorption maxima at 472 nm in hexane. Meyers and Bligh, (1981) has reported that solvent extracted astaxanthin was quantified by absorbance at 470 nm. The total carotenoids extracted from shrimp waste of *Xiphopenaeus kroyeri* were refered as astaxanthin by Holanda and Netto, 2006.

In the present study maximum yield of carotenoids was obtained with wet waste using acetone. Vimla and Paul (2009) has reported that maximum yield of carotenoids from *Penaeus monodon* waste was obtained with acetone compared to other solvents used for extraction. Britton (1985) has suggested the use of water miscible polar organic solvents usually acetone, methanol or ethanol, for extraction of carotenoids from tissues containing water. The use of polar solvents are generally good extraction media for xanthophylls whereas non polar solvents are not recommended as their penetration through the hydrophobic mass that surrounds the pigment is limited (Delgodo-Vargus *et al.,* 2000). De Ritter and Purcell (1981) postulated that complete extraction of carotenoids from plant tissues could be achieved with samples of low moisture content by use of slightly polar and non polar solvents. Use of organic solvents for recovery of carotenoids from shrimp waste has been limited to analytical purposes only (Britton, 1985; Meyers and Bligh, 1981).

Sachindra *et al.* (2006a) studied the recovery of carotenoids from shrimp waste in organic solvents. They have reported that a 50:50 mixture of isopropyl alcohol and hexane gave the highest yield (43.91µg/gwet waste) of carotenoid compared to acetone (40.60 µg/g wet waste). On the contrary in the present study highest carotenoid yield was obtained with acetone (87.14 µg/g) when compared to 60:40 mixture of hexane : isopropanol (70.27µg/g). This difference may be due to difference in species or due to high polarity of acetone.

Holanda and Netto (2006) reported that astaxanthin recovery from shrimp waste using a mixture of solvents is more efficient than oil extraction. This is in agreement with the present study also, but their report that enzymatic hydrolysis of shrimp shell waste enhanced carotenoid extraction is against the present result. The present study shows that deproteinisation by enzyme or alkali decreases the extraction yield of carotenoids from shrimp shell waste. It is observed that when hydrolysis is carried out, the pigment is getting leached into the medium and thereby decreasing the content of carotenoid in the residue left after the hydrolysis.

Stepnowski *et al.* (2004) studied the recovery of astaxanthin from shrimp waste water using fish scales by scale/astaxanthin sorption process, an effective innovative method of waste water treatment. They have reported that drying of such astaxanthin loaded scales caused significant loss of astaxanthin activity. The present study also showed that on drying the shell colour was found to be bleached and the yield of astaxanthin was found to be very low from dried samples.

Several reports are available on the yield of astaxanthin from different species of deep sea shrimps. Of these, highest yield of 14.8 mg/100 g dry waste was reported by Sahidi and Synowiecki (1991) from the waste of *P. borealis* shrimp. Saito and Reiger (1971) obtained a yield of 11.1 mg of astaxanthin/100 g *S. melantho*. Holanda and Netto (2006) reported that an yield of 9.17 mg astaxanthin/100g dry waste of *Xiphopenaeus kroyeri.* In the present study we obtained an yield of 87.14 μg/g wet waste from *Aristeus alcocki*. This is equivalent to 29.78 mg/100 g dry waste. The present study thus reveals that, of the different species of deep sea shrimps *Aristeus alcocki* shell waste is an excellent source of astaxanthin.

At present *P. borealis* shrimp waste is commercially exploited for the production of natural astaxanthin (M/s Bioprawns, Norway). Since astaxanthin content in *Aristeus alcocki* shell waste is double the content of astaxanthin in *P.*

borealis there is ample scope for exploiting *Aristeus alcocki* shell waste for the commercial production of natural astaxnthin.

5.3. DETERMINATION OF DIFFERENT COMPONENTS IN SHRIMP SHELL WASTE EXTRACT BY THIN LAYER CHROMATOGRAPHY

In the present study TLC of the extract showed three distinct bands of astaxanthin, astaxanthin monoester and astaxanthin diester. The R_f values obtained were 0.33, 0.60 and 0.78, respectively. The R_f values obtained for astaxanthin monoester and astaxanthin diester are in agreement with the results reported by Kobayashi and Sakamoto (1999), 0.60 for astaxanthin monoester and 0.75–0.85 for astaxanthin diester. The R_f value of astaxanthin obtained in the present study is in accordance with the R_f value obtained for the standard astaxanthin and also the R_f value reported by Todd (1998).

Meyers (1993) reported that, in nature esterified forms of astaxanthin are more predominant in the exoskeleton of crustaceans. Either of the esterified forms monoester or diester predominates in shrimp exoskeleton and free astaxanthin occurs mainly in internal organs (Dall, 1995; Manasveta *et al.,* 1993; Okada *et al.,* 1994). Sahidi *et al.* (1998) and Sachindra *et al.*(2005a) have reported that astaxanthin and its esters are the major carotenoids in the marine crustaceans. Coral-Hinostroza and Bjerkang (2002) reported that astaxanthin diesters, astaxanthin monoester and free astaxanthin comprised approximately 70%, 12%, and 10% of total carotenoids, respectively in carotenoids of red crab, Langostilla (*Pleuroncodes planipes*). Breithaupt (2004) observed that homogenous diester astaxanthin was the predominant compound, followed by mixed diester astaxanthin in the carotenoid extract from *Pandalus borealis.* Sachindra *et al.* (2006b) reported that astaxanthin and its mono and diesters (63.5-92.2%) were the major carotenoids in *Aristeus alcocki* and *Solonocera indica,* two important deep sea species from Indian waters. Quantitative study of carotenoid distribution in those species have revealed that the levels of esterified astaxanthin were higher than the free form of astaxanthin and the levels of astaxanthin esters were higher (61.7-70.8%) in *A.*

alcocki compared to *S. indica* (43.8-58.4%). Khanfari *et al.* (2007) also reported that astaxanthin, astaxanthin monoester and diester and β-carotene are the major pigments in *Penaeus semisulcatus* shell extract. Maoka and Akimoto (2008) reported that astaxanthin diester was the major component in the carotenoid extract from the carapace of spiny lobster *Panulirus japonicus.*

Results of the present study revealed that the carotenoid extract from *Aristeus alcocki* shell waste contained astaxanthin, astaxanthin mono and diesters. These were present in the proportion of 1:1:2 showing the predominance of astaxanthin diester.

5.4. FATTY ACID COMPOSITION IN ASTAXANTHIN MONOESTER AND ASTAXANTHIN DIESTER

GLC analysis revealed that astaxanthin monoester contained 49.29 % saturated fatty acids, 30.43 % monounsaturated fatty acids (MUFAs) and 20.28 % PUFAs where as astaxanthin diester contained 41.94 % saturated fatty acids, 29.91 % monounsaturated fatty acids and 29.85 % PUFAs. In monoester, the main fatty acids esterified with astaxanthin were palmitic acid (18.382 %), oleic acid (14.4 %) and poly unsaturated fatty acids (PUFAs): EPA (4.83%) and DHA (6.58%). In diester, the main fatty acids esterified with astaxanthin were palmitic acid (20.472 %), oleic acid (18.197 %), PUFAs *viz.* EPA (8.797%) and DHA (11.36%).The fatty acid profiles are in agreement with the findings of Maoka and Akimoto (2008), who reported that saturated fatty acids constitute 45.55 %, monounsaturated fatty acids constitute 30.25 % and PUFAs 29.3 % in the two monoester fractions of spiny lobster *Panulirus japonicus.* Whereas in astaxanthin diester saturated fatty acids, monounsaturated fatty acids and PUFAs constituted 47.95 %, 22.80% and 22.95 % respectively.

Crustaceans accumulated carotenoid in both free and esterified forms and esterification increased the stability of carotenoid in the lipid matrix. Maoka and Akimoto (2008) reported that 66 % of carotenoids were esterified in spiny lobster *Panulirus japonicus*. The major fatty acids esterified with

astaxanthin were identified as docosahexaenoicacid (DHA;C22:6), eicosapentaenoic acid (EPA; C20:5), eicosatetraenoic acid (C 20:4), octadecanoic acid (C18:0), octadecenoic acid (C18:1), heptadecanoic acid (C17:0), hexadecanoic acid (C16:0), hexadecenoic acid (C16:1), tetradecanoic acid (C14:0) and dodecanoic acid (C12:0). Among them, stearic acid, oleic acid and palmitoelic acid were found to be major components followed by PUFAs such as DHA, EPA and eicosatetraenoic acid. Similar results were obtained in the present study with palmitic acid, oleic acid and stearic acid being the major fatty acids in mono and diester followed by PUFAs, DHA and EPA. The fatty acid profiles of astaxanthin ester from shrimp *Pandalus borealis* reported by Renstrom and Jensen (1981) and of brown shrimp *Crangon vulgaris* reported by Snauwaert *et al.* (1973) and of spear shrimp shells *Parapenaeopsis hardwickii* (Lin *et al.*, 2005) also supported the findings of present study. In *Pandalus borealis* C12:0, C14:0, C16:0, C16:1, C18:1, C22:1, were the major fatty acids and oleic acid being the predominant one in addition to PUFAs, DHA and EPA. In red crab Langostilla, palmitic acid and oleic acid were predominant in the diester fraction while EPA and DHA were the predominant fatty acids in monoester. Five kinds of fatty acids, dodecanoate, tetradecanoate, hexadecanoate, hexadecenoate and octadecenoate, were detected from astaxanthin monoesters and diesters of krill *Euphausia superba* (Takachi *et al.,* 2003). Sachindra *et al.* (2006b) reported that unsaturated fatty acid constituted 60.5% of the carotenoid extract from head of *A. alcocki*, while saturated fatty acids (83.5 %) were predominant in the carotenoid extract from the carapace of *S. indica.*

The present study thus reveals that the carotenoid extract from *Aristeus alcocki* shell waste is mainly composed of free astaxanthin, astaxanthin monoester and astaxanthin diester in the proportion 1:1:2. The fatty acid composition of the monoester reveals that saturated fatty acids, MUFA and PUFA are in the ratio 5:3:2 whereas in diester they are in the ratio 4:3:3.

5.5. *IN VITRO* ANTIOXIDANT ACTIVITY

In the present study astaxanthin extract from shrimp shell waste showed significant hydroxyl radical scavenging activity, superoxide anion scavenging activity and inhibition of lipid peroxidation. The standard antioxidants quercetin and catechin showed antioxidant activity at microgram levels whereas astaxanthin present in shrimp shell extract showed *in vitro* antioxidant activity at nanogram levels. This clearly indicates the high antioxidant potential of astaxanthin from *Aristeus alcocki* shell waste. This powerful antioxidant function may be due to unique molecular structure of astaxanthin. Natural compounds are excellent singlet oxygen quenchers as well as lipid peroxidation chain breakers. Astaxanthin is such a natural compound whose dual antioxidant capacity may be attributed to the activity of polyene chain. Higher the number of conjugated double bonds along the polyene chain, higher will be the antioxidant activity. Cardounel *et al.* (2003) reports that such compounds can entrap free radicals, neutralize singlet and triplet oxygen. Wang *et al.* (2000) reports that carotenoids can take part in protecting against the damage from free radicals and singlet oxygen reactive species. Sewer *et al.* (1998) showed that astaxanthin has a strong quenching capability against damage from singlet oxygen *in vitro.* This quenching capability has been shown to be 80 times stronger than α-tocopherol and twice as strong as β-carotene (Wang *et al.,* 2000; Sewer *et al.,* 1998). Sewer *et al.* (1998) argued that the strong singlet oxygen quenching capability of astaxanthin is due to to its unique molecular structure, by virtue of which singlet oxygen associated carbon centered radicals of astaxanthin can form more stable resonance structures by the attachment of carbonyl groups and hydroxyl groups to the β-ionone ring of astaxanthin.

Palozza and Krinsky (1992a) have reported that astaxanthin can remove the chain carrying lipid peroxyl radicals in the liposomal suspension more efficiently than β-carotene but less efficiently than α-tocopherol, because the hydrogen bonds by the carbonyl group in the β-ionone ring of astaxanthin and

hydrophobic association by the polyene chain allows AST to exhibit its strong antioxidant effect both *in vitro* and *in vivo.*

Bell *et al.* (2000) in a feeding study with salmon showed that the antioxidant synergism of vitamin E and astaxanthin reduced malondialdehyde formation in an *in vitro* stimulation of microsomal lipid peroxidation. Kurashige *et al.* (1990) and Miki (1991) reported that in rats deprived of vitamin E the resistance of lipids to oxidation was restored by feeding the animals with astaxanthin.

Thus the present study as well as other reports clearly indicate the powerful antioxidant potential of astaxanthin. Oxygen derived free radicals or reactive oxygen species (ROS) formed in the body during energy producing metabolic process, play an important role in pathophysiology of a number of diseases (Cuzzocrea *et al.,* 2001). Normally oxygen free radicals are neutralised by natural antioxidants. However, ROS become a problem when either a decrease in their removal or their overproduction occurs, resulting in oxidative stress. This stress and the resultant damage have been implicated in many diseases and a wealth of preventive drugs and treatments are currently being studied. Thus astaxanthin exhibiting multiple antioxidant activity will find utility in applications like antioxidant therapy, which is based on reducing oxidative stress in the target tissues. Since synthetic astaxanthin is a mixture of all types of isomers astaxanthin from natural sources is preferred for using it as an antioxidant. Astaxanthin from natural sources is abundant in the isomer showing highest biological activity (3R,3'R; 3S 3'S).

Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis* has been reported by Kobayashi and Sakamoto (1999). They showed that astaxanthin esters function as powerful antioxidant agent under both hydrophilic and hydrophobic conditions. The IC_{50} values of astaxanthin diester, astaxanthin monoester and free astaxanthin from *Haematococcus pluvialis* for singlet oxygen quenching activity in 50 % (v/v) hexane in ethanol were 7.4 µg/ml, 8.6 µg /ml and 9.4 µg /ml respectively.

Kamath *et al.*(2008) has reported that the IC_{50} values for free radical scavenging activity of *Haematococcus pluvialis* astaxanthin esters *in vitro* were 8.0 µg /ml. In the present study the *in vitro* IC_{50} values reported for *in vitro* antioxidant activity of astaxanthin from *Aristeus alcocki* shell waste are in the range of ng/ml. This clearly indicates the astaxanthin extract from *Aristeus alcocki* is a more powerful antioxidant agent than the astaxanthin present in the *Haematococcus pluvialis.* This may be due to a higher proportion of astaxanthin diester and a higher content of poly unsaturated fatty acids (20 % PUFAs in monoester and 30 % PUFAs in diester) in the carotenoid extract obtained from *Aristeus alcocki* shell.

5.6. ANTIINFLAMMATORY ACTIVITY

Results of the present investigations reveal that astaxanthin exhibited significant anti-inflammatory activity in acute inflammations in mice in a dose dependent manner. This confers well with the findings of Ohgami *et al.*(2003) where a dose dependent anti-inflammatory effect with astaxanthin was noted by supression of nitric oxide (NO), prostaglandin E2 (PGE2) and tumour necrosis factor (TNF-α) production through directly blocking nitric oxide synthase (NOS) enzyme activity. Carrageenan induced acute inflammation in animals is one of the most suitable test procedures to screen anti-inflammatory agents. Several inflammatory mediators account for the edema formation caused by carageenan injection. The development of carrageenan induced edema is biphasic, the first phase is attributed to the release of histamine, 5-HT and kinins and occurs within an hour of injection and is partly due to the trauma of injection, while, the second phase is related mainly to prostaglandins (PGs) which is measured around 3h (Larsen and Henson, 1983; Vane and Booting, 1987). The increased synthesis of PGs is due to increased release of arachidonic acid from the membrane phospholipids and the up regulation of cyclooxygenase-2 (Subbaramaiah *et al.,* 1997*).* Exposure of outer bacterial toxins stimulates cellular inflammatory responses and releases factors such as nitric oxide (NO) (Chen *et al.,* 2001*;* Boujedaini *et al.,* 2001*)*, prostaglandin E2 (PGE2) (Bellot *et al.*, 1996; Murakami *et al.,* 2000; Hoekzema *et al*., 1992),

cytokines including tumour necrosis factor (TNF-α) that promote inflammatory responses.

Kurashige *et al.*,1990 reported that carrageenan induced swelling of the paw of rats fed with astaxanthin was significantly lower than that of control. This explains the anti-inflammatory role of astaxanthin.

Bennedsen *et al.*(1999) and Wang *et al.* (2000) reported that dietary astaxanthin was found to help fight symptoms of ulcer disease from *Helicobacter pylori* which causes inflammation of gastric tissues. Kim *et al.*(2005) reported that astaxanthin is effective in protection against gastric lesions induced by the use of non steroid anti-inflammatory drugs such as naxopen. Mahmoud *et al.*(2004) reported that suppression of T-cell activation makes astaxanthin as effective as commonly used antihistamines and hence may have a role in novel antiasthmatic formulations.

Lee *et al.* (2003) demonstrated that astaxanthin inhibits NO production and inflammatory gene expression iκB kinase dependent NFκB activation. The present study demonstrates that natural astaxanthin from *Aristeus alcocki* shrimp waste inhibits carrageenan induced inflammatory response in mice. This anti-inflammatory effects of astaxanthin from *Aristeus alcocki* shell has important implications for the development of anti-inflammatory drugs from shrimp shell waste.

5.7. CARDIOPROTECTIVE ACTIVITY

Myocardial infarction (MI) induced by isoproterenol [L-β-(3,4-dihydroxy phenyl)-2-isopropylaminoethanol hydrochloride], a β-adrenergic agonist, has been reported to show many metabolic and morphologic aberrations in the heart tissue of the experimental animals similar to those observed in human MI (Nirmala and Puvanakrishnan, 1996a).

