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INVESTIGATING THE IMPACT OF AMINO-ACID SUPPLEMENTATION ON ENHANCEMENT OF BETA CAROTENE CONTENT IN FENUGREEK PLANT.

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ABSTRACT Plants are capable of synthesizing β-carotene but unfortunately humans are not able to do so. However, they are capable of converting β-carotene absorbed from their diets to vitamin A. Hence, an increase in β-carotene content in plants can be an alternative to increase the vitamin A content & prevent from certain diseases content in diet of individuals. Therefore, foliar application of amino acid solution is a technique used for investigating the effect of amino acid supplementation on enhancement of beta carotene content in green leafy vegetables.

In this study, fenugreek (*Trigonella foenum graecum*) seeds are treated with proline, tyrosine, tryptophan and proline, tyrosine, tryptophan combination (1:1:1). It was observed that growth and beta carotene content were higher in the treated plants. Exclusive treatment with Proline, tyrosine and the tryptophan resulted in an increase in growth and beta carotene content when compared to control which is lesser than that of proline-tyrosine-tryptophan combination treated seeds.

KEYWORDS : β-carotene, Proline, Tyrosine, tryptophan, Trigonella foenum graecum.

INTRODUCTION:

Fenugreek is one of the oldest medicinal plants known to humanity and has been used in both Eastern and Western herbal medicine for hundreds of years. Fenugreek has light green leaves and small white flowers and is an annual herb. It is from the family of peas (Fabaceae) and is sometimes referred to as Trigonella foenum-graecum (Greek hay). At two to three feet high, the fenugreek plant stands upright, and the seed pods produce 10-20 thin flat, yellow-brown, pungent, and aromatic seeds.

Similar to celery, maple syrup, or burnt sugar, fenugreek seeds have a very sour flavor and are sometimes used to produce medicine. When baked, however, fenugreek has a much more friendly flavor. The seeds, which are normally dried and ground, are the most commonly used component of fenugreek. In cooking as well the leaves are also used.

In Argentina, France, India, North Africa, England, and the United States, fenugreek is widely cultivated today. The seeds and fresh leaf shoots nowadays originate only from cultivated plants.

Fenugreek is often included in foods as an ingredient in spice blends, most of which are found in Indian dishes, such as curried dishes. It's also used in imitation maple syrup, foods, beverages and tobacco as a flavouring agent. The plant's leaves can be used in salads, and Indian cookery uses both fresh and dried leaves.

Recent scientific research has found that seeds of fenugreek can help reduce blood cholesterol. It is the unique fiber composition of the herb and the high saponin content that are believed to be responsible for the glucose-lowering and cholesterol-lowering effects of both.

The fenugreek leaves contain the substance choline and the seeds are an excellent source of beta carotene antioxidants.

In order to help heal inflammation, Fenugreek can be taken by mouth or used to form a paste that is applied to the skin. Antimicrobial, antioxidant, antidiabetic and antitumor activities are known to occur with fenugreek extract and oil. It has a long history as an ingredient in traditional medicine, cultivated in North Africa, the Middle East, Egypt and India. Fenugreek extracts can be found in soaps and cosmetics during the production process.

Fenugreek is now used as a traditional or folk remedy for diabetes and loss of appetite, as well as to stimulate the production of milk in women who are breastfeeding. Among various other possible advantages, it is also applied to the skin for inflammation.

Fenugreek seeds contain hormone precursors that in nursing mothers may increase the production of milk and are widely used for insufficient lactation.

Benefits and Uses of Fenugreek:

Enhances digestive problems and levels of cholesterol:

Many digestive problems, such as upset stomach, constipation and inflammation of the stomach, may be helped by fenugreek. The water-soluble fibre in fenugreek, for example, helps relieve constipation, among other foods. It also works to treat digestion and, owing to its anti-inflammatory effects, is often integrated into an ulcerative colitis diet treatment plan.

Heart problems, such as hardening of the arteries and elevated blood levels of some fats, including cholesterol and triglycerides, also appear to benefit from Fenugreek. Indeed, a study from India showed that the administration of 2.5 grams of fenugreek twice daily for three months to individuals suffering from non-insulin-dependent diabetes mellitus naturally significantly reduced cholesterol along with triglycerides without affecting HDL cholesterol.

Boosts libido in men:

For men suffering from hernias, erectile dysfunction, and other male issues, such as baldness, Fenugreek is also used. That's because sexual arousal and testosterone levels can be increased by fenugreek.

In a study published in Phytotherapy Research, 60 men with no history of erectile dysfunction between the ages of 25 and 52 years were supplemented with either placebo or 600 milligrammes of fenugreek extract per day for six weeks. The participants noted their outcomes with fenugreek through self-evaluation, reporting that the supplement had a positive effect on their libidos.

Promotes Breastfeeding Milk Flow:

Fenugreek also supports women who breastfeed who can suffer a poor supply of milk. Fenugreek can increase the supply of breast milk to a woman because it acts as a galactagogue, which is a milk supply enhancement substance. This activates the milk ducts and, in as little as 24 hours, will increase milk production.

Helps with dietary disorders:

Fenugreek has been shown to increase appetite, which results in restorative and nutritional properties, beyond enhancing flavor. To investigate the effects of a fenugreek seed extract on feeding behavior, a study published in Pharmacology Biochemistry and Behavior was designed. To establish food intake and desire to feed, as well as metabolic-endocrine shifts, experiments were conducted. The results showed that chronic oral administration of the fenugreek extract significantly increased food intake and the motivation to eat. The report also indicated, however, that the treatment does not prevent anorexia or the decreased motivation to eat.

1.1 BETA-CAROTENE :

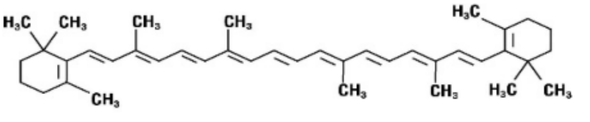
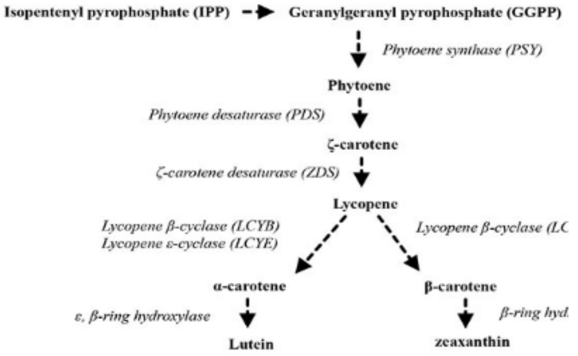


Fig: Structure Of Beta- Carotene

The carotenoid is beta-carotene. Carotenoids are pigments present in plants that exist naturally and are primarily responsible for the vivid colors of certain fruits and vegetables. Beta-carotene, for instance, is responsible for giving the orange hue to carrots. Beta-carotene is either converted into vitamin A (retinol) once absorbed, which the body may use in a number of forms, or it serves as an antioxidant to help shield cells against the negative effects of free radicals that are harmful. Beta-carotene and other carotenoids contain as much as 50 percent of vitamin A in a traditional diet.

Beta-carotene is known to be an antioxidant and is a vitamin A precursor as well. This compound helps to retain healthy skin and plays a crucial role in eye protection as well. The risk of coronary heart disease, stroke, macular degeneration, and other age-related disorders may be reduced by individuals eating the required amounts of beta-carotene.



Genes and enzymes of carotenoid biosynthesis in plants

1.2 Role of beta carotene

1.2.1. Vitamin A

Beta-carotene is a pro-vitamin converted into vitamin A by the body. In fruits and vegetables, β-Carotene is the most abundant source of vitamin A. Alpha-carotene and β-cryptoxanthin are the only two carotenoids with vitamin A involvement that is not abundant in fruit. β-Carotene in both conventional diets and dietary supplements is an important source of vitamin A. In the small intestinal enterocyte, through cleavage of the β-carotene molecule, β-carotene can be converted into vitamin A predominantly as retinyl ester (20-75 percent). The bulk of vitamin A conversion takes place not in the liver, but in the mucosa of the intestine.