During myocardial infarction the diagnostic cardiac marker enzymes (GOT, GPT, LDH, CPK) are released from damaged heart tissue into the blood. A significant rise in cardiac marker enzyme in serum is noted in the

present study in Group II. This confers well with the findings of Ithayarasi *et al.* (1996), Sathish *et al.* (2002), Farvin *et al.* (2004) and Sophia (2007). Nirmala and Purvanakrishna (1996a) also reported that increased activities of serum creatinine phosphokinase (CPK), lactate dehydrogenase (LDH), glutamate oxaloacetate transaminase (GOT), and increase in corticosteroids, blood urea and nitrogen were some of the changes noted in MI. Feeding astaxanthin @ 10mg/kg body weight along with normal diet to the experimental rats of Group IV prior to isoproterenol administration decreased the activities of these marker enzymes as compared to Group II isoproterenol-injected rats fed on normal diet alone. Astaxanthin is also reported to block GOT, GPT and TBARS levels in a study with rats where liver damage is induced by CCL_4 (Kang *et al.,* 2001).

The loss of membrane integrity is one of the changes noted in MI (Nirmala and Puvanakrishnan, 1996a). Reactive oxygen-derived free radicals play a crucial role in the pathogenesis of isoproterenol-induced MI (Nirmala and Puvanakrishnan, 1996b). The action of astaxanthin can be compared to similar lipophilic agents. Astaxanthin is polar and aligns parallel to the lipid component of the cell membrane facilitating its antioxidant and anti inflammatory effects. Astaxanthin with the moieties of its polyene chain and terminal rings rigidify membranes and modify their permeability (Matsushita *et al.*,2000; Goto *et al.,* 2001; Barros *et al.,* 2001).This property is more evident in its function as an antioxidant agent in both cytoplasmic and membranous regions of cells (Kobayashi and Sakamoto, 1999).

Glutathione and ascorbic acid levels were found to be decreased in isoproterenol administered groups. This is in accordance with the earlier findings of Farvin *et al.* (2004). Glutathione is a scavenger of superoxide radicals, hydroxyl radicals and singlet oxygen. Ascorbic acid is a water soluble antioxidant and readily scavenges reactive oxygen species. It is suggested that reactive oxygen-derived free radicals play a crucial role in the pathogenesis of isoproterenol-induced MI (Nirmala and Puvanakrishnan, 1996b). The

administration of astaxanthin increased the levels of ascorbic acid and GSH in Group IV compared to Group II and this protects the heart membrane from oxidative damage.

Isoproterenol acts as a cardiotoxic agent and causes destruction of heart muscle by increased production of free radicals (Singal *et al.,* 1982; Noronha-Dutra *et al.,* 1984). Astaxanthin supplementation to human subjects may decrease *in vivo* lipid peroxidation in healthy men. Astaxanthin's ability to quench singlet oxygen and scavenge free radicals has been demonstrated by a number of *in vitro* studies (Kurashige *et al.,*1990; Miki, 1991; Lim *et al.,* 1992; Oshima *et al.,* 1993; Jorgensen and Skibsted, 1993; Nakagawa *et al.,* 1997; Woodall *et al.,* 1997).

Malondialdehyde is produced in biological systems by lipid peroxidation. MDA is not the major product of lipid peroxidation, but a degradation product. MDA reacts with nitrogenous bases of DNA to form DNA adducts (Vuillaume, 1987). An increase in MDA levels in the heart tissues of Group II compared to Group IV was observed in the present study. Astaxanthin administration for a period of 45 days decreased the level of lipid peroxidation in group IV.

The mechanism of action of isoproterenol in inducing myocardial necrosis is a multiple step process (Ravichandran *et al.*, 1990). The primary disturbance of isoproterenol-induced MI has been reported to be, enhanced adenylate cyclase activity resulting in increased cAMP formation, which in turn would lead to the higher lipid accumulation in the myocardium (Subhash *et al*., 1978). The antioxidant enzymes *viz.* GPx, GST, GR, CAT, SOD are lower in Group II rats compared to normal controls. This result in the present study is supported by similar observations of Nirmala and Pvanakrishnan (1996a), Ithayarasi *et al.*(1997) and Farvin *et al.* (2004). An increased oxidative stress is noted in isoproterenol administered groups due to reduction in levels of these antioxidant enzymes. The lipid peroxides and free radical accumulation enhanced in myocardial cells. Thus GSH dependent antioxidant enzymes have a direct relation to isoproterenol induced myocardial damage.

Glutathione has a series of protective functions, it reduces peroxides by GPx. Another protective function of GSH is to conjugate with many foreign substances (xenobiotics). These conjugates are formed by the action of enzymes called glutathione transferases. GST catalyses the addition reaction of the SH group of glutathione with many compounds like chlorinated hydrocarbons and others. Similiarly, GST can bind to many lipophilic drugs and could be expected to bind with isoproterenol. Astaxanthin supplementation helped to maintain the activities of these enzymes to normal levels compared to group II.

Antiperoxidative enzymes SOD and CAT decreased in the heart tissue of isoproterenol induced rats compared to controls. Increased generation of free radicals during myocardial damage can lead to the inactivation of enzyme activities. Rats fed with astaxanthin showed a decrease in lipid peroxidation and increase in the levels of SOD and CAT. The particular configuration of astaxanthin molecule with both hydroxyl and carbonyl groups at each end, exhibits greatest antioxidant activity, peroxyl radical chain breaking abilities, scavenging free radicals better than other antioxidants. Kamath *et al.*(2008) reported that pre-treatment with astaxanthin esters resulted in significant increase in the level of antioxidant enzyme *viz.* catalase, superoxide dismutase, and glutathione peroxidase in stomach homogenate of rats in a study with anti-ulcer properties of astaxanthin in protecting gastric mucosa.

Membrane bound Na^+-K^+ ATPase is an important enzyme utilizing the energy of ATP hydrolysis for transport of several cations. It is also known that an inhibition of this enzyme results in depletion of intracellular Mg^{2+} and increase in Ca^{2+} load (Kramer *et al.,* 1991). The change in the intracellular levels of calcium and magnesium is the major factor in the pathogenesis of various disorders like neurological disorders, hypertension, diabetes, coronary artery disease and stroke, tumours etc. Wang *et al. (*1993) reported an increase in Ca^{2+} level when Na^+-K^+ ATPase was inhibited. Increased intracellular calcium can trigger apoptosis or cell death. The results of the present study

indicate that Na^+-K^+ ATPase levels are decreased in isoproterenol administered groups. This is due to the damage of the myocardial membrane, the enzyme being a membrane bound enzyme, its activity is inhibited. Feeding astaxanthin caused a decrease in the inhibition of enzyme activity in group IV compared to group II.

The powerful cardioprotective effect of astaxanthin can be explained based on multiple independent mechanisms that may coexist in the biological system (Gross and Lockwood, 2005). It has been well documented that bioactive carotenoids such as astaxanthin possess potent antioxidant effects, quench singlet oxygen (Miki, 1991), inhibit lipid peroxidation of membranes both *in vitro* and *in vivo* (Kurihara *et al.,* 2002; Hix *et al.,* 2004a) and exhibit lipoxygenase inhibiting property. Recent evidences show that carotenoids like astaxanthin induced an upregulation of the gap junction protein connexin 43 (Cx43) as well as increased functional gap junctional intercellular communication (GJIC) *in vitro* (Betram, 1999; Hix *et al.,* 2004b). Recent studies show that astaxanthin inhibit macrophage infiltration and leukocyte apoptosis in atherosclerotic plaques formed in arteries of hyperlipidemic rabbits (Li *et al.,* 2004). Additional effects of astaxanthin such as antiinflammatory property (Lee *et al.,* 2003) by inhibiting the proinflammatory mediators can also supplement the cardioprotective potency of astaxanthin.

5.8. IMMUNOSTIMULATORY EFFECT

Immunomodulating effects of astaxanthin in the present study is done based on the evaluation of immune system using different assays. Immune response cells are more sensitive to oxidation and antioxidants like astaxanthin can act as a positive effector in protecting those cells from free radical attack. Due to the various antioxidant capabilities of astaxanthin it can be beneficial in many pathological conditions by its immunopotentiating action. The present study was done by evaluating the immune system in mice fed with astaxanthin @ 10 mg/kg feed.

Feeding astaxanthin caused a significant increase in the proliferation of spleen cells. Lymphocyte proliferation assay using MTT is a rapid colourimetric assay and has a number of advantages over the conventional assays. The test is based on the capacity of mitochondrial enzymes succinate dehydrogenase to transform the tetrazolium salt of MTT in to a blue colour product formazan that can be quantified spectrophotometrically (Mosmann, 1983; Bounous *et al.,* 1992). T-cells contribute the major effector mechanisms in cell mediated immunity (Graubert and Ley, 1996). Astaxanthin was found to stimulate the spleen cell proliferation in presence of mitogens. Immune cells respond to mitogens such as PHA by rapid blastogenesis (Kumar *et al.,* 1999). Immunomodulating properties of astaxanthin observed in present study is in agreement with previous studies done in mice. Jyonouchi *et al.* (1991) studied the immunomodulating effects of carotenoids on mouse lymphocytes *in vitro* by assay of mitogen responses of spleen cells, thymocyte proliferation, interleukin 2 production and antibody production *in vitro* in response to sheep red blood cells. Proliferation rate of splenocytes were increased in presence of astaxanthin.

Administration of astaxanthin to mice in the present study also enhanced the number of plaque forming cells in the spleen and antibody titre in the circulation which are functions of B-cells. Jyonouchi *et al.* (1991) has also reported that spleen cells produced significantly more antibody forming cells (plaque forming cells, immunoglobulins M and G) in presence of astaxanthin. A significant increase in the production of antibodies *in vitro* in response to SRBC was also observed in presence of astaxanthin (Jyonouchi *et al.,* 1991).

Bone marrow cells from astaxanthin fed mice also showed an enhanced proliferation rate *in vitro*. This indicates that astaxanthin administration stimulates the formation of blood forming cells. Bone marrow cells positive for non specific esterases were found to increase after astaxanthin treatment which indicates increased maturation of cells of lymphoid linkage (Nelson and Davey, 1992).

Astaxanthin was reported to show more significant effects in the bioassays compared to other carotenoids like β-carotene. Findings of Okai and Okai (1996) proved that astaxanthin shows considerable immunomodulating activities in *in vitro* cell culture experiments. Carotenoids like beta-carotene, canthaxanthin and astaxanthin caused significant stimulatory effects on cell proliferative response of spleen cells and thymocytes from Balb/c mice. Astaxanthin exhibited highest activity on the polyclonal antibody production of murine spleen cells. The different mechanisms responsible for the immunomodulating properties can be explained based on the earlier studies. Astaxanthin enhanced the *in vitro* antibody production to T-cell dependent antigen and maximum enhancing action was exerted when it was present at the initial period of antigen priming. The direct interactions between T-cells and B-cells are required for the activation of antibody production by astaxanthin. Carotenoids like astaxanthin without pro vitamin A activity augment *in vitro* specific antibody production to T-cell dependent antigen, partly through affecting the initial stage of antigen presentation without facilitating polyclonal B-cell activation or autoantibody production (Jyonouchi *et al.,* 1993). Jyonouchi *et al.* (1995) found that astaxanthin has a significant effect on human immunoglobulin production in response to T-dependent stimuli. Jyonouchi *et al.* (1996) also reported that astaxanthin enhances murine T-helper (Th) cell clone mediated antibody production. It also suppresses interferon-gamma (IFN-gamma) production by Th cells and increased the number of antibody secreting cells.

The present study, thus reveals that oral feeding of astaxanthin from *Aristeus alcocki* shell waste stimulates immune function in mice through enhanced proliferation and function of immunocompetant cells.

5.9. ANTITUMOUR ACTIVITY

A significant reduction in the viability of ascites tumour cells DLA was noted in the current study. Time and dose dependent effects of carotenoids on proliferation, cell cycle and apoptosis of human breast cancer cell line MCF –

7 were reported by Li *et al.* (2002). They showed that carotenoids could inhibit the proliferation of human breast cancer MCF – 7 cell line *in vitro* and the action of carotenoids worked through different pathways. Muller *et al.*(2002) reported that carotenoids showed direct cytotoxicity against T-lymphoblast cell line Jurkat E6.1. through apoptosis. The cytotoxic action of astaxanthin against DLA may be through induction of apoptosis or through a different pathway.

In the present study antitumour activity was noted for astaxanthin extract from shell waste. The increase in life span was about 67 % compared to control when astaxanthin was administered at 5 mg astaxanthin/kg body weight. At this concentration reduction in solid tumour by volume and weight was more than 80%. Jyonouchi *et al.,* (2000) has also reported antitumour activity of astaxanthin. They reported that astaxanthin is a carotenoid without pro vitamin A activity and exert its antitumour activity through the enhancement of immune responses. The antitumour activity noted in ascites tumour may be due to suppression of tumour cell growth by the astaxanthin. They postulated that the tumour cells induce a tumour antigen that induced T cell mediated immune response. The antitumour activity is also paralleled with higher cytotoxic T lymphocyte activity and interferon-gamma production by tumour draining lymph node and spleen cells. Their study also revealed that feeding astaxanthin has significantly reduced tumour size and weight in Balb/c mice. In the present study, the decrease in tumour volume in astaxanthin injected groups was noted in a dose dependent manner. Higher concentrations of astaxanthin decreased both ascites tumour and solid tumour. These effects may be due to the immunomodulating properties of astaxanthin. Repeated injections of astaxanthin in predetermined doses increased the total astaxanthin levels which also helped in increasing the immune response, which in turn is responsible for the antitumour activity. Kurihara *et al.* (2002) reported that daily oral administration of astaxanthin @ 1 mg/kg/day, p.o., for 14 days markedly attenuated the promotion of hepatic metastasis in DBA/2 mice induced by restraint stress. They suggested that astaxanthin improves antitumour immune responses by inhibiting lipid peroxidation induced by stress.

The bioactivities of astaxanthin can also be explained based on its antioxidant property. Kozuki *et al.,* (2000) reported that anitumour activity of astaxanthin can be due to its antioxidant property. Carotenoids like alpha carotene, beta carotene, lycopene, beta cryptoxanthin, zeaxanthin, lutein, canthaxanthin, astaxanthin show antitumour activity against rat ascites hepatoma cells. In the present study the tumour volume and tumour weight was less in mice injected with 5 mg/kg body weight astaxanthin. Astaxanthin can prevent reactive oxygen species potentiated invasive capacity of ascites tumour cells and this highlights the antioxidative action of astaxanthin in suppressing the action of ascites.

5.10. ANTICANCER PROPERTY

The present study demonstrates that the astaxanthin from shrimp shell waste of *Aristeus alcocki* inhibits the growth of cervical cancer cells HeLa. A dose dependent effect on the proliferation of cells were noted upto a concentration of 20 µg/ml astaxanthin in the present study.

Muller *et al.* (2002) has reported that carotenoids induce apoptosis in the T-lymphoblast cell line JurkatE6.1. Apoptosis was characterised by chromatin condensation, nuclear fragmentation, DNA degradation, PARP cleavage and caspase-3-activation. They also noted that the apoptosis may be due to the action of carotenoid itself or due to an early degradation product of carotenoids. Li *et al.*(2002) in a study investigating the effects of carotenoids on the proliferation, cell cycle, apoptosis and expression of bcl-2 gene in breast cancer cell MCF – 7 showed that astaxanthin inhibited the proliferation of human breast cancer cell line MCF – 7 *in vitro* and it also down regulated the expression of bcl-2 gene. They suggested that the action of carotenoids may be worked through different pathways. The cytotoxic action of astaxanthin on cervical cancer cell line HeLa obtained in the present study may be due to induction of apoptosis as suggested by Muller *et al.* (2002) or the action may be worked through different pathways as suggested by Li *et al.* (2002).

The carotenoid extract from shrimp shell waste of the deep sea shrimp *Aristeus alcocki* contains astaxanthin, astxanthin monoester and diester forms. The total astaxanthin present in the extract from this species is double the content of astaxanthin reported in the only commercially exploited astaxanthin shrimp species, the arctic shrimp *Pandalus borealis*. So this deep sea species *Aristeus alcocki* can be considered as a better source of astaxanthin for commercial exploitation than *Pandalus borealis*. In the present study the carotenoid extract from *Aristeus alcocki* showed a predominance of astaxanthin diester. The monoester and diester contained 20 % PUFAs and 30 % PUFAs as their component fatty acids. The higher proportion of diesters and comparatively higher proportion of PUFAs in diester forms may have contributed to the potent antioxidant action of astaxanthin exploited from this candidate species. The antioxidant activity of astaxanthin from *Haematococcus pluvialis,* the richest source of natural astaxnthin, is reported in microgram levels. But in the present study the antioxidant activity of astaxanthin from *Aristeus alcocki* was obtained at nanogram levels. This strong antioxidant potential of *Aristeus alcocki* astaxanthin may be due to predominance of diester forms and synergistic effect of astaxanthin and PUFAs present in the astaxanthin mono and diester forms. This potent antioxidant activity well explains the other bioactive potencies shown by astaxanthin extract from *Aristeus alcocki* shell waste.

6. SUMMARY

Shrimp processing waste is the single largest industrial waste in the country causing diverse environmental problems. This industrial waste can be utilized for isolation of novel biopotent compounds like natural carotenoids mainly astaxanthin. The pigments are reported to have antioxidant properties and hence thought to be beneficial in cardiovascular, immune, anti-inflammatory and neuro degenerative diseases and cancer. The extractability of carotenoids from shell waste of deep sea shrimp *Aristeus alcocki* using different organic solvents and vegetable oils were studied. Extraction was tried using wet and dried waste with and without deproteinisation. Waste was subjected to deproteinisation using alkali and enzyme (pancreatin). Of the different extraction media and samples tried maximum yield of carotenoids (87.14 ± 4.55µg/g) was obtained with non deproteinised wet waste using acetone. The study thus revealed that astaxanthin recovery using organic solvents was more efficient than vegetable oil extraction. The non deproteinised samples gave better yield than deproteinised samples. Acetone is the best solvent for extracting astaxanthin from shrimp shell waste in wet condition. The astaxanthin yield from *Aristeus alcocki* shell waste is double the content of astaxanthin in *P. borealis* waste, the only commercially exploited (M/s Bioprawns, Norway) shrimp species for astaxanthin production. The present study thus reveals that of the different species of deep sea shrimps *Aristeus alcocki* shell waste is an excellent source of astaxanthin. Hence there is ample scope for exploiting *Aristeus alcocki* shell waste for the commercial production of natural astaxnthin

Determination of different components in the carotenoid extract from *Aristeus alcocki* shell waste was studied using TLC. The extract contained astaxanthin, astaxanthin mono and diesters in the proportion of 1:1:2. Astaxanthin diester was the predominant fraction in the carotenoid extract from *Aristeus alcocki* shell waste. The fatty acid composition of the astaxanthin esters were determined using GLC. The present study reveals that the

monoester and diester contained saturated fatty acids, MUFA and PUFAs in the ratio 5:3:2 and 4:3:3 respectively. The main fatty acids esterified with astaxanthin were palmitic acid, oleic acid and poly unsaturated fatty acids (PUFAs) especially EPA and DHA.

The *in vitro* antioxidant activity of astaxanthin extract from shrimp shell waste showed significant hydroxyl radical scavenging activity, superoxide anion scavenging activity and inhibition of lipid peroxidation. Compared to standard antioxidants quercetin and catechin showing antioxidant activity at microgram levels the *in vitro* IC_{50} values reported for *in vitro* antioxidant activity of astaxanthin from *Aristeus alcocki* shell waste are in the range of nanogram levels. Natural compounds are excellent singlet oxygen quenchers as well as lipid peroxidation chain breakers. This powerful antioxidant function may be due to unique molecular structure of astaxanthin. Astaxanthin is such a natural compound whose dual antioxidant capacity may be attributed to the activity of polyene chain. The study also revealed that the astaxanthin extract from *Aristeus alcocki* is a more powerful antioxidant agent than the astaxanthin present in the *Haematococcus pluvialis.* This may be due to a higher proportion of astaxanthin diester and a higher content of poly unsaturated fatty acids (20 % PUFAs in monoester and 30 % PUFAs in diester) in the carotenoid extract obtained from *Aristeus alcocki* shell.

Carrageenan induced paw edema in mice was used for determining the acute anti-inflammatory activity of astaxanthin. The reduction in edema was noted in a dose dependent manner. Astaxanthin concentrations at 0.5mg/Kg body weight and 1.0 mg/Kg body weight inhibited the inflammation by 47.83 and 67.11 percent. The inhibition of inflammation at 1.0mg/Kg body weight was greater than that produced by the standard reference drug diclofenac.

The present study demonstrates that natural astaxanthin from *Aristeus alcocki* shrimp waste inhibits carrageenan induced inflammatory response in mice. This anti-inflammatory effects of astaxanthin from *Aristeus alcocki* shell

has important implications for the development of anti-inflammatory drugs from shrimp shell waste.

The PUFAs present in the astaxanthin diester, predominant fraction of the astxanthin extract also might have contributed to such beneficial effects observed in present study.

Cardioprotective effect of astaxanthin was examined in isoproterenol induced myocardial infarction in rats. The animals were grouped as I & II- fed on normal diet; Groups-III & IV which were fed on normal diet containing astaxanthin @ 10 mg/kg feed for a period of 45 days. Myocardial infarction was induced in animals of II (fed on normal diet) and in IV (fed on normal diet containing astaxanthin @ 10 mg/kg feed) by subcutaneous injection of isoproterenol [6mg (dissolved in physiological saline) per 100g body weight] twice at an interval of 24h at the end of 45 days. Simultaneously, the control animals (I and III) were injected with physiological saline alone.

The administration of isoproterenol to Group II rats resulted in the induction of myocardial infarction as was evident from the increased levels of marker enzymes namely LDH, CPK, GPT, GOT and CK-MB. The prior administration of astaxanthin @ 10 mg/kg along with feed to the group IV animals decreased the activities of these marker enzymes as compared to group II isoproterenol-injected rats. The lower activities of the marker enzymes in the astaxanthin treated group IV compared to that of control group II, highlights the cardioprotective effect of the astaxanthin.

Glutathione and ascorbic acid levels and the levels of the antioxidant enzymes *viz.* GPx, GST, GR, CAT, SOD were found to be decreased in isoproterenol administered groups. The administration of astaxanthin increased the levels of ascorbic acid, GSH and the levels of the antioxidant enzymes in Group IV compared to Group II and this protects the heart membrane from oxidative damage. An increased oxidative stress is noted in isoproterenol administered groups due to reduction in levels of the antioxidant enzymes. An

increase in MDA levels in the heart tissues of group II compared to group IV was observed in the present study. Astaxanthin administration for a period of 45 days decreased the level of lipid peroxidation in group IV.

Antiperoxidative enzymes SOD and CAT decreased in the heart tissue of isoproterenol induced rats compared to controls. Rats fed with astaxanthin showed a decrease in lipid peroxidation and increase in the levels of SOD and CAT. The particular configuration of astaxanthin molecule with both hydroxyl and carbonyl groups at each end, exhibits greatest antioxidant activity, peroxyl radical chain breaking abilities, scavenging free radicals better than other antioxidants.

The loss of membrane integrity is one of the changes noted in MI. The results of the present study indicate that Na^+-K^+ ATPase levels are decreased in isoproterenol administered groups. This is due to the damage of the myocardial membrane, the enzyme being a membrane bound enzyme its activity is inhibited. Astaxanthin with the moieties of its polyene chain and terminal rings rigidify membranes and modify their permeability. Feeding astaxanthin caused a decrease in the inhibition of enzyme activity in group IV compared to group II.

The powerful cardioprotective effect of astaxanthin can be attributed to the multiple independent mechanisms that may coexist in the biological system. Bioactive carotenoids such as astaxanthin possess potent antioxidant effects, singlet oxygen quenching ability and inhibition of lipid peroxidation of membranes both *in vitro* and *in vivo*. Carotenoids like astaxanathin increased functional gap junctional intercellular communication (GJIC) *in vitro* and inhibit macrophage infiltration and leukocyte apoptosis in atheroscelerotic plaques. Additional effects of astaxaxnthin such as antiinflammatory property by inhibiting the proinflammatory mediators can also supplement to the cardioprotective potency of astaxanthin.

The immunostimulatory effect of astaxanthin was determined by assaying the Splenic T-lymphocyte mitogen response, bone marrow cell

proliferation assay, plaque formation cell assay, circulating antibody titre, and alpha-naphthyl acetate esterase activity in bone marrow cells at the end of 45 days feeding of the control mice with normal diet and the test animals with normal diet containing astaxanthin @ 10 mg/kg feed.

In the astaxanthin treated animals, the spleen cell proliferation was found to be stimulated in the presence of mitogen. Enhanced proliferation of bone marrow cells was also observed in treated animals compared to control animals. These findings indicate induction of proliferation of bone marrow stem cells either directly or indirectly, stimulating the release of factors that are involved in the regulation of hemopoesis. The treated animals also showed an increase in number of plaque forming cells in the spleen, and antibody titre in the circulation which are the functions of B-cells. Bone marrow cells positive for non specific esterases were found to increase after astaxanthin treatment which indicates increased maturation of cells of lymphoid linkage. The present study, thus reveals that oral feeding astaxanthin from *Aristeus alcocki* shell waste stimulates immune function in mice through enhanced proliferation and function of immunocompetant cells.

The antitumour activity of astaxanthin extract from *Aristeus alcocki shell waste* was studied using Dalton's lymphoma ascites in BALB/c mice. The results showed that the astaxanthin showed *in vitro* cytotoxic activity against DLA in a dose dependent manner. The % viability of the DLA cells was reduced to 4.34 % at higher concentration of 15µg astaxanthin/ml. Antitumour activity of astaxanthin from *Aristeus alcocki* shell waste *in vivo* was also exhibited in a dose dependent manner. Astaxanthin increased the life span from 15 days to 25 days. This account for 67 % increase in the life span in DLA bearing mice. This significant increase in life span was noted at a dose of 5 mg astaxanthin /kg body weight. At this concentration reduction in solid tumour by volume and weight was more than 80%.These effects may be due to the immunomodulating properties of astaxanthin. Repeated injections of astaxanthin in predetermined doses increased the total astaxanthin levels which

also helped in increasing the immune response which inturn is responsible for the antitumour activity. Astaxanthin also improves antitumour immune responses by inhibiting lipid peroxidation. Astaxanthin can prevent reactive oxygen species potentiated invasive capacity of ascites tumour cells and this highlights the antioxidative action of astaxanthin in suppressing the action of ascites. In the present study astaxanthin extract from *Aristeus alcocki* shell waste showed a dose dependent effect on the proliferation of cervical cancer cells *in vitro* upto a concentration of 20 µg/ml astaxanthin. The cytotoxic action of astaxanthin on cervical cancer cell line HeLa obtained in the present study may be due to induction of apoptosis or the action may be worked through different pathways.

The carotenoid extract from shrimp shell waste of the deep sea shrimp *Aristeus alcocki* contains astaxanthin, astxanthin monoester and diester forms. Astaxanthin is the most potent antioxidant known till date with some unique properties suitable for use as a drug or food supplement in the treatment of cardiovascular,immune,neurodegenerative diseases and cancer. This potent antioxidant activity well explains the other bioactive potencies shown by this novel compound in the present study. Many of the synthetic medicines available for the treatment of these disorders have the risk of serious side effects and safer alternatives are essential. Effective utilization of shrimp shell waste will enhance its status as a biomedical research material, for development of natural medicine without side effects. Eventhough the astaxanthin extraction in the present study is based on the deep sea shrimp *Aristeus alcocki* shell waste, any crustacean waste could be effectively utilized as the cheapest raw materials for astaxanthin recovery.

7. REFERENCES

A.O.A.C. 1990. *Official Methods of Analysis.* Fifteenth edition. Association of Official Analytical Chemists, Washington

Aghdassi, E. and Allard, J.P. 2000. Breath alkanes as a marker of oxidative stress in different clinical conditions. *Free Radic. Biol. Med*. 28: 880–886

Ahluwalia, G.S., Jayaram, H.N., Plowman, J.P., Cooney, D.A., and Johns, D.G. 1984. Studies on the mechanisms of activity of 2-B-D-ribofuranosyl thiazole-4-carboxamide. *Biochem. Pharmacol.* 33: 1195-1203

Ajith, T.A. and Janardhanan, K.K. 2001. Antioxidant and anti-inflammatory activities of methanolic extract of *Phellinus rimosus* (Berk). *Indian. J. Exp. Biol.*39: 1166-1169

Allen, R.G. and Tresini, M. 2000. Oxidative stress and gene regulation *Free Radic. Biol. Med.* 28: 463-499

Almgren, K. 1966. Ecology and distribution in Sweden of algae belonging to Haematococcaceae. *Svensk Botanisk Tidskrift BD.* 60(H.1): 49-73

Ames, B. N. 1998. Micronutrients prevent cancer and delay aging. *Toxicol. Lett.* 102: 5-18

Ames, B. N. and Shigenaga, M. K. 1992.Oxidants are a major contributor to aging. *A. N. Y. Acad. Sci*. 663: 85-96

Ames, B. N., Shigenaga, M. K., and Hagen, T. M. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. nat. Acad. Sci. USA*. 90(17): 7915-7922

Anderson, J., Gowri, M., Turner, J., Nichols, L., Diwadkar, V.A., Chow, C.K. and Oeltgen, P.R. 1999. Antioxidant supplementation effects on low-density lipoprotein oxidation for individuals with Type 2 diabetes mellitus. *J. Am. Coll. Nutr.*18: 451-461

Anderson, L.K. 1975. *Extraction of carotenoid pigment from shrimp processing waste*. US Patent.No.3906112

Anderson, M. 2001. *Method of inhibiting 5-α reductase with astaxanthin to prevent and treat beningn prostate hyperplasia (BPH) and prosate cancer in human males.* US Patent.No.6227417

Andersson, M., Nieuwerburgh, L.V. and Snoeijs, P. 2003. Pigment transfer from phytoplankton to zooplankton with emphasis on astaxanthin production in the Baltic Sea Food web. *Mar. Ecol. Prog. Ser.* 254: 213-224

Anon. 2004. The new youth preserve from under the sea. *Woman's. Wld. Mag.*

Anon. 2006. Extraction and storage stability of astaxanthin from shrimp (*Penaeus monodon*) waste. http://www.pcierd.dost.gov.ph/food.

Aoi, W., Naito, Y., Sakuma, K., Kuchide, M., Tokuda, H., Maoka, T., Tokoyuni, S., Oka, S., Yasuhara, M. and Yoshikawa, T. 2003. Astaxanthin limits exercise induced skeletal and cardiac muscle damage in mice. *Antiox. Redox. Signal.*5: 139-144

Armenta, R.E. and Guerrero, I. L., I. 2009. Amino acid profile and enhancement of the enzymatic hydrolysis of fermented shrimp carotenoproteins. *Fd. Chem.* 112(15): 310-315

Armenta-,López R, Guerrero, I. L., and Huerta, S. 2002. Astaxanthine extraction from shrimp waste by lactic fermentation and enzymatic hydrolysis of the carotenoprotein complex. *J. Fd. Sci.* 67(3): 1002–1006

Athar, M. 2002. Oxidative stress and experimental carcinogenesis. *Ind. J. Exp. Biol.* 40: 656-667

Aw, T.Y. 1999. Molecular and cellular responses to oxidative stress and changes in oxidation-reduction imbalance in the intestine. *Am.J. Clin. Nutr*. 70: 557–565

Babu, C.M., Chakrabarti, R. and Sambasivarao, K.R.S. 2008. Enzymatic isolation of carotenoid-protein complex from shrimp head waste and its use as a source of carotenoids. *Fd. Sci. Technol*. 41(2): 227-235

Baeck, H. H. and Cadwallader, K. R. 1995. Enzymatic hydrolysis of crayfish processing byproducts. *J. Fd. Sci.* 60: 929-935

Balachandran, K.K. 1976. On the carotenoid pigments in Indian prawns. *Fish. Technol.* 13: 121-125

Bancroft, J.D. and Cook, H.C. 1994. *Manual of Histological Techniques,* Churchill Livingstone, London, p.171

Barbosa, M.J., Morais, R. and Choubert, G. 1999. Effect of carotenoid source and dietary lipid content on blood astaxanthin concentration in rainbow trout *(Oncorhychus mykiss)*. *Aquaculture.* 176: 331-334

Barratt, A. and Montano, R., 1986. Shrimp heads—a new source of protein. *INFOFISH Markg. Dig.* 4(86): 21

Barros, M.P., Pinto, E., Colepicolo, P. and Pedersen, M. 2001. Astaxanthin and peridinin inhibit oxidative damage in Fe^{2+} -loaded liposomes: Scavenging oxyradicals or changing membrane permeability? *Biochem. Biophys. Res. Comm.* 288: 225-232

Behl, C. 1999. Alzheimer's disease and oxidative stress:implications for novel therapeutic approaches. *Prog. Neurobiol.* 57: 301-323

Belitz, H.D. and Grosch, W. 1999. *Food Chemistry*. Springer Gazching FRG. 232 p.

Bell, J.G., McEvoy, J., Tocher, D.R. and Sargent, J.R. 2000. Depletion of alpha-tocopherol and astaxanthin in Atlantic salmon, (Salmo salar) affects autoxidative defense and fatty acid metabolism. *J. Nutr.* 130(7): 1800-1808

Bellot, J.L., Palmero, M., Galcia-Cabanes, C., Espi, R., Hariton, C. and Oris, A. 1996. Additive effect of nitric oxide and prostaglandin E2 synthesis inhibitors in endotoxin induced uvetis in the rabbit. *Inflamm. Res.* 45: 203-208

Bennedsen, M., Wang, X., Willen, R., Wadstrom, T. and Andersen, L.P. 1999. Treatment of H. pylori infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. *Immunol. Lett.* 70: 185–189

Benny, K.J., Nair, C.R.S. and Padmanavan, A. 1994. Matrix diffusion controlled transdermal delivery of atenolol from chitosan film. *Nutrients and Bioactive Substances in Aquatic Organisms*.(eds. Devadasan, K., Mukundan, M.K., Antony, P.D., Nair, P.G.V., Perigreen, P.A. and Joseph, J.). Society of Fishery Technoogists (India), Kochi, pp 76-81

Bernhard, K. 1990. Synthetic astaxanthin. The route of a carotenoid from research to commercialization. *Carotenoids: Chemistry and Biology* (eds. Krinsky, N. I., Mathews-Roth, M.M. and Taylor, R.F.). Plenum Press, New York, pp. 337-363

Betram, J.S. 1991. Cancer chemoprevention by retinoids and carotenoids: roposed role of gap junctional communication. *Vitamins and Minerals in the Prevention and Treatment of Cancer*. (ed. Jacobs, M.M.). Boca Raton, F.L.CRC Press, pp. 31-50

Betram, J.S. 1999. Carotenoids and gene regulation. *Nutr. Rev.*57: 182-191

Beutler, W.H. and Bernes, J.B. 1996. *Nature* 209: 90

Beutner, S., Bloedorn, B. and Frixel, S. 2001. Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: Carotenoids, avonoids, phenols and indigoids. The role of β-carotene in antioxidant functions. *J. Sci. Fd. Agric*. 81:559–568

Bezerra, R,D., Abadie-Guedes, R., Melo, F. R. M., Paiva, A. M. D., Amâncio-dos-Santos, A. and Guedes, R.C.A. 2005. Shrimp carotenoids protect the developing rat cerebral cortex against the effects of ethanol on cortical spreading depression. *Neurosci. Lett.* 391(1-2): 51-55

Bianchet, M.A., J. Hullihen, P.L. Pedersen, and L.M. Amzel. 1998. The 2.8-Å structure of rat liver F1-ATPase: configuration of a critical intermediate in ATP synthesis/hydrolysis. *Proc. nat. Acad. Sci. USA* 95: 11065-11070

Biesalski, H.K. 1999. The role of antioxidative vitamins in primary and secondary prevention of coronary heart disease. *Int. J. Vitam. Nutr. Res.*69: 179-186

Black, H. 1998. Radcal interception by carotenoids nd effects on UV carcinogenesis. *Nutr. Cancer.* 31: 212-217

Blostein, R. 1968. Relationship between erythrocyte membrane phosphorelation and adenosine triphosphate hydrolysis. *J. Biol. Chem.* 243 (8): 1957-1965

Blumberg, J.B. and Halpner, A.D. 1999. Antioxidant status and function: relationships to aging and exercise. *Antioxidant Status, Diet, Nutrition, and Health.* (ed. Papas, A.M.). Boca Raton: CRC Press. pp. 251-274

Boileau T.W.M., A.C. Moore, J.W. Erdman,Jr. 1999. Carotenoids and vitamin A. *Antioxidant Status, Diet, Nutrition, and Health.* (ed. Papas, A.M.). Boca Raton: CRC Press.