1.2.2. Immune response

Beta-carotene is a pro-vitamin converted into vitamin A by the body. In fruits and vegetables, β-Carotene is the most abundant source of vitamin A. Alpha-carotene and β-cryptoxanthin are the only two carotenoids with vitamin A involvement that is not abundant in fruit. β-Carotene in both conventional diets and dietary supplements is an important source of vitamin A. In the small intestinal enterocyte, through cleavage of the β-carotene molecule, β-carotene can be converted into vitamin A predominantly as retinyl ester (20-75 percent). The bulk of vitamin A conversion takes place not in the liver, but in the mucosa of the intestine.

1.2.3. Antioxidant

The fact that LDL is an important circulating carrier of β-carotene and lycopene and that these carotenoids are capable of trapping peroxyl radicals and quenching singlet oxygen confirms this theory. β-Carotene is a peroxyl radical scavenger, especially at low oxygen stress. Other carotenoids can also show the behavior. Carotenoid interactions with peroxyl radicals can occur through an unstable adduct of β-carotene radicals. It has been shown that carotenoid adduct radicals are strongly resonance stabilized and are expected to be relatively unreactive. In order to create non-radical products, they can further undergo decay and may terminate radical reactions by binding to the attacking free radicals. By interacting quicker with peroxyl radicals, carotenoids serve as antioxidants than unsaturated acyl chains do. Carotenoids are killed in this process.

1.2.4. Cancer

The main anticancer agent in fruits and vegetables may be β-carotene. It may be hypothesised that beneficial effects of β-carotene arise at physiological or dietary intake levels, whereas if pharmacological amounts are given, adverse effects in certain subpopulations may be observed.

1.2.5. Lung cancer

Most of the interaction between β-carotene and lung cancer has been studied, and the evidence has been more clear. Proof of β-carotene as a chemoprotective agent in fruits and vegetables is obtained from prospective epidemiological studies in which 11 out of 15 found an important inverse association between the consumption of β-carotene and/or the amount of plasma and lung cancer danger. The smoke has been invoked as a pro-oxidant, and it has been proposed that cigarette smoke can lead to oxidative degradation of β-carotene in the presence of high concentrations of β-carotene, contributing to the formation of oxidized metabolites that could promote carcinogenesis.

1.3 BEER-LAMBERT LAW:

The Beer-Lambert law (or Beer's law) is the linear relationship between absorbing organisms' absorbance and concentration. Normally, the general Beer-Lambert rule is written as:

A = a () * b * c

Where A is the measured absorbance, a () is a coefficient of absorptivity depending on wavelength, b is the path length, and c is the concentration of the analyte. The Beer-Lambert law is written in concentration units of molarity while operating as:

A = * b * c

Where is the molar absorptivity coefficient depending on the wavelength with units of M-1 cm-1 Data in percent transmission (I/I0 * 100) or in absorbance [A = log (I/I0)] are commonly mentioned the latter is particularly handy.

OBJECTIVE:

1) Beta carotene extraction, and estimation in fenugreek plants.
2) To investigate the effect of supplementation with amino acids on the improvement of the content of beta carotene in fenugreek plants.

LITERATURE REVIEW

According to Dr. Richard Palmquist, Fenugreek, director of integrative health services at Centinela Animal Hospital in Inglewood, Calif., was discovered by Ayurvedic medicine practitioners to have therapeutic properties thousands of years ago. He states that it is useful for many factors, including the treatment of metabolic and digestive conditions such as diabetes, designed to reduce blood sugar.

Fenugreek helps to slow down stomach sugar absorption and activate insulin. The spice is recognised as a phlegm mover in Traditional Chinese Medicine and is said to break up trapped forces within the body and cool inflammation.