Bone, R.A., Landrum, J.T. and Tarsis, S.L. 1985. Preliminary identification of the human macular pigment. *Vision. Res.* 25: 1531-1535

Borlongan, C., Kanning, K., Poulos, S.G., Freeman, T.B., Cahill, D.W. and Sanberg, P.R. 1996. Free radical damage and oxidative stress in Huntington's disease. *J. Fla. Med. Assoc.* 83: 335-341

Borowitzka, M. A., Huisman, J.M. and Osborn, A. 1991. Culture of the astaxanthin-producing green alga *Haematococcus pluvialis* 1. Effects of nutrients on growth and cell type. *J. Appl. Phycol.* 3(4): 295-304

Boujedaini, N., Liu, J., Thuillez, C. and Cazini. Menasah-Nyagan, A.G. 2000. *In vivo* regulation of vasomotoricity by nitric oxide an prostanoids during gestation. *Eur. J. Pharmacol.* 427: 143-149

Bounous, O.I., Campernoh, R.P. and Brown, J. 1992. Comparison of MTT colurimetric assay and tritiatedthymidine uptake by lymphocyte proliferation assay using chicken splenocytes. *Avian. Dis.* 36:1022-1027

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72*:* 248-254

Breimer, L.H. 1990. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. *Mol. Carcinog.* 3: 188-197.

Breithaupt, D.E. 2004. Identification and quatification of astaxanthin esters in shrimp (*Pandalus borealis*) and in microalga (*Haematococcus pluvialis*) by liquid chromatography-mass spectrometry using negative ion atmospheric pressure chemical ionization. *J. Agric. Fd. Chem.* 52: 3870-3875

Britton, G. 1985. General carotenoid methods.*Methods in Enzymology.Vol III.*(eds. Law, J.H., Rilling, H.C.). Academic Press, New York, pp. 113-149

Britton, G. 1995. Structure and properties of carotenoids in relation to function. *FASEB J.* 9:1551–1558

Britton, G., Armit, G.M., Lau, S.Y.M., Patel, A.K. and Shone, C.C. 1981. Carotenoproteins. *Carotenoid Chemistry and Biochemistry,* (eds. Britton, G. and Goodwin, T.W.). Oxford: Pergamon Press, pp. 237-251

Britton, G., Liaaen-Jensen. S., Pfander, H., Mercadante, A. Z. and Egeland, E. S. 2004. *Carotenoids handbook*. Basel: Birkhäuser

Buege, J. A. and Aust, S. D. 1978. *Methods in enzymology* Vol. II. (ed. Fleisher, S and Packer, L.). Acad. Press, New York, pp. 305-306.

Cano-Lopez, A., Simpson, B. K. and Haard, N. F. 1987. Extraction of carotenoprotein from shrimp process waste with the aid of typsin from Atlantic cod. *J. Fd. Sci.* 52: 503-504

Cardounel, A. J., Dumitrescu, C., Zweier, J. L. and Lockwood, S. F. 2003. Direct superoxide anion scavenging by disodium disuccinate astaxnthin derivative: Relative efficacy of individual stereoisomers versus the statistical mixture of stereoisomers by electron paramagnetic resonance imaging. *Biochem. Biophys. Res. Commun.* 307(3): 704-712

Casillas, A., Hiura, T., Li, N. and Nel, A.E.T. 1999. Enhancement of allergic inflammation by diesel exhaust particles: permissive role of reactive oxygen species. *Ann. Allerg. Asthma. Im.*83: 624-629

Chakrabarti, R. 2002. Carotenoprotein from tropical brown shrimp shell waste by enzymatic process. *Fd. Biotechnol.* 16(1): 81-90

Chang, K. L. and Tsai, G. 1997. Response surface optimization and kinetics of isolating chitin from pink shrimp (*Solenocera melantho)* shell waste. *J. Agric. Fd. Chem.* 45: 1900-1904

Charest, D.J., Bulaban, M.O., Marshall, M.R. and Cornell, J.A. 2001. Astaxanthin extraction from crawfish shells by supercritical CO_2 with ethanol as cosolvent. *J.Aqua Fd. Prod. Technol*. 10: 79-93

Chen, H. and Meyers, S.P.1982. Extraction of astaxanthin pigment from Crawfish waste using a soy oil process. *J. Fd. Sci*. 47(3): 892-896

Chen, H. and Meyers, S.P.1983 .Ensilage treatment of crawfish wastefor improvement of Astaxanthin pigment extraction. *J. Fd. Sci.* 48(5): 1516-1520

Chen, H.M. and Meyers, S.P. 1984. A rapid quantitative determination of astaxanthin pigment concentrate in oil extraction. *J. Am. Oil. Chem. Soc.* 61: 1045–1047

Chen, Y.C., Shen, S.C., Lee, W.R., Hou, W.C. Yang, I.L. and Lee, T.J. 2001. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cycloxygenase 2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *J. Cell. Biochem.* 82: 537-548

Chew, B.P., Park, J.S., Wong, M.W. and Wong, T.S. 1999a. A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice *in vivo. Anticancer. Res.*19: 1849-1854

Chew, B.P., Wong, M.W., Park, J.S. and Wong, T.S. 1999b. Dietarybeta-carotene and astaxanthin but not canthaxanthin stimulate splenocyte function in mice. *Anticancer. Res.*19 (6): 5223-5227

Chien, Y.H., Pan, C.H. and Hunter, B. 2003. The reistance to physical stress by *Penaeus monodon* fed diets supplemented with astaxanthin. *Aquaculture.*216: 177-191

Chihara, G., Hamuro, J., Maeda, Y.Y. and Fuuoka, F. 1970. Fractionation and purification of the polysaccharides with antitumor activity, especially lentinan from *Lentinus edodus* (Berk) Sing. (an edible mushroom). *Cancer. Res.* 30: 2776-2781

Christen, W., Ajani, U.A. and Glynn, R.J.1999. Prospective cohort study of antioxidant vitamin supplement use and the risk of age related maculopathy. *Am. J. Epidemiol.* 6: 141-166

Christiansen, R., Lie, O. and Torrissen, O.J. 1995. Growth and survival of Atlantic salmon *Salmo salar L.* fed different dietary levels of astaxanthin. First feeding fry. *Aquac. Nutr.* 1: 189-198

Cianci, M., Rizkallah, P. J., Olczak, J., Raftery, N.E., Chayen, N.E., Zagalsky, P.F. and Helliwell, J. R. 2001. Structure of lobster apocrstacyanin A1 using softer X-rays. *Acta.Cryst.* 57: 1219-1229

Cianci, M., Rizkallah, P. J., Olczak, J., Raftery, N.E., Chayen, N.E., Zagalsky, P.F. and Helliwell, J. R. 2002. The molecular basis of the colouration mechanism in lobster shell: Beta-crustacyanin at 3.2-A resolution. *Proc. Nat. Acad. Sci.*USA. 99: 9795

Comhair, S., Bhathena, P.R., Dweik, R.A., Kavuru, M. and Erzurum, S.C. 2000. Rapid loss of superoxide dismutase activity during antigen-induced asthmatic response. *Lancet.* 355(9204): 624

Cooper, D., Eldridge, A.L. and Peters, J.C. 1999. Dietary carotenoids and certain cancers, heart disease, and age related macular degeneration: a review of recent research. *Nutr. Rev.* 57: 201-214

Coral-Hinostroza, G.N. and Bjerkang, B. 2002. Astaxanthin from the red crab langostilla (*Pleuroncodes planipes*): Optical R/S isomers and fatty acid moieties of astaxanthin esters. *Comp. Biochem. Physiol.* 136B: 437-444

Cotter, M.A., Love, A., Watt, M.J., Cameron, N.E. and Dines, K.C. 1995. Effects of natural free radical scavengers on peripheral nerve and neurovascular function in diabetic rats. *Diabetologia.* 38: 1285-1294

Cremades,O., Ponce, E., Corpas, R., Gutiérrez, J. F., Jover, M., Alvarez-Ossorio M.C., Parrado, J. and Bautista, J. 2001. Processing of crawfish (*Procambarus clarkii*) for the preparation of carotenoproteins and chitin. *J. Agric. Fd Chem.* 49(11): 5468–5472

Cross, C. E., B. Halliwell, E.T., Borish, W.A., Pryor, B.N. Ames, R.L. Saul, J.M. McCord, and D. Harman. 1987. Oxygen radicals and human disease. *Ann. Intern. Med.* 107: 526-545

Cuzzocrea, S., Riley, D.P., Caputi, A.P. and Salvemini, D .2001. Antioxidant therapy: A new pharmacological approach in shock, inflammation and ischemia/ reperfusion injury. *Pharmacol. Rev*. 539: 139-159

Dall, W. 1995. Carotenoids versus retinoids (Vitamin A) as essential growth factors in penaeid prawns (*Penaeus semisulcatus*). *Mar. Biol.* 124: 209-213

Danesh, J. 1999. Smoldering arteries? Low-grade inflammation and coronary heart disease. *J. Amer. Med. Assoc.* 282: 2169-2171

Darley-Usmar, V. and Halliwell, B. 1996. Blood radicals: reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. *Pharm. Res*. 13(5): 649-662

Davies, B.H. 1985. Carotenoid metabolism in animals: A biochemist's view. *Pure Appl. Chem.* 57: 679

Davies, K. J. 1995. Oxidative stress: the paradox of aerobic life. *Biochem. Soc. Symp*., 61: 1-31

Dawson, V. and Dawson, T. 1996. Nitric oxide neurotoxicity. *J. Chem. Neuroanat.* 10: 179-190

De Dios-Naranjo, C., Ponce-Aliquicira, E., Cruz-Sosa, F. and Guerrero-Lagarreta, I. 2002. Carotenoid pigment extraction from blue crab (*Callenectus sapidus*) wastes using organic solvent systems and lactic acid fermentation. Annual Meeting and Food Expo, 18th June 2002. Anaheim, California. *Abstract*: 15

De Rijk, M., Breteler, M.M., Den Breeijen, J.H., Launer, L.J., Grobbee, D.E., Van Der Meche, F.G., Hofman, A. 1997. Dietary antioxidants and Parkinson disease. The Rotterdam study.*Arch. Neurol.* 54: 762-765

De Ritter, E. and Purcell, A.E. 1981. Carotenoid analytical methods. *Carotenoids as Colourants and Vitamin A Precursors* (eds. Bauernfeind, J.C.). Academic Press, New York, USA, pp. 815-882

Defelice, S.L. 1995. The nutritional revolution: its impact on food industry R and D. *Trends. Fd. Sci. Technol.* 6: 59-61

Dekkers, J. VanDoornen, L.J. and Kemper, H.C. 1996. The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. *Sports. Med.* 21: 213-238

Delgrado-Vargus, F., Jimenez, A.R. and Peredes-Lopez, O. 2000. Natural pigments: carotenoids, anthocyanins and betalains: characteristics, biosynthesis, preparation and stability. *CRC Crit. Rev. Fd. Sci. Nutr.* 40: 173-289

Deodhar, S.D. 1989. C-Reactive protein: The best laboratory indicator available for monitoring disease actiivity. *Cleveland. Clinic. J. Med.* 56(2): 126-129

Dhanya, C.R., Indu, A.R., Deepadevi, K.V. and Kurup, P.A. 2003. Inhibition of membrane Na^{+}-K^{+} ATPase of the brain, liver and RBC in rats administered di(2-ethyl hexyl) phthalate (DEHP) a plasticizer used in polyvinyl chloride (PVC) blood storage bags. 41: 814-820

Di Mascio P., M. E. Murphy, H. Sies. 1991. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am. J. Clin. Nutr.* 53:194-200

Di Mascio, P., T.P.A. Devasagayam, S. Kaiser, H. Sies. 1990. Carotenoids, toco-pherols and thiols as biological singlet molecular oxygen quenchers. *Biochem. Soc. Transactions*. 18: 1054-1056

Donia, M., Hamann, M.T. 2003. Marine natural products and their potential applications as antiinfective agents. *The Lancet*. 3: 338-348.

Dore, J., 1999. *Astaxanthin in Health*. Internal report. Mera Pharmaceuticals Inc.

Drexel, H., Amann, F.W. and Beran, J. 1994. Plasma triglycerides and three lipoprotein cholesterol fractions are independent predictors of the extent of coronary atherosclerosis. *Circulation.* 90: 2230-2235

Droop, M.R.1953. On the ecology of flagellates from some brackish and fresh water rockpools of Finland. *Acta. Bota. Fen.* 51: 1-52

Droop,M.R. 1961. *Haematococcus plivialis and its allies.* III organic nutrition. *Rev. Algol.* 5(4): 247-259

Duetsch, L. 2007. Evaluation of the effect of Neptune krill oil on chonic inflammation and arthritic symptoms. *J. Am. Coll. Nutr.* 16(1): 39-78

Dworski, R., Murray, J.J., Roberts, L.J. 2nd., Oates, J.A., Morrow, J.D., Fischer, L. and Sheller, J.R. 1999. Allergen-induced synthesis of F(2)-isoprotenes in atopic asthmatics.Evidence for oxidative stress. *Am. J. Resp. Crit. Care.* 160: 1947-1951

Ebadi, M., Srinivasan, S.K. and Baxi, M.D. 1996. Oxidative stress and antoxidant therapy in Parkinson's disease. *Prog. Neurobiol.* 48: 1-19

Egeland, E, S. 1993. Carotenoids in combs of capercaillie (*Tetrao urogallus)* fed defined diets. *Poult. Sci*. 72: 747-751

Elizabeth, K. and Rao, M.N.A. 1990. Oxygen radical scavenging activity of curcumin. *Int. J. Pharmacol.* 58: 237-240

Ellis, J.N. 1978.*Proceedings of the World Symposium on Fin Fish Nutrition and Feed Technology,* Hamburg, West Germany

Ernst, H. 2002. Recent advances in industrial carotenoid synthesis. *Pure & Appl. Chem.* 74: 1369-1382

Esterbauer, H., J. Gebicki, H. Puhl, and G. Jurgens. 1992. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic. Biol. Med.* 13: 341-390.

Fachinetti, F., Dawson, V. and Dawson, T. 1998. Free radicals as mediators of neuronal injury. *Cell. Mol. Neurobiol.* 18: 667-682

Fahey, R. C. and Sundquist, A. R. 1991. Evolution of glutathione metabolism. *Adv. Enzymol. Related Areas Mol. Biol*. 64: 1-53

Fahn, S. and Cohen, G. 1992. The oxidant stress hypothesis in Parkinson's disease: evidence supporting it. *Ann. Neurol.* 32: 804-812

Farvin, S.K.H., Anandan, R., Kumar, H.S., Shiny, K.S., Sankar, T.V. and Thankappan, T.K. 2004. Effect of squalene on tissue defense system in isoproterenol-induced myocardial infarction in rats. *Pharmacol. Res.* 50: 231-236

Felix Valenzuela, L., Higuera Ciapara, I., Goycoolea Valencia, F., Arguelles-Monal, W. 2001. Supercritical CO2/ethanol extraction of astaxanthin from blue crab (*Callinectes sapidus*) shell waste. *J. Fd. Process. Engng*. 24: 101-112

Ferrante, R., Browne, S.E., Shinobu, L.A., Bowling, A.C., Baik, M.J., MacGarvey, U., Kowall, N.W., Brown, R.H. Jr. and Beal, M.F. 1997. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J. Neurochem.* 69: 2064-2074

Fiske and SubbaRow. 1925. Determination of inorganic phosphorus. *J. Biol. Chem.* 66: 375-400

Foss, P., Renstrøm, B., and Liaaen-Jensen, S. 1987. Natural occurrence of enantiomeric and meso astaxanthin. 7. Crustaceans including zooplankton. *Comp. Biochem. Physiol.* 86: 313-314

Frei, B. 1995. Cardiovascular disease and nutrient antioxidants: role of low-density lipoprotein oxidation. *Crit. Rev. Fd. Sci. Nutr*. 35: 83–98

Fridovich, I. 1995. Superoxide radical and superoxide dismutases. *Ann. Rev. Biochem.* 64: 97-112

Fuchs, J. 1998 .Potentials and limitations of the natural antioxidants RRR-alpha-tocopherol, L-ascorbic acid and beta-carotene in cutaneous photoprotection. *Free Radic. Biol*. Med. 25: 848–873

Fuesetani, N. 2000. *Drugs from the Sea*. (ed. Fuesetani, M.) Basel : Karger, pp. 1-5

Furr, H.C. and Clark, R.M. 1997. Intestinal absorption and tissue distribution of carotenoids. *Nutr. Biochem.* 8: 364-377

Gagne, N. and Simpson, B.K. 1993. Use of proteolytic enzymes to facilitate the recovery ofchitin from shrimp waste. *Fd. Biotechnol.* 7: 253-263

Geetha, A., Sankar, R., Thankamani, M. and Devi, C.S. 1990. Alpha-tocopherol reduces dexoruvicin induced toxicity in rats-histological and biochemical evidence. *Ind. J. Pharmacol.* 34:94-100

Gershon, D. 1999. The mitochondrial theory of aging: Is the culprit a faulty disposal system rather than indigenous mitochondrial alterations? *Exp. Gerontol*. 34: 613–619

Gerster, H. 1991. Review: antioxidant protection of the ageing macula. *Age Ageing*. 20: 60-69.

Ghadge, G., Lee, J.P., Bindokas, V.P. Jordan, J., Ma, L., Miller, R.J. and Roos, R.P. 1997. Mutant superoxide dismutase-1-linked familial amyotrophic lateral sclerosis: molecular mechanisms of neuronal death and protection. *J. Neurosci*. 17: 8756-8766

Gildberg, A. and Stenberg, E. 2001. A new process for advanced utilization of shrimp waste. *Process. Biochem.* 36: 809-812

Gil-Huan, A. and Eui-Sung, C. 2003. Preparation of the red yeast, *Xanthophyllomyces dendrorhous,* as feed additive with increased availability of astaxanthin. *Biotech. Letters*. 25(10): 767-771

Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. nat. Acad. Sci.USA.*90: 7915-7922

Gopakumar, K. 1993. Shrimp and crab shell utilization. *Fishing Chimes*. 67- 69

Gopakumar, K. 1997. *Tropical Fishery Products.* First edition. Oxford and IBH Publishing Co. Pvt.Ltd, New Delhi, 190 p

Gopakumar, K. and Nair,M.R. 1975. Lipid composition of five species of Indian prawns. *J. Sci. Fd. Agric.* 26: 319-325

Gopal, S.T.K., Thankamma, R., Nair, K.G.R., Prabhu, P.V. and Madhavan, P. 1991. Use of chitosan adhesive for the manufacture of corrugated fibreboard. *Fish. Technol*. 28(2): 154

Goto, S., Kogure, K., Abe, K., Kimata, Y., Kitahama, K., Yamashita, E. and Terada, H. 2001. Efficient radical trapping at the surface and inside the phospholipids membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochim. Biophys. Acta.* 1512: 251-258

Gouveia, L., Rema, P., Pereira, O. and Empis, J. 2003. Colouring ornamental fish (*Cyprinus carpio* and *Carassius auratus*) with microalgal biomass. *Aquaculture. Nutr.* 9: 123-129

Gradelet, S., Astrog, P., Leclere, J., Chevalier, J., Vernevaut, M.F. Siess, M.H. 1996. Effects of cantaxanthin, astaxanthin, lycopene and lutein on liver xenobiotic-metabolizing enzymes in the rat. *Xenobiotica.* 26: 49-63

Gradelet, S., Le Bon, A.M., Berges, R., Suschetet, M. and Astrog, P. 1998. Dietary carotenoids inhibit aflatoxin B1-induced liver preneoplastic foci and DNA damage in the rat: role of the modulation of aflatoxin B1 metabolism. *Carcinogenesis.* 19: 403-411

Grant, W.B. 1997. Dietary links to Alzheimer's disease. *Alz. Dis. Rev.* 2: 42-55

Graubert, T.A. and Ley, T.J. 1996. How do lymphocytes kill tumor cells? *Clin. Cancer. Res.* 2(5): 785-789

Green, L. 1995. Asthma and oxidant stress: nutritional, environmental, and genetic risk factors. *J. Am. Coll. Nutr.* 14: 317-324

Gross, G. J. and Lockwood, S.F. 2004. Cardioprotection and myocardial salvage by a disodium disuccinate astaxanthin derivative (Cardax). *Life Sci.* 75(2): 215-224

Gross, G. J. and Lockwood, S.F. 2005.Acute and chronic administration of disodium disuccinate astaxanthin (Cardax^{TM}) produces marked cardioprotection in dog hearts. *Mol. Cell. Biochem.*272: 221–227

Gross, G. J., Hazen, S.L. and Lockwood, S.F. 2006. Seven day oral supplementation with Cardax (disodium disuccinate astaxanthin) provides signifcant cardioprotection and reduces oxidative stress in rats. *Mol. Cell. Biochem.* 283(1-2): 215–224

Guerin, M., Huntley, M. E. and Olaizola, M. 2003. Haematococcus astaxanthin : applications for human health and nutrition. *Trends. Biotechnol*. 21(5): 210-216

Guillou, A., Khalil, M. and Adambounou, L. 1995. Effects of silage preservation on astaxanthin forms and fatty acid profiles of processed shrimp (*Pandalus borealis)* waste. *Aquaculture*. 130: 351-360

Gupta, S.K. and Gupta, P.C. 2006. *General and Applied Ichthyology (Fish and Fisheries*). S.Chand and Company Ltd, , Ram Nagar, New Delhi, 1130 p

Habig, W.H., Pabst, M.J. and Jackoby, W.B.C. 1974. Glutathione-S-transferases: the first enzymatic step in enzymatic stepping mercapturic acid formation. *J. Biol. Chem.* 249: 7130-7139

Haefner, B. 2003. Drugs from the Deep. *Drug Discov. Today.* 8: 536-544

Hall, E., Andrus, P.K., Oostveen, J.A., Fleck, T.J. and Gurney, M.E. 1998. Relationship of oxygen radical –induced lipid peroxide damage to disease onset and progression in a transgenic model of familial ALS. *J. Neurosci. Res.* 53: 66-77

Halliwell, B. 1992. Reactive oxygen species and central nervous system. *J. Neurochem.*59: 1609-1623

Halliwell, B. 1996. Oxidative stress, nutrition, and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radic. Res*. 25(1): 57-74.

Handayani, A.D., Sutrisno., Indraswati, N. and Ismadji, S. 2008. Extraction of astaxanthin from giant tiger (*Panaeus monodon*) shrimp waste using palm oil: Studies of extraction kinetics and thermodynamics. *Bioresour. Technol.* 99(10): 4414-4419

Hansen, K.B., Tauson, A.H. and Inborr, J. 2001. Effect of supplementation with the antioxidant astaxanthin on reproduction, pre-weaning growth performance of kits and daily milk intake in mink. *J. Reprod. Fertil. Suppl*. 57: 331-334

Hara, S., Omata, T., Tanaka, Y., Hibino, H. and Totani, Y. 2001.Concentration of esterified astaxanthin in euphasid oil. *J. Oleo. Sci.*50: 73–76

Harman, D. 1981. The aging process. *Proc. Natl. Acad. Sci. USA.* 78: 7124-7128

Head, K.A. 2001. Natural therapies for ocular disorders, part two: cataracts and glaucoma. *Altern. Med. Rev.* 6: 141-166

Hellenbrand, W., Boeing, H., Robra, B.P. Seidler, A., Vieregge, P., Nischan, P., Joerg, J., Oertel, W. H., Schneider, E. and Ulm, G. 1996. Diet and Parkinson's disease. II: A possible role for the past intake of specific nutrients. Results from a self-administered food-frequency questionnaire in a case-control study. *Neurology.* 47: 644-650

Hemnani, T. and Parihar, M.S. 1998. Reactive oxygen species and oxidative DNA damage. *Ind. J. Physiol. Pharmacol*. 42: 440-452

Hennekens, C.H. 1999. Antioxidant vitamins and cardiovascular disease. *Antioxidant Status, Diet, nutrition, and Health.*(ed. Hennekens, C.H.). Boca Raton, CRC Press, pp. 463-477

Higuera-Ciapara, L., Felix-Valenzuela, F. and Goycoolea, F. 2006. Astaxanthin: A review of its chemistry and applications. *Critical Rev. Fd Sci. Nutr.* 46(2): 185-196

Hirano, S., Hakura, C., Seino, H., Akiyama, Y., Nouaka, I., Kambara, N. and Kawakami, T. 1990. Chitosan as an ingredient for domestic animal feeds. *J. Agric. Fd. Chem.*38: 214-217

Hirayama, O., Nakamura, K., Hamada, S. and Kobayashi, Y. 1994. Singlet quenching ability of naturally occurring carotenoids. *Lipids.* 29: 149-150

Hix, L.M., Lockwood, S.F. and Betram, J.S. 2004a. Upregulation of connexin 43 protein expression and increased gap junctional communication by water soluble disodium disuccinate astaxanthin derivatives. *Cancer. Lett.* 211: 25-37

Hix, L.M., Lockwood, S.F. and Betram, J.S. 2004b. Bioactive carotenoids: Potent antioxidants and regulators of gene epression. *Redox. Rep.*9: 181-191

Hoekzema, R., Verhagen, C., Van Haren, M. and Kijlstra, A. 1992. Endotoxin induced uvetis in the rat: significance of intraocular interleukin-6. *Invest. Opthamol. Vis. Sci.* 33: 532-539

Holanda, H.D.D. and Netto, F.M.. 2006. Recovery of components from shrimp (*Xiphopenaeus kroyeri*) processing waste by enzymatic hydrolysis. *J. Fd. Sci*. 71(5): 298-303

Hughes, D.A. 1999. Effects of dietary antioxidants on the immune function of middle-aged adults. *Proc. Nutr. Soc.* 58: 79–84

Hussein, G., Nakamura, M., Zhao, Q., Iguchi, T., Goto, H., Sankawa, U. and Watanabe, H. 2005. Antihypertensive and neuroprotective effects of astaxanthin in experimental animals. *Biol. Pharm. Bull.* 28(1): 47-52

Inborr, J. 1998. Haematococcus the poultry pigmentor. *Feed. Mix.* 6(2): 31-34

Inoue, T., Simpson, K.L., Tanaka, Y. and Sameshima, M. 1988. Condensed astaxanthin of pigmented oil from crawfish carapace and its feeding experiment. *Bull. Japn. Soc. Sci. Fish*. 54: 103–106

Ithayarasi, A.P., Padmavathy, V.N. and Devi, C.S. 1996. Effect of alpha-tocopherol on Isoproterenol induced myocardial infarction in rats-elecrocardiographic, biochemical and histological evidences. *Ind. J. Physiol. Pharmacol.* 40:297-302

Iwamoto, T., Hosoda, K., Hirano, R., Kurata, H., Matsumoto, A., Miki, W., Kamiyama, M., Ikatura, H., Yamamoto, S. and Kondo, K. 2000. Inhibition of low-density lipoprotein oxidation by astaxanthin. *J. Atheroscler. Thromb.* 7: 216-222

Jacques, P. 1999. The Potential preventive effects of vitamins for cataract and age related macular degeneration. *Int. J. Vitam. Nutr. Res.*69: 198-205

Je, J.Y., Park, P.J. and Kim, S.K.2004.Radical scavenging activity of hetero-chitooligosaccharides. *Eur. Fd. Res. Technol.* 219: 60-65

Jenson, C., Birk, E., Jokumsen, A., Skibsted, L.H. and Bertelsen, G. 1998. Effect of dietary levels of fat, α-tocopherol and astaxanthin on colour and lipid oxidation during storage of frozen rainbow trout (*Oncorhychus mykiss)* and during chill storage of smoked trout. *Z. Lebensm. Unters. Forsch. A.*207: 189-196

Jeon, Y.J. and Kim, S.K. 2002. Antitumour activity of chitosan oligosaccharides produced in an ultra filtration membrane reactor system. *J. Microbiol. Biotechnol.* 12: 503-507

Jeon, Y.J., Park, P.J. and Kim, S.K. 2001.Antimicrobial effect of chitooligisaccharides produced by bioreactor. *Carbohyd. Polym.* 44: 71-76

Jewell, C. and O'Brien, N. 1999. Effect of dietary supplementation with carotenoids on xenobiotic metabolizing enzymes in the liver, lung, Kidney and small intestine of the rat. *Br. J. Nutr.*81: 235-242

Jilal, I. and Fuller, C.J. 1995. Effect of vitamin E, vitamin C and beta-carotene on LDL oxidation and atheroscelorosis.*Can. J. Cardiol.* 11: 97-103.

Joffe, S., Thomas, R. 1989. Phytochemicals: a renewable global resource. *Biotech News Information*. 1: 697-700.

Johnson, E.A. and An, G.H. 1991. Astaxanthin from microbial sources. C*rit. Rev. Biotechnol.* 11: 297-326

Jorgensen, K. and L. Skibsted. 1993. Carotenoid scavenging radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. *Z. Lebensm. Unters Forsch.* 196:423-429.

Jyonouchi, H., Hill, R.J., Tomita, Y. and Good, R.A. 1991. Studies of immunomodulating actions of carotenoids. I. Effects of beta-carotene and astaxanthin on murine lymphocyte functions and cell surface marker expression in in vitro culture system. *Nutr Cancer.* 16(2): 93-105

Jyonouchi, H., Sun, S. and Gross, M. 1995a. Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: astaxanthin, a carotenoid without vitamin A activity, enhances in vitro immunoglobulin production in response to a T-dependent stimulant and antigen. *Nutr. Cancer* 23: 171–183

Jyonouchi, H., Sun, S., and Mikokami, M. 1996. Effects of various carotenoids on cloned, effector-stage T-helper cell activity. *Nutr. Cancer.* 36: 59–65

Jyonouchi, H., Sun, S., Iijima, K. and Gross, M.D. 2000. Antitumour activity of astaxanthin and its mode of action. *Nutr. Cancer*. 36: 59–65

Jyonouchi, H., Sun, S., Tomita, Y. and Gross, M.D. 1995b. Astaxanthin, a carotenoid without vitamin A activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen. *J. Nutr.* 124: 2483–2492

Jyonouchi, H., Zhang, L. and Tomita, Y. 1993. Studies of immunomodulating actions of carotenoids. II. Astaxanthin enhances in vitro antibody production to T-dependent antigens without facilitating polyclonal B-cell activation. *Nutr. Cancer.* 19: 269–280

Jyonouchi, H., Zhang, L., Gross, M. and Tomita, Y. 1994. Immunomodulating actions of carotenoids: enhancement of *in vivo* and *in vitro* antibody production to T-dependent antigens. *Nutr. Cancer*. 21: 47–58

Kakkar, P., Das, B. and Vishwanathan, P.N. 1984. A modified spectrophotometric assay of superoxide dismutase. *Ind. J. Biochem. Biophys.* 21: 130-132

Kalinowski, C.T., Robaina, L.E., Fernandez-palacios, H., Schuchardt, D. and Izquierdo, M.S. 2005. Effect of different carotenoid sources and their dietary levels on red porgy (*Pagrus pagrus*) growth and skin colour. *Aquaculture*. 244: 223-231

Kamath, B.S., Srikanta, B.M., Dharmesh, S.M., Sarada, R. and Ravishankar G.A. 2008. Ulcer preventive and antioxidant properties of astaxanthin from *Hematococcus pluvialis.Eur. J. Pharmacol* .590(1-3): 387-395

Kang, C.D. and Sim, S.J. 2008. Direct extraction of astaxanthin from Haematococcus culture using vegetable oils. *Biotech. Lett.* 30(3): 441-444

Kang, J.O., Kim, S.J. and Kim, H. 2001. Effect of astaxanthin on the hepatotoxicity, lipid peroxidation and antioxidative enzymes in the liver of CCl4-treated rats. *Methods. Find. Exp. Clin. Pharmacol.* 23(2): 79-84

Kapoor, B.G. and Khanna, B. 2004. *Icthyology Handbook.* Narosa Publishing House, New Delhi, 1059 p

Karrer, P. and Jucker, E. 1950. *Carotenoids.* Elsevier Publishing Company, New York, 384 p

Katayama, T., Hirata, K. and Chichester, C.O. 1971. The biosynthesis of astaxanthin-IV. The carotenoids in the prawn, *Penaeus japonicus. Bull. Japan. Soc. Fd. Sci. Technol.* 37(7): 614-620

Katayama, T., Kunisaki, T., Shimaya, M., Simpson, K.L. and Chichester, C.O. 1973. *Comp. Biochem. Physiol.* 46: 269-272

Kawakami, T., Tsushima, M., Katabami, Y., Mine, M., Ishida, A. and Matsuno, T. 1998. Effect of B, B-carotene, B-echinenone, astaxanthin, fucoxanthin, vitamin A and vitamin E on the biological defense of sea urchin *Pseudocentrotus depressus*. *J. Exp. Mar. Biol. Ecol.* 226: 165-174

Kelley, C.E. and Harmon, A.W. 1972. Method of determining carotenoid contents of Alaska pink shrimp and representative values for several shrimp products. *Fish. Bull.* 70: 11-17

Khanfari, A., Saberi, A., Azar, M., Vosooghi., Gh., Jamili, Sh. and Sabbaghzadeh, B. 2007. Extraction of astaxanthin esters from shrimp waste by chemicaland microbial methods. *Iranian. J. Environmental Health. Sci. Engng*. 4(2): 93-98

Kim, J.H., Kim, Y.S., Song, G.G., Park, J.J. and Chang, H.I. 2005. Protective effects of astaxanthin on naxopen-induced gastric antral ulceration in rats. *Eur. J. Pharmacol.* 514 (1): 53-59

Kim, S., Mendis, E. and Sahidi, F. 2008. Marine fisheries by products as potential nutraceuticals: an overview. *Marine Neutraceuticals and Functional Foods.*(eds. Barrow, C. and sahidi, F.). CRC Press, Boca Raton, pp. 1-23

Kobayashi, M. and Sakamoto, Y. 1999. Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis. Biotechnol. Lett.* 21: 265-269

Kobayashi, M., Kurimura, Y., Sakamoto, Y. and Tsuji, Y. 1997. Selective extraction of astaxanthin and chlorophyll from the green alga *Haematococcus pluvialis*. *Biotech. Tech.* 11(9): 657-660

Kozo, F. 1997. *Extraction of astaxanthin from shell of lobster or shrimp or crab and apparatus therefore*. Japanese patent No. JP 9301950A

Kozuki, Y., Miura, Y. and Yagasaki, K. 2000. Inhibitory effects of carotenoids on the invasion of rat ascites hepatoma cells in culture. *Cancer. Lett.* 151(1): 111-115

Kramer, H.J., Lehnert, M., Michel, H. and Predel, H.G.1991.Endogenous natriuretics and oubain like factors; their roles in body fluid volume and BP regulation. *Am. J. Hyperten.*1:81-89

Krappi, J., Rissanen, T. H., Nyyssonen, J., Olsson, A. G., Voutilainen, S. and Salonen, J. T. 2007. Effects of astaxanthin supplementation on lipid peroxidation. *Int. J. Vit. Nutr. Res*. 77(1): 3-11

Kritchevsky, S.B. 1999. b-Carotene, carotenoids and the prevention of coronary heart disease. *J. Nutr*. 129: 5–8

Kumar, P.V., Kuttan, R. and Kuttan,G. 1999. Effect of "Rasayanas", a herbal drug preparation on immune responses and its significance in cancer treatment. *Indian J. Exp. Biol*. 37: 27-31

Kuo, H. C., Lee, T.C., Kamata, T. and Simpson, K.L. 1976. *Alimenta.* 15: 47-51

Kurashige, M., E. Okimasu, M. Inoue, and K. Utsumi. 1990. Inhibition of oxidative injury of biological membranes by astaxanthin. *Physiol. Chem. Phys. & Med. NMR* 22: 27-38.