It has been shown that amino acids promote plant growth and yield: 11.51 mg/L proline on Zea mays L. (Hamed & El-Wakeel 2004), on peppermint 25 mg/L of alanine or tyrosine (Menthapiperita) (Refaat & Naguib2008) and on Daturainnoxia Mill 100, 200 or 400 mg/L of ornithine and phenylalanine (Phe) (Habba 2009).

Gamal El-Din & AbdElWahed (2005) showed that plant height, number of leaves, fresh and dry weight of aerial vegetative sections and chamomile flower head (Matricaria chamomilla) were increased by foliar application of 50 mg/L ornithine and 100 mg/L proline or Phe. Shukry et al. (2008) suggested that Phaseolus vulgaris (L.) grain yield and vegetative characters gained from asparagine and glutamine.

Glutamine foliar application at 100-200 mg/L significantly increased plant height, leaf number, fresh leaf weight, fresh and dry weight, leaf area, bulb length, bulb diameter, and weight, as well as onion yield and bulb quality (Amin et al. 2011). Bálványos et al. (2002) reported that 66 mg/L Phe addition maximized growth and alkaloid (lobeline) development of Lobelia inflata L. hairy root cultures. Increased plant height, number of leaves, stem diameter, dry weight of shoots, fresh and dry weight of fruit, photosynthetic pigments, and capsaicin and pepper dihydrocapsaicin content by foliar application of Phe at 250 mg/L (Capsicum annum L.) (Rashad et al. 2002).

Proline and tyrosine are the starting materials for the synthesis of beetroot pigments, betacyanine and betaxanthine, according to Hess (2008).

As the amount of salinity increased relative to those plants irrigated with tap water (Sadak and Hisham et al), irrigation of sunflower plants with filtered seawater caused marked incremental decreases in chlorophyll a, chlorophyll b, carotenoids, and complete photosynthetic pigments in leaves. In several crops, such as sunflower (Sadak et al., 2012), flax (Sadak & AbdElhamid 2013 and Sadak & Dawood, 2014), sunflower (Khan et al., 2014), and canola (Dawood & Sadak 2014), the deleterious effects of salinity stress on leaf chlorophyll and β-carotene content have been recorded.

Ali et al. (2007) mentioned that the exogenous application of 30mM proline increased photosynthetic pigments of maize plants grown under water deficit stress. Proline treatments had the ability to alleviate the adverse effects of salinity on photosynthetic pigments.

Yan et al., (2011) mentioned that proline not only functioned as a nutrient but also possessed some defensive mechanisms for damaged plants under salt stress, these mechanisms were, promoting photosynthesis, maintaining enzyme activity, and scavenging ROS. Ali et al., (2007) explained the beneficial effect of proline applied was due to its promotive effects on photosynthetic capacity by overcoming stomata limitations, enhancing biosynthesis of photosynthetic pigments, or protecting photosynthetic pigments from water stress-induced degradation. Despite the positive effects of applied proline for inducing tolerance, some reports concerning the inhibitory effects of high concentrations of proline are also available (Yamada et al., 2005; Ashraf and Foolad, 2007, Ali et al., 2013 and Khan et al., 2014). However, the application of 7.5 mM proline caused significant decreases in all components of photosynthetic pigments. Sivakumar et al. (1998) demonstrated a negative effect of an exogenous application of 100 mM proline in the level of Rubisco activity in three plant species.

Moreover, Millimolar concentrations of proline have been demonstrated to cause disruptive effects on chloroplast and mitochondria membranes in Arabidopsis because of increased levels of reactive oxygen intermediates (Hare et al., 2002).