Kurihara, H., Koda, H., Asami, S, Kiso, Y. and Tanaka, T. 2002. Contribution of the antioxidative property of astaxanthin to its protective effect on the promotion of cancer metastasis in mice treated with restraint stress. *Life. Sci.*70: 2509-2520

Kushner, I. 1990. C-reactive protein and the acute phase response. *Hospital Practice*. 30: 13-28

Landrum, J.T., Bone, R.A., Moore, L. L. and Gomez, C.M. 1999. Analysis of zeaxanthin distribution within individual human retinas. *Methods. Enzymol*. 299: 457–467

Larsen G. L. and Henson P. M. 1983. Mediators of inflammation. *Ann. Rev. Immunol.* 1: 335-344

Lauver, D.A., Lockwood, S.F., Luccessi, B.R. 2005. Disodium disuccinate astaxanthin (Cardax) attenuates complement activation and reduces myocardial injury following ischemia/reperfusion. *J. Pharmacol. Exp. Ther.*314(2): 686-692

Lawlor, S. M. and O'Brien, N. M. 1995. Astaxanthin: antioxidant effects in chicken embryo fibroblasts. *Nutr. Res*., 15:1695-1704

Lee, J., Jiang, S. and Watson, R.R. 2000. Carotenoid supplementation reduces erythema in human skin after simulated solar radiation exposure. *Proc. Soc. Exp.Biol. Med*. 223(2): 170–174

Lee, S.H., Roh, S.K., Park, K. H. and Yoon, K. 1999. Effective extraction of astaxanthin pigment from shrimp using proteolytic enzymes. *Biotechnol. Bioprocess Engng*.4(3): 199-204

Lee, S.J., Bai, S.K., Lee, K.S., Namkoong, S., NaIIJ, Ila, K.S., Ilan, J.A., Yim, S.V., Chang,K., Kwon, Y.G., Lee, S.K. and Kim, Y.M. 2003. Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing IαB kinase-dependent NF-αB activation. *Moll. Cells.*16: 97-105

Li, H., Tyndale, S.J., Heath, D.D. and Letcher, R.J. 2005. Determination of carotenoids and all-trans-retinol in fish eggs by liquid chromatography-electrospray ionization-tandem mass spectrometry. *J. Chrom.* 816: 49-56

Li, W., Hellsten, A., Jacobsson, L.S., Blomqvist, H.M., Olsson, A.G. and Yuan, X.M. 2004. Alpha-tocepherol and astaxanthin decrease macrophage infiltration, apoptosis and vulnerability in atheroma of hyperlipidaemic rabbits. *J. Mol. Cell. Cardiol.* 37: 919-948

Li, Z., Wang, Y. and Mo, B. 2002. The effects of carotenoids on the proliferation of human breast cancer cell and gene expression of bcl-2. *Zhonghua Yu Fang Yi Xue Za Zhi.* 36(4): 254-257

Lignell, A. 1999. *Medicament for improvement of duration of muscle function or treatment of muscle disorders or disease*. World Patent. No. 9929313. Astacarotene AB, Sweden

Lim, B.P., A. Nagao, J. Terao, K. Tanaka, T. Suzuki, K. Takama .1992. Antioxidant activity of xantophylls on peroxyl radical-mediated phospholipid peroxidation. *Biochim. Biophys. Acta.* 1126(2): 178-184

Lin, W., Chien, J. and Chen, B. 2005. Determination of carotenoids in spear shrimp shells (*Parapenaeopsis hardwickii*) by liquid chromatography. *J. Agric. Fd. Chem.* 53(13): 5144-5149

Liu, X. and Osawa, T. 2007. Cis astaxanthin and especially 9-cis astaxanthin exhibits a higher antioxidant activity *in vitro* comparedto all-trans isomer. *Biochem. Biophys. Res. Commun. Biochem. Biophys. Res. Commun.* 357(1): 187-193

Lockwood, S.F. and Gross, G.J. 2005. Disodium disuccinate astaxanthin (Cardax): antioxidant and anti-inflammatory cardioprotection. *Cardiovasc. Drug. Rev.* 23(3): 199-216

Lopez, M., Arce, L., Garrido, J., Rio, S. and Vakarcel, M. 2004. Selective extraction of astaxanthin from crustaceans by use of supercritical carbon dioxide. *Talanta.*64: 726-731

Lopez-Cervantes, J., Sanchez-Mahado, D.I., Gutierrez-Coronado, M.A. and Rios-Vazquez, N.J. 2006. Quantification of astaxanthine in shrimp waste hydrolysate by HPLC. *Biomed. Chrom.*20: 981-984

Lorenz, R.T. and Cysewski, G.R. 2000. Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends Biotechnol.* 18: 160–167

Lyle, B.J., Mares-Perlman, J.A., Klein, B.E.K., Klein, R. and Greger, J.L. 1999. Antioxidant intake and risk of incident age related nuclear cataracts in the Beaver Dam Eye Study. *Am. J. Epidemiol*. 149: 801–809

Ma, Y., Mizuno, T. and Ito, H. 1991. Antitumour activity of some polysaccharides isolated from a Chinese mushroom "Hungmo" the fruiting body of *Hohnbuehelia serotina. Agric. Biol. Chem.* 44: 2701-2710

Machley, A.C and Chance, B. 1954. *Methods of Biochemical Analysis.* Vol.I.(ed. Glick, D.). Inter science, New York, pp.357-360

Madhavan, P. 1992. *Chitin, Chitosan and their Novel Applications*. Popular Science lecture Series-April 1992. Sponsored by the Department of Biotechnology, ministry of Science and Technology, Government of India. Organised by CIFT, Kochi, 45 p

Madhavan, P. and Nair, K.G.R. 1974. Utilization of prawn waste-Isolation of chitin and its conversion to chitosan. *Fish. Technol*. 11: 50-55

Madhavan, P. and Nair, K.G.R. 1975. Chitosan from squilla. *Fish. Technol*. 12: 81-82

Madhavan, P. and Nair, K.G.R. 1977. *Proc. 1st International Conference on Chitin/Chitosan,* Boston, U.S.A. May, 1977, 444

Mahmoud, F.F., Haines, D.D., Abul, H.T., Abal, A.T., Onadeko, B.O. and Wise, J.A. 2004. *In vitro* effects of astaxanthin combined with ginkgolide B on T lymphocyte activation in peripheral blood mononuclear cells from asthmatic subjects. *J.Pharmacol. Sci.* 94: 129-136

Manasveta, P., Worawattanamateekul, W., Latscha, T. and Clark, J.S. 1993. Correction of black tiger prawn (*Penaeus monodon Fabricius*) colouration by astaxanthin. *Aquat. Engng.* 12: 203-213

Mandeville, S., Yaylayan, V., Simpson, B.K. and Ramaswamy. 1991. Isolation and purification of carotenoid pigments, lipids and flavour active components from raw commercial shrimp waste. *Fd. Biotechnol.* 5: 185-195

Maoka, T. and Akimoto, N. 2008. Carotenoids and their fattyacid esters of spiny lobster *Panulirus japonicus. J. Oleo. Sci.* 57(3): 145-152

Markesbery, W.R. and Carney, J.M. 1999. Oxidative alternations in Alzheimer's disease. *Brain. Pathol.* 9: 133-146

Marnett, L.J. 1987. Peroxyl free radicals: potential mediators of tumor initiation and promotion. *Carcinogenesis*. 8: 1365-1373

Masatoshi, M.. and Junji,S. 1999. Method of simultaneously producing astaxanthin and chitosan from shell waste. Japanese Patent No.JP 11049972A2.

Mathew, P. and Nair, K.G.R. 2006. Ensilation of shrimp waste by *Lactobacillus fermentum. Fish. Technol.* 43: 59-64

Mathew, P.T., Nair, K.G.R., Madhavan, P. and Prabhu, P.V. 1989. Effect of chitin and prawn shell on the growth of albino rat. *Fish. Technol*. 26: 36

Matsuno, T. 2001. Aquatic animal carotenoids. *Fish. Sci.* 67:771-783

Matsuno, T. and Maoka, T. 1988. The carotenoid of crab *Paralithodes brevipes. Bull. Japan. Soc. Sci. Fisheries.* 54: 1437-1442

Matsuno, T., Nagata, S. and Chiba, K. 1975. Comparitive biochemical studies on carotenoids V.Comparitive studies on carotenoids in fresh water and marine striped mullet. *Bull. Japan. Soc. Sci. Fish.* 41: 459-464

Matsuno, T., Watanabe, T. and Nagata, S. 1974. Carotenoid pigments of crustacean I. The carotenoid pigment of *Scyllarides squamosus* and *Parribacus antarticus. Bull. Japan. Soc. Sci. Fish.* 40: 619-624

Matsushita, Y., Suzuki, R., Nara, E., Fokoyama, A. and Miyashite, K. 2000. Antioxidant activity of polar carotenoids including astaxanthin-β-glucoside from marine bacterium on PC liposomes. *Fish. Sci.*66: 980-985

Mc Vean, M., Kramer-Stickland, K. and Liebler, D.C. 1999.Oxidants and antioxidants in ultraviolet induced nonmelanoma skin cancer.*Antioxidant Status, Diet, Nutrition, and Health.* Boca Raton, CRC Press, pp. 400-430

McCord, J.M. and Fridovich, I. 1969. Superoxide dismutase and enzymatic function of erythrocuprein. *J. Biol. Chem.* 244: 6049-6055

McCoy, M. 1999. Astaxanthin market a hard one to crack. *Chem.News.* 77: 15-17

Mehrotra, N.N. 1992. *A handbook of Practical and Clinical Immunology* (eds. Talwar, G.P. and Gupta, S.K.), CBS Publishers, New Delhi. Vol.I. pp.242-269

Meland, N. 2008. *Astaxanthin and Health.* Igene Biotechnology Inc, 9 p

Metcalfe, L.D., Schimitz, A.A. nd Ratta, J.R. 1966. Rapid preparation of fatty acid esters from lipids for chromatograph analysis. *Anal. Chem.*38: 514-519

Meyers, S.P. 1993. The biological role of astaxanthin in salmonids and other aquatic species. *Proceedings of the First International Symposium on Natural Colors for Foods, Nutraceuticals, Beverages and Confectionary* (ed. Francis, F.J.). The Herald Organization,S.I.C. Publishing Company,Amherst,USA

Meyers, S.P. and Bligh, D. 1981. Characterisaton of astaxantin pigment from heat processed craw fish waste. *J.Agric.Fd. Chem*.29: 505-508

Meyers, S.P. and Chen, H.M. 1985. *Process for utilization of shellfish waste.* US Patent. No. 4505936

Meyers, S.P. and Rutledge, J.E. 1971. Shrimp meal – a new look at an old product. *Feedstuffs*. 43(49): 31-32

Miki,W., Hosada, K., Kondo, K. and Itakura, H. 1998. *Astaxanthin-Containing Drink*. Japanese Patent, No.10155459

Miki. W. 1991.Biological functions and activities of animal carotenoids. *Pure Appl Chem*. 63:141–146

Mitchell, J., Paiva, M., Heaton, M.B. 1999. Vitamin E and beta-carotene protect against ethanol combined with ischemia in anembryonic rat hippocampal culture model of fetal alcohol syndrome. *Neurosci. Lett.* 263: 189-192

Mizani, M., Aminlari, M. and Khodabandeh, M. 2005. An effective method for producing a nutritive protein extract powder from shrimp head waste. *Fd. Sci. Tech. Int.* 11: 49-54

Moller, P., Wallin, H., and Knudsen, L. E. 1996. Oxidative stress associated with exercise, psychological stress, and life-style factors. *Chem. Biol. Interact*., 102(1): 1-36

Moody, C.S., and H.M. Hassan. 1982. Mutagenicity of oxygen free radicals. *Proc. Natl. Acad. Sci. USA.*79: 2855-2859

Moorjani, M.N., Achinta, V. and Khasim, D.I. 1975. Parameters affecting viscosity of chitosan from prawn waste. *J. Fd. Sci. Technol*. 12: 187-191

Moorjani, M.N., Khasim, D.I., Rajalakshmi, S and Amla, B.L. 1977. Chitosan of high viscosity and protein as valuable by-products. *Proc. 1st International Conference on Chitin/Chitosan,* Boston, U.S.A. May, 1977

Mortensen, A., Skibsted, L. H., Sampson, J., Rice-Evans, C., and Everett, S. A. 1997. Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. *FEBS Letters*. 418: 91-97

Mossmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods* 65: 55-61

Muller, K., Carpenter, K.L., Challis, I.R., Skepper, J.N. and Arends, M.J. 2002. Carotenoids induce apoptosis in the T-lymphoblast cell line *Jurkat* E6.1. *Free. Radic. Res.* 36(7): 791-802

Murakami, A., Nakamura, Y. and Tanaka, T. 2000. Suppression by citrus auraptene of phorbol ester and endotoxin induced inflammatory responses: role of attenuation of leukocyte activation. *Carcinogenesis.*21: 1843-1850

Murillo, E. 1992. Hypercholesterolemic effect of canthaxanthin and astaxanthin in rats. *Arch. Latinoam. Nutr*. 42: 409–413

Naguib, Y.M.A. 2000. Antioxidant activities of astaxanthin and related carotenoids. *J. Agri. Fd. Chem*. 48: 1150 – 1154

Nagyvary, J.J., Falk, J.D., Hill, M.L., Scmidt, M.L., Wilkino, A. and Bradbury, E.L. 1979. The hypolipidemic activity of chitosan and other polysaccharides in rats.*Nutr. Rep. Int.* 20: 677-684

Nair, K.G.R. Madhavan, P. 1975. Deacetylation of Chitin – I. *Proceedings of Symposium of Fish Processing Industry in India, February 13-14, 1975,* Association of Food Scientists and Technologists, Mysore (India), pp 98

Nair, K.G.R. Madhavan, P. 1984. Chitosan for removal of mercury from water. *Fish. Technol*. 21(2): 110-112

Nair, K.G.R. Madhavan, P. 1989. Advances in chitin research. *Recent Trends in Processing Low Cost Fish* (eds. Balachandran, K.K., Perigreen, P.A., Madhavan, P. and Surendran, P.K.). Society of Fishery Technologists (India), Kochi, pp. 174

Nair, K.G.R., Madhavan, P. and Gopakumar, K. 2002. Advances in fishery by-products technology. *Textbook of Fish Processing Technology*. First edition. Indian Council of Agricultural Research, New Delhi, pp. 467-483

Nair, K.G.R., Mathew, P.T., Madhavan, P. and Prabhu, P.V. 1987. Chitin preparation from prawn shell. Indian. *J. Poult. Sci.* 22: 40

Naito, Y., Uchiyama, K., Aoi, W., Hasegawa, G., Nakamura, N., Yoshida, N., Maoka, T., Takahashi, J. and Yoshikawa, T. 2004. Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice. *Biofactors.* 20(1): 49-59

Nakagawa, K., S. Kang, D. Park, G. Handelman, and T. Miyazawa. 1997. Inhibition by beta-carotene and astaxanthin of NADPH-dependent microsomal phospholipid peroxidation. *J. Nutr. Sci. and Bitamin.* 43(3):345-355.

Nelson, D.A. and Davey, F.R., *Haematology* (eds. Bentler, E., Litchman, M.A., Coller, B.S. and Kipps, T.J.). McGraw Hill, New York, pp. 1-70

Nieuwerburgh, L.V., Wänstrand, I., Liu, J. and Snoeijs, P. 2005. Astaxanthin production in marine pelagic copepods grazing on two different phytoplankton diets. *J. Sea. Res.* 53(3): 147-160

Nirmala, C. and Puvanakrishnan, R. 1996a. Effect of curcumin on certain lysosomal hydrolases in Isoproterenol induced myocardial infarction in rats. *Biochem. Pharmacol.* 51:47-51

Nirmala, C. and Puvanakrishnan, R. 1996b. Protective role of curcumin against Isoproterenol induced myocardial infarction in rats. *Mol. Cell Biochem.* 159:85-93

No, H. K., Meyers, S. P. and Lee, K. S. 1989. Isolation and characterization of chitin from crawfish shell waste. *J. Agric. Fd. Chem.* 37: 575-579

No, H.K. and Meyers, S.P. 1992. Utilization of crawfish processing wastes as carotenoid, chitin and chitosan source. *J. Korean Soc. Fd. Nutr.* 21: 319–326

Nobre, B., Marcelo, F., Passos, R., Beirao, L., Palavra, A., Gouveia, L. and Mendes, L. 2006. Supercritical carbon dioxide extraction of astaxanthin and other carotenoids from the microalga *Haematococcus pluvialis*. *Eur. Fd. Res. Technol.* 223: 787-790

Noguchi, N., and E. Niki. 1999. Chemistry of active oxygen species and antioxidants. *Antioxidant Status, Diet, Nutrition, and Health* (ed. Papas, A.M..). Boca Raton: CRC Press

Noronha-Dutra, A.A., Steen, E.M. and Woolf, N. 1984. The correlation between catecholamine and lipid peroxidation induced in heart cells. *Basic. Res. Cardiol.* 80: 133-136

O'Connor, I. and O'Brien, N. 1998. Modulation of UVA light-induced oxidative stress by beta-carotene, lutein and astaxanthin in cultured fibroblasts. *J. Dermatol. Sci.* 16: 226–230

Odeberg, M.J., Lignell, A., Petterson, A. and Hoglund, P. 2003. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. *Eur. J. Pharm. Sci.* 19(4): 299-304

Oghami, K., Shiratori, K., Kotake, S., Nishida, T, Mizuki, N., Yazawa, K. and Olino, S. 2003. Effects of astaxanthin on lipopolysaccharide-induced inflammation *in vitro* and *in vivo*. *Invest. Ophthalmol. Vis. Sci.* 44(6): 269-2701

Ohkawa, H., Ohishi, N. and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analyt. Biochem.*95: 351-358

Okada, S., Nur-E-Borhan, S.A. and Yamaguchi, K. 1994. Carotenoid composition in the exoskeleton of commercial black tiger prawns. *Fish. Sci.* 60: 213

Okai, Y. and Okai, H.K. 1996. Possible immunomodulating activities of carotenoidsin *in vitro* cell culture experiments. *Int. J. Immunopharmacol.* 18: 753-758

Olaizola, M. 2000. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000 – liter outdoor photobioreactors. *J. Appl. Phycol.* 12: 499-506

Omara-Alwala, T.R., Chen, H.M., Ito, Y.M., Simpson, K.L. and Meyers, S.P., 1985. Carotenoid pigment and fatty acid analysis of crawfish oil extracts. *J. Agric. Fd. Chem*. 33: 260–263

Oshima, S., F. Ojima, H. Sakamoto, Y. Ishiguro, and J. Terao. 1993. Inhibitory effect of ß -carotene and astaxanthin on photosensitized oxidation of phospholipid bilayers. *J. Nutr. Sci. Vitaminol.* 39: 607-615

Osterlie, M., Bjerkeng, B., Liaaen-Jensen, S. 2000. On bioavailability and deposition of bent Z-isomers of astaxanthin.*Pigments in Food Technology*. (eds. Mosquera, M.M.I., Galan, M.J. and Mendez, D.H.). Proceedings of the First International Congress, PFT, pp. 157-161

Pacheco. N., Garnica-González, M., Ramírez-Hernández, J.Y., Flores-Albino, B., Gimeno, M., Bárzana, E. and Shirai, K. 2009. Effect of temperature on chitin and astaxanthin recoveries from shrimp waste using lactic acid bacteria. *Bioresour. Technol.* 100(11): 2849-2854

Palozza, P. and Krinsky, N.I. 1992b. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. *Arch. Biochem.Biophys*. 297: 291–295

Palozza, P., and Krinsky, N.I. 1992a. Antioxidant effects of carotenoids *in vivo* and *in vitro*: an overview. *Meth. Enzymol*. 213: 403-420

Papas, A.M. 1999. Determinants of antioxidant status in humans. *Antioxidant Status, Diet, Nutrition, and Health.* (ed. Papas, A.M.). Boca Raton: CRC Press, pp. 21-36

Parada, J. and Anguilera, J. M. 2007. Food microstructure affects the bioavailability of several nutrients. *J. Fd. Sci*. 72(2): 21-32

Parajo, J.C., Santos, V. and Vazquez, M. 1998. Production of carotenoids by *Phaffia rhodozyma* growing on media made from hemicellulosic hydrolysates of *Eucalyptus globulus* wood. *Biotechnol. Bioengng.* 59: 501-506

Park, P.J., Je, J.Y., and Kim, S.K.2003a. Free radical scavenging activity of chitooligosaccharides by electron spin resonance spectrometry. *J. Agric. Fd. Chem.* 51: 4624-4627

Park, P.J., Je, J.Y., and Kim, S.K.2003b. Angiotensin I converting enzyme (ACE) inhibitory activity of hetero-chitooligosaccharides prepared from partially different deacetylated chitosans. *J. Agric. Fd. Chem.* 51: 4930-4934

Park, P.J., Je, J.Y., Byun, H.G., Moon, S.H. and Kim, S.K. 2004a. Antimicrobial activity of hetero-chitosans and their oligosaccharides with different molecular weights. *J. Microbiol. Biotechnol.* 14: 317-323

Park, P.J., Je, J.Y., Jung, W.K., Ahn, C.B. and Kim, S.K. 2004b. Anticoagulant activity of hetero-chitosan and their oligosaccharide sulfates. *Eur. Fd. Res. Technol.* 219: 529-533

Patterson, J.W. and Lazarrow, A. 1955. *Methods of Biochemical Analysis,* Vol. 2, (ed. Glick, D.). Interscience, New York, pp. 259-278.

Peterson, D.H., Jager, H.K., Savage, G.M., Washburn, G.N. and Westers, H. 1966. *Trans. Am. Fish. Soc.* 95: 408-414

Phelps, D.L. 1987. Current perspectives on vitamin E in infant nutrition. *Am. J. Clin. Nutr*. 46: 187-191.

Pintea, A., Bele, C., Anderi, S. and Socaciu, C. 2003. HPLC analysis of carotenoids in four varieties of *Calendula officinalis* L. flowers. *Acta. Biologica. Szegediensis.* 47(1): 37-40

Prabhu, P.V., Radhakrishnan, A.G. and Iyer, T.S.G. 1976. Chitosan as a water clarifying agent. *Fish. Technol*. 13(1): 69-72

Pratico, D. 2002. Alzheimer's disease and oxygen radicals: new insights. *Biochem. Pharmacol.* 15: 563-567

Quaglia, F. B. and Orban, E. 1987. Influence of the degree of hydrolysis on the solubility of the protein hydrolysates from sardine (*Sardina pilchardus*). *J. Sci. Fd. Agric.* 38: 271-276

Quan, C. and Turner, C. 2009. Extraction of astaxanthin from shrimp waste using pressurized hot ethanol. *Chromatographia.* 70(1-2): 247-251

Radhakrishnan, A.G. and Prabhu, P.V. 1971. Chitosan preparation from prawn waste. *Res. Ind.* 16(4): 265

Ramachandran, P., Viswanathan, T.V., George, M.C. and Mercy, A.D. 1997. Nutritive and microbiological evaluation of prawn waste rice bran silage for ruminants. *J. Vet. Sci.* 28: 1-5

Rao, B., Bai, M.V. and Naseema, K. 1994. Studies on safety and potential of chitosan as a biomaterial. *Nutrients and Bioactive Substances in Aquatic Organisms*.(eds. Devadasan, K and others). Society of Fishery Technologists (India), Kochi, pp. 82

Ravichandran, L. V., Puvanakrishnan,R. and Joseph K.T. 1990. Alterations in the heart lysosomal stability in Isoproterenol induced myocardial infarction in rats. *Biochem. Int.* 22:387-396

Rebeca, B., Pena-Vera, M.T. and Diaz-Castaneda, M . 1991. Production of fish protein hydrolysates with bacterial proteases: yield and nutritional value. *J. Fd. Sci.* 56: 309-314

Reiter, R. J. 1998. Oxidative damage in the central nervous system: protection by melatonin. *Prog. Neurobiol.* 56(3): 359-384

Reiter, R. J., Carneiro, R. C., and Oh, C. S. 1997. Melatonin in relation to cellular antioxidative defense mechanisms. *Horm. Metab. Res*. 29(8): 363-372

Rengel, D., Diez-Navajas, A., Serna-Rico, A., Veiga, P., Muga., A. and Milicua, J.C. 2000. Exogenously incorporated ketocarotenoids in large unilamellar vesicle-Protective activity against perooxidation. *Biochim. Biophys. Acta.* 1463(1): 179-187

Renstrom, B. and Liaaen-Jensen, S. 1981. Fatty acid composition of some esterified carotenols.*Comp. Biochem. Physiol.* 69B: 625-627

Ridker, P.M. 2000. Novel risk factors for coronary disease.*Adv. Intern. Med.* 45: 391-418

Roe, J.H. 1954. *Methods of Biochemical Analysis,* Vol. 1, (ed. Glick, D.). Interscience, New York, pp. 115-139

Rona, G., Chappel, C.L., Balazs, T. and Gudry, R. 1959. An infarct like myocardiallesim and other toxic manifestations produced by isoproterenol in the rat. *Arch.Pathol*. 67: 443

Rotruck, J.J., Pope, A.L., Gantha, H. E. and Swanson, A. B. (1973). *Science.* 179: 588-590

Rutledge, J.E. 1971. Decalcification of crustacean meals. *J. Agric. Fd. Chem*. 19(2): 236

Sachindra, N.M. and Mahendrakar, N.S. 2005. Process optimization for extraction of carotenoids from shrimp waste with vegetable oils. *Bioresour. Technol*. 96: 1195-1200

Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. 2005a. Carotenoids in different body components of Indian shrimps. *J. Sci. Fd. Agric*. 85: 167-172

Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. 2006a. Recovery of carotenoids from shrimp waste in organic solvents. *Waste. Mgmt*. 26: 1092-1098

Sachindra, N.M., Bhaskar, N. and Mahendrakar, N.S. 2006b. Carotenoids in *Solonocera indica* and *Aristeus alcocki*, deep-sea shrimp from Indian waters. *J. Aqua. Fd. Prod. Technol.*15(2): 5 - 16

Sachindra, N.M., Bhaskar, N. Siddegowda, G.S., Sathisha, A.D.and Suresh, P.V. 2007. Recovery of carotenoids from ensilaged shrimp waste. *Bioresour.Technol.* 98(8): 1642-1646

Sachindra, N.M., Bhaskar, N., Sakhare, P.Z., Mahendrakar, N.S. and Narasimha Rao, D.2001. *An improved process for recovery of carotenoids from crustacean waste.* Indian Patent No.95. *Delhi*

Sachindra,N.M., Bhaskar, N., Mahendrakar,N.S 2005b. Carotenoids in crabs from marine and fresh waters of India. *Lebens Wiss. Technol*.38: 221-225

Shahidi, F. and Synowieki, J. 1991. Isolation and characterisation of nutrients and value added products from snow crab (*Chinoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *J. Agric. Fd. Chem.* 39: 1527-1532

Shahidi, F., Metusalach. Brown, J.A. 1998. Carotenoid pigments in seafoods and aquaculture. *CRC Crit. Rev. Fd. Sci.* 38(1):1-67

Saito, A. and Reiger, L.W. 1971.Pigmentation of brook trout (*Salvelinus fontinalis*) by feeding dried crustacean waste. *J. Fish. Res. Bd. Can.* 28: 509-512

Sambasivan, M. 1992. *Chitosan and its haemostatic activity*. Popular Science Lecture Serier-April 1992. Sponsored by the Department of Biotechnology, Government of India. Organised by CIFT, Kochi, 8 p.

Sampalis, F., Bunea, R., Pelland, M.F., Kowalski, O., Duguet, N. and Dupuis, P. 2003. Evaluation of the effects of Neptune krill oil on the management of premenstrual syndrome and dysmenorrhea. *Ageing Rev. Med.* 8(2) :171-179

Sang, W., Li, H., Liu, B., Zhou, X. and Jin, Z. 2002. Extraction and its partial characterization of pigments from shrimp shell. Annual Meeting and Food Expo, 18th June, 2002. Anaheim, California. *Abstract*: 42

Santos, M.F., and Mesquita, J.F. 1984. Ultrastructural study of *Haematococcus lacustris* (Girod) Rostafinski (Volvocales) I. Some aspects of carotenogenesis. *Cytologia.* 49: 215-228

Sathish, V., Vimal, V., Ebenezar, K.K. and Devaki, T. 2002. Synergistic effect of nicorandil and amlaodipine on mitochondrial function during Isoproterenol induced myocardial infarction in rats. *J. Pharm. Pharmacol.* 54:133-137

Savoure, N., Briand, G., Amory-Touz, M. C., Combre, A., Maudet, M. and Nicol, M. 1995. Vitamin A status and metabolism of cutaneous polyamines in the hairless mouse after UV irradiation: action of b-carotene and astaxanthin. *Int. J. Vitam. Nutr. Res*. 65: 79–86

Schmidi, M.K. 1993. Food products for medical purposes. *Trends. Fd. Sci. Technol.* 4: 163-168

Seddon, J. M., Ajani, U.A. and Sperduto, R.D. 1994. Dietary carotenoids, vitamin A, C, E and advanced age-related macular degeneration. *J. Am. Med. Assoc.* 272: 1413-1420

Segner, H., Arend, P., Peppinghausen, K.V. and Smith, H. 1989. The effect of feeding astaxanthin to *Oreochromis niloticus* and *Colisa labiosa* on the histology of liver. *Aquaculture.*79: 381-390

Sen, D.P. 2005. *Advances in Fish Processing Technology*. First edition. Allied Publishers. Pvt.Ltd, New Delhi, 818 p.

Sewer, M.B., Barclay, T.B. and Morgan, E.T. 1998. Down-regulation of cytochrome P450 mRNAs and proteins in mice lacking a functional NOS2 gene. *Mol. Pharmacol.* 54:273–279

Shahidi, F., Han, X. Q. and Synowiecki, J. 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Fd. Chem*. 53: 285-293

Shahidi, F., Metusalach and Brown, J.A., 1998. Carotenoid pigments in seafoods and aquaculture. *CRC Cri. Rev. Fd. Sci*. 38: 1–67

Shahidi, F., Synowiecki, J. and Naczk, M. 1992. Utilization of shellfish processing discards. *Seafood Science and Technology* (eds.Bligh, E.G.). Fishing News Books, pp.301-304

Sherief, P.M., George, M.C., Nair, J.R., Pillai, D., Joseph, S.M. and Senan, V.P. 2004. Antibacterial activity in the extract of accessory nidamental glands of squid and cuttlefish. *Proceedings of MBR 2004 National Seminar.* Proceeding of the National Seminar on New Frontiers in Marine Bioscience Research, 2004. NIOT, Chennai, pp.47-51

Shibata, A., Kiba, Y., Akati, N., Fukuzawa, K. and Terada, H. 2001. Molecular characteristics of astaxanthin and beta-carotene in the phospholipids monolayer and their distributions in the phospholipids bilayer. *Chem. Phys. Lipids.* 113: 11-22

Shimidzu, N., Goto, M. and Miki, W. 1996. Carotenoids as singlet oxygen quenchers in marine organisms. *Fish. Sci*. 62: 134–137

Simonian, N. and Coyle, J. 1996. Oxidative stress and neurodegenerative diseases. *Annu. Rev. Pharmacol. Toxicol.* 36: 83-106

Simpson, B.K. and Haard, N.F. 1985. The use of enzymes to extract carotenoprotein from shrimp waste. *J. Appl. Biol*. 7: 212-222

Simpson, B.K., Dauphin, L. and Smith,J.P. 1993. Recovery and characteristics of carotenoprotein from lobster (*Homarus americanus*) waste. *J. Aquatic. Fd. Prod. Technol.* 1(2): 129-146

Simpson, K. L. 1982. Carotenoid pigments in seafood. *Chemistry and Biochemistry of Marine Food Products* (eds. Martin, R. E., Flick, G. J., Hebard, C. E. and Ward, D.R.). The AVI Publishing Company Inc, Westport, Connecticut, pp.115-136

Simpson, K.L., Kamata, T., Collins, J.G. and Collins, J.H. 1976. *Proceedings of Tropical and Subtropical Fish. Tech. Conference,* Corpus Christi, Texas, pp.395-411

Simpson, P.J., and B.R. Lucchesi. 1987. Free radicals and myocardial ischemia and reperfusion injury. *J. Lab. Clin. Med.* 110: 13-30

Singal, P.K., Kapur, N., Dhillon, K.S., Beamish, R.E. and Dhalla, N.S. 1982. Role of free radicals in catecholamine-induced cardiomyopathy. *Can. J. Physiol. Pharmacol.* 60: 1390-1397

Singh, B.P., Singhal, L. and Chauhan, R.S. 2007. Immunotoxicity of crbaryl in chicken. *Ind. J. Exp. Biol.* 45: 890-895

Snauwaert, F., Tobback, P.P. and Maes, E. 1973. Carotenoid pigments in brown shrimp (*Crangon vulgaris Fabr*). *Lebensm. Wiss. Technol.* 6: 43-47

Snodderly, D.M. 1995. Evidence for protection against age related macular degeneration by carotenoids and antioxidant vitamins. *Am. J. Clin. Nutr.* 62(6):1448-1461

Snow, K. and Seddon, J. 1999. Does age related macular degeneration and cardiovascular disease share commom antecedents? *Ophthalmic. Epidemiol.*6: 125-143

Sophia, M.J. 2007. Biological effect of feeding cuttlefish liver oil to rats. Ph.D thesis, Mahatma Gandhi University, Kottayam, p.150

Spinelli, J. and Mahnken, C. 1978. Carotenoid deposition in pen reared salmonids fed diet containing oil extracts of red crab (*Pleuronnocodes planipes). Aquaculture.* 13: 213-216

Spinelli, J., Lehman, L. and Wieg, D. 1974. Composition, processing, and utilization of red crab (*Pleuroncodes planipes*) as an aquacultural feed ingredient. *J. Fish. Res. Bd. Can.* 31: 1025-1029

Spinelli, J.1978. *Proceedings of the World Symposium on Fin Fish Nutrition and Feed Technology,* Hamburg, West Germany, pp.383-392

Stahl, W., Heinrich, U., Jungmann, H., Sies, H. and Tronnier, H. 2000. Carotenoids and carotenoids plus vitamin E protect against ultraviolet light–induced erythema in humans. *Am.J. Clin. Nutr*. 71: 795–798

Steel, R.E. 1971. M.S thesis, Oregon state University, Corvallis, Oregon.

Steinberg, D., S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Eng. J. Med.* 320: 915-924

Stepnowski, P., Ólafsson G., Helgason, H. and Jastorff, B. 2004. Recovery of astaxanthin from seafood wastewater utilizing fish scales waste. *Chemosphere.* 54(3): 413-417

Subash, D., Narinder, K.K. and Nityanaand, S. 1978. Effect of isoprenaline on lipid profile and cardiac enzymes in rats. *Ind. J. Exp. Biol.* 16:376-378

Subbaramaiah, K., Zakim, D., Weksler, B.B. and Dannenberg, A.J. 1997. Inhibition of cyclooxygenase: a novel approach to cancer prevention. *Proc. Soc. Exp. Biol. Med.* 216:201-210

Sugano, M., Fujikawa, T., Hiratsuji, Y., Nakashima, K., Fukuda, N. and Hasegawa, Y. 1980. A novel use of chitosan as a hypocholesterolemic agent in rats. *Am. J. Clin. Nutr.* 33:787

Suzuki, Y., Ohgami, K., Shiratori, k., Jin, X.H., Ilieva, I., Kooyama, Y., Yazawa, K., Yoshida, K., Kase, S. and Ohno, S. 2006. Suppressive effects of astaxanthin against rat endotoxin-induced uveitis by inhibiting the NF-kappaB signalling pathway. *Exp. Eye. Res.* 82: 275-281

Synowiecki, J. and Al-Khateeb, N.A. 2000. The recovery of protein hydrolysate during enzymatic isolation of chitin from shrimp *Crangon crangon* processing waste discards. *Fd. Chem.* 68: 147-152

Synowiecki, J. and Al-Khateeb, N.A. 2003. Production, properties and some new applications of chitin and its derivatives. *Crit.Rev.Fd.Sci. Nutr.* 43:145-171.

Tagami, M., Yamagata, K., Ikeda, K., Nara, Y., Fujino, H., Kubota, A., Numano, F. and Yamori, Y. 1998. Vitamin E prevents apoptosis in cortical neurons during hypoxiaand oxygen reperfusion. *Lab. Invest.* 78: 1415-1429

Takaichi, S., Matsui, K., Nakamura, M., Muramatsu, M. and Hanada, S. 2003. Fatty acid of astaxanthin esters in krill determined by mild mass spectrometry. *Comp. Biochem. Physiol.* 136B: 317-322

Takayama, F., T. Egashira, Y. Kudo, and Y. Yamanaka. 1992. Chemiluminescence-HPLC assay of phosphatidylcholine hydroperoxide generated by ischemia-reperfusion in the liver of rats. *Biochem. Pharmacol.* 44: 2412-2414

Talwar, G.P. 1974. *Hand book of Practical Immunology*, National Book Trust, New Delhi. pp.329-336

Tanaka, T., Kawamori, T., Ohnishi, M., Makita, H., Mori, H., Satoh, K. and Hara, A. 1995a. Suppression of azomethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. *Carcinogenesis*. 16: 2957–2963

Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh, K. and Hara, A. 1995b. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. *Cancer Res.* 55: 4059–4064

Tanaka, T., Morishita, Y., Suzuki, M., Kojima, T., Okumura, A. and Mori, H. 1994. Chemoprevention of mouse urinary bladder carcinogenesis by naturally occurring carotenoid astaxanthin. *Carcinogenesis.* 15: 15-19

Tapiero, H., Townsend, D.M. and Tew, K.D. 2004. The role of carotenoids in the prevention of human pathologies. *Biomed. Pharmacoth.*58: 100-110

Terao, J. 1989. Antioxidant activity of beta-carotene-related carotenoids in solutions. *Lipids*. 24(7): 659-661

Tinkler, J.H., Bohm, F., Schalch, W. and Truscott, T.G. 1994. Dietary carotenoids protect human cells from damage. *J. Photochem. Photobiol.* 26: 283-285

Tlusty, M. and Hyland, C. 2005. Astaxanthin deposition in the cuticle of juvenile American lobster (*Homarus americanus)*: implications for phenotypic and genotypic colouration. *Mar. Biol.* 147: 113-119

Todd, L. 1998. Thin layer chromatography (TLC) system for Natu Rose Cartenoids. *Natu Rose. Technol. Bull.* 003: 1-3

Tomita, Y., Jyonouchi, H., Engelman, R.W., Day, N.K. and Good, R.A. 1993. Preventive action of carotenoids on the development of lymphadenopathy and proteinuria in MRL-1pr/1pr mice. *Autoimmunity.* 16: 95-102

Torissen, O.J., Hardy, R.W. and Shearer, K. 1989. Pigmentation of salmonids – carotenoid deposition and metabolism. *CRC Crit. Rev. Aquat. Sci.* 1: 209–225

Torrisen, O.J. and Chriatiansen, R. 1995. Requirements of carotenoids in fish diets. *J. Appl. Ichthyol.* 11: 225-230

Torrisen, O.J., Tideman, E., Hansen, F., Raa, J., 1981. Ensilaging in acid—a method to stabilize astaxanthin in shrimp processing byproducts and improve uptake of this pigment by rainbow trout (*Salmo gairdneri*). *Aquaculture.* 26: 77–83

Tracy, R.P. 1999. Inflammation markers and coronary heart disease. *Curr. Opin. Lipidol*. 10: 435–441

Tso, M. and Lam, T. T. 1996. Method of retarding and ameliorating central nervous system and eye damage. US Patent #5527533

Tsukada, K., Matsumoto, T., Aizawa, K., Tokoro, A., Naruse, R., Suzuki, S. and Suzuki, M. 1990. Antimetastatic and growth inhibitory effects of N-acetyl chito-hexaose in mice bearing lwis lung carcinoma. *Jpn. J. Cancer. Res.* 81: 259-265

Uchiyama, K., Naito, Y., Hasegawa, G., Nakamura, N., Takahashi, J. and Yoshikawa, T. 2002. Astaxanthin protects beta-cells against glucose toxicity in diabetic db/db mice. *Redox. Rep.* 7(5): 290-293

Vane, J. and Booting, R. 1987. Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB J.* 1: 89-95

Vassallo-Agius, R., Imaizumi, H., Watanabe, T., Yamazaki, T., Satoh, S. and Kiron, V. 2001. The influence of astaxanthin supplemented dry pellets on spawning of striped jack. *Fish. Sci.* 67: 260-270

Venugopal, V. 2008. *Marine Products for Healthcare: Functional and Bioactive Neutraceutical compounds from the Ocean.*CRC Pess, New York, 528 p.

Vimala, S. and Paul, V.I. 2009. Utilization of crustacean fishery waste as a source of carotenoids. *J. Exp. Zool. Ind.* 12(2): 377-380

Vuillaume, M. 1987. Reduced oxygen species mutation induction and cancer initiation. *Mutation Res*. 186: 43-72

Waagabo, R., Hamrel, K., Bjerkas, E., Berge, R., Wathne, E., Lie, O. and Torstensen, B. 2003. Cataract formation in Atlantic salmon, *Salmo salar* L., smolt relative to dietary pro-and antioxidants and lipid level. *J. Fish. Dis.* 26: 213-229

Wang, W.H., Geibel, J. and Giebisch,G.1993.Mechanism of apical K+ channel modulation in principal renal tubule cells. Effect of inhibition of basolateral Na+ - K+ ATPase. *J.Gen. Phys.* 101: 673-694

Whathene, E., Bjerken, B., Storeabakken, T., Vassvik, V. and Odaland, A.B.1998. Pigmentation of Atlantic salmon (*Salmo salar)* fed astaxanthin in all meals or in alternating meals. *Aquaculture*. 159: 217-231

Wilkie, D.W. 1972. the carotenoid pigmentation of *Pleuroncodes planipes* Stimpon (Crustacea: Decapoda Galatheidae). *Comp. Biochem. Physiol.*42: 731-734

Woodall, A., G. Britton and M. Jackson. 1997. Carotenoids and photoprotection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: Relationship between carotenoid structure and protective ability. *Biochim. Biophys. Acta.* 1336(3):575-586

Yamada, S., Tanaka, Y., Sameshima, M. and Ito, Y. 1990. Pigmentation of prawn (*Penaeus japonicus*) with carotenoids. I. Effect of dietary astaxanthin, beta-carotene and canthaxanthin on pigmentation. *Aquaculture.* 87: 323- 330

Yamaguchi, K., Murakami, M., Nakano, H., Konosu, T., Yamamoto,H., Kosaka, M. and Hata, K., 1986. Supercritical carbon dioxide extraction of oil from Antarctic krill. *J. Agric. Fd. Chem*. 34: 904–907

Yokoyama, A. and Miki, W. 1995. Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum. FEMS Microbiol. Lett.* 128: 139-144

Yu, Z. F., Bruce-Keller, A. J., Goodman, Y., and Mattson, M. P. 1998. Uric acid protects neurons against excitotoxic and metabolic insults in cell culture, and against focal ischemic brain injury *in vivo*. *J. Neurosci. Res*. 53(5): 613-625

Yuan, J.P. and Chen,F. 1997. Identification of astaxanthin isomers in *Haematococcus lacustris* by HPLC-photodiode array detection. *Biotech. Tech.* 11(7): 455-459

Yuan, J.P., Gong, X. D. and Chen, F. 1996. Separation and identification of astaxanthin esters and chlorophylls in *Haematococcus lacustris* by HPLC. *Biotechnol. Tech.* 10: 655-660

Yuan, J.P., Gong, X. D. and Chen,F. 1997. Separation and analysis of carotenoids and chlorophylls in *Haematococcus lacustris* by high performance liquid chromatography photodiode array detection. *J. Agric. Fd. Chem.* 45:1952-1956

Zagalsky, P.F., Eliopoulos, E.E. and Findlay, J.B. 1990. The architecture of invertebrate carotenoproteins. *Comp. Biochem. Physiol.* 97B: 1-18

Zhang, L.X. Cooney, R.V. and Bertram, J.S. 1991. Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action. *Carcinogenesis.* 12(11): 2109-2114

Zikakis, J.P., Saylor, W.W. and Austin, P.R. 1982. *Chitin and Chitosan* (eds. Hirano, S. and Tokura, S.). Japanese Society of Chitin and Chitosan, Tottori, pp. 233-236

BIOACTIVITY OF CAROTENOIDS FROM SHRIMP SHELL WASTE

By

SINDHU.S, **M.F.Sc.**

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree

DOCTOR OF PHILOSOPHY

IN

FISH PROCESSING TECHNOLOGY

Faculty of Fisheries

Kerala Agricultural University

2010

DEPARTMENT OF PROCESSING TECHNOLOGY

COLLEGE OF FISHERIES

PANANGAD, COCHIN-682 506

8. ABSTRACT

Shrimp processing waste is the single largest industrial waste in the country causing diverse environmental problems. A study was carried out to assess the extractability of astaxanthin from shrimp waste in different organic solvents and vegetable oils. Extraction was tried using wet and dried waste, with and without deproteinisation. Waste was subjected to deproteinisation using alkali and enzyme (pancreatin). The different solvent systems tried were ether:acetone:water (15:75:10 v/v/v), acetone, hexane:isopropanol (3:2 v/v) and 90% acetone v/v. Astaxanthin in the extract was quantified by measuring the OD at 470 nm in hexane. Extraction was also done using vegetable oils *viz.* coconut oil, soybean oil and sunflower oil. Quantification of astaxanthin in pigmented oil was done by measuring the absorbance at 485 nm using 2155 as extinction coefficient.

Astaxanthin yields from deproteinised samples were significantly lower than those from non deproteinised samples. The highest astaxanthin yield of 87.14 ± 4.55µg/g was obtained with non deproteinised wet waste extracted using acetone. The astaxanthin yield was significantly lower when oil was used as the extraction medium. Of the three oils coconut oil gave the highest yield. The results showed that acetone is the best solvent for extracting astaxanthin from shrimp shell waste in wet condition. The astaxanthin content in *Aristeus alcocki* shell waste is double that of *Pandalus borealis* shell waste, which is currently used as the commercial source of astaxanthin. The deep sea species *Aristeus alcocki* can thus be considered as a better source of astaxanthin for commercial exploitation than *Pandalus borealis*.

TLC analysis of the shell waste extract showed that it contains free astaxanthin, astaxanthin monoester and astaxanthin diester in the ratio 1:1:2. GLC identification of the fatty acids esterified with astaxanthin revealed that saturated fatty acids, MUFA and PUFA are in the ratio 5:3:2 in monoester, whereas in diester they are in the ratio 4:3:3. The main fatty acids in monoester and diesters are palmitic acid, oleic acid, stearic acid and PUFAs: DHA and EPA.

The *in vitro* antioxidant activity of the astaxanthin extract showed significant hydroxyl radical scavenging activity, superoxide anion scavenging activity and inhibition of lipid peroxidation. The IC_{50} values obtained were 56.43 ± 1.06 ng/ml, 27.91 ± 0.54 ng/ml and 26.54 ± 0.42 ng/ml, respectively. The antioxidant activity of astaxanthin from *Aristeus alcocki* was obtained at nanogram levels. This powerful antioxidant function may be due to the unique molecular structure of astaxanthin and synergistic effect of astaxanthin and PUFAs present in the astaxanthin monoester and diester fractions.

The astaxanthin extract from shrimp shell waste significantly reduced carageenan induced paw edema in mice, percentage inhibition being 47.83 and 67.11 percent at astaxanthin concentrations of 0.5 mg/kg body weight and 1.0 mg/kg body weight, respectively. The inhibition of inflammation at 1.0mg/kg body weight was greater than that produced by the standard reference drug diclofenac. Cardioprotective effect of astaxanthin was examined in isoproterenol induced myocardial infarction in rats. Levels of diagnostic marker enzymes, LDH, CPK, GOT, GPT, CK, CK-MB in plasma, lipid peroxides, ascorbic acid, reduced glutathione and the activities of glutathione-dependent antioxidant enzymes GPx, GR, GST and antiperoxidate enzymes CAT, SOD and the membrane bound enzyme Na^+ - K^+ ATPase in the heart tissues of experimental groups of rats were determined. The prior administration of astaxanthin @ 10mg/kg feed for 45 days significantly prevented the isoproterenol-induced elevation in the levels of diagnostic marker enzymes in plasma, induction of lipid peroxidation and alterations in the level of reduced glutathione and in the activities of glutathione dependent antioxidant enzymes and antiperoxidative enzymes of experimental rats. Feeding astaxanthin caused a decrease in the inhibition of Na^+ - K^+ ATPase activity against isoproterenol induced myocardial infarction. The powerful cardioprotective effect of astaxanthin can be attributed to the multiple independent mechanisms *viz.* antioxidant effects, singlet oxygen quenching ability and inhibition of lipid peroxidation of membranes, increased functional gap junctional intercellular communication, anti-inflammatory effects etc.

Immunostimulatory action of astaxanthin extract was evaluated in experimental mice. Astaxanthin administration was found to enhance the proliferation of spleen cells and bone marrow cells. Esterase activity was found to be enhanced in bone marrow cells indicating increased maturation of cells of lymophoid linkage. Astaxanthin also enhanced number of antibody forming cells and circulating antibody titre. Thus astaxanthin exhibits strong immunomodulating properties.

A significant reduction in the viability of ascites tumour cells DLA *in vitro* was noted in the current study. The % viability was reduced to 4.34 % at a concentration of 15µg astaxanthin/ml. The cytotoxic action of astaxanthin against DLA may be through induction of apoptosis or through a different pathway. Antitumour activity of astaxanthin was studied by ascite and solid tumour models in mice. An increase in life span of about 67 % was noted in DLA bearing mice administered with astaxanthin at 5 mg/kg body weight. The tumour volume and tumour weight were significantly lower in mice injected with 5 mg/kg body weight astaxanthin. *In vitro* studies revealed that astaxanthin from shrimp shell waste of *Aristeus alcocki* inhibited the proliferation of cervical cancer cells HeLa in a dose dependent manner.