Tyrosine is hydroxy phenyl amino acid that is used to build neurotransmitters and hormones. High concentrations of tyrosine had a negative effect on the in vitro growth of Alternanthera species affecting the formation of shoots and roots, but the addition of this amino acid in the culture medium increased the biosynthesis of betacyanin in the studied two species. The involvement of tyrosine in the metabolism of natural compounds such as alkaloids and other nitrogen products such as betacyanin has been well established in the literature. But to-date little is known about its mechanism of control and regulation, and the role in secondary metabolism of the two medicinal plant species tested. (Kleinowski et al.)

The role of Tryptophan is well known: it has an indirect role in the growth via its Influence on auxin synthesis. Phillips (2005) reported that alternative routes of IAA synthesis exist in plants, all starting from Tryptophan. Tryptophan has more positive effect on plant growth as compared to pure auxins (Zahir et al 2009).

MATERIALS & METHOD:

MATERIAL REQUIRED:

Seeds (fenugreek) soil, plastic glass, amino acid solution, ethanol, petroleum ether, 95% ethanol, 80% ethanol, separating funnel, measuring cylinder, distil water, marker.

METHOD:

3.1. Seed collection

Fenugreek seeds (250gms each) were collected from the local market. The samples were collected in polythene zip-lock pouches.

3.2 Soil collection

Fine granular black soil (approximately 2kg) was collected from the Garden of Biyani Girls College. The soil was collected in a large polythene bag and then was equally distributed in 6 conical flasks (500 ml) for sterilization.

3.3 Preparation of Amino Acid Solution

Amino acid solutions (Proline, Tryptophan, and tyrosine) of 10 mM concentration were prepared (Appendix). The amino acids selected were proline, tyrosine& tryptophan.

3.4 Seeds Treatment

Seeds of mustard and fenugreek were treated (1ml/seed) for 1 hour. Following treatment, the seeds were blotted dry using a blotting paper.

3.5 Sowing of seeds

In order to sow pre-soaked fenugreek seeds pot experiment were conducted. The fenugreek and mustard seeds were sown in plastic pots (4cm radius x 8cm in height), in which 100 gm soil was filled. And to each plastic pot 50 pre socked seeds were sown and sprinkled with water.

3.6 Extraction of β-carotene

The extraction of β-carotene was carried out according to the method of the Association of Official Analytical Chemists (AOAC).

In to a conical flask containing 25ml of 95% ethanol, 1g of the macerated sample was placed and maintained at a temperature of 70-80°C in a water bath for 20minutes with periodic shaking. The supernatant was decanted, allowed to cool and 5ml of distilled water was added to it and further cooled in a container of ice water for about 5 minutes. The mixture was transferred into a separating funnel and 12ml of petroleum ether (pet-ether) was added and 10ml of the cooled ethanol was poured over it. To achieve a homogenous mixture, the funnel was swirled softly and it was then allowed to stand until two different layers were collected. While the top layer was gathered, the bottom layer ran off into a beaker. The bottom layer was transferred into the funnel and re-extracted with 5ml pet-ether for 3-4 times until the extract became fairly yellow. The entire pet-ether was collected into a conical flask and transferred into a separating funnel for re-extraction with 25ml of 80% ethanol. The final extract was measured and poured into sample bottles for further analysis.

3.7 Analysis of β-carotene

As the extraction of beta carotene was a liquid-liquid extraction hence the absorbance of the extracts was measured using a spectrophotometer (model 22UV/VIS) at a wavelength of 457nm. A cuvette containing pet-ether (blank) was used to calibrate the spectrophotometer to zero points. Samples were put in cuvettes for each extract and readings were taken when the figure became steady in the view window. For every sample, the procedure was repeated 5-6 times and average readings were reported. The carrot extraction of b-carotene was simply done by liquid-liquid extraction and the UV absorption was measured at 457 nm.

3.8 Calculation of β-carotene

The β-carotene concentration was determined using the Bear-Lamberts Law or the Beer-Lambert-Bouguer law, which refers to the light attenuation of the material properties by which the light passes. According to the law, absorbance (A) is proportional to the concentration© of the pigment, as represented by the equation:

A L (if concentration© is constant).

A=ECL; C=A/EL

Where: C= concentration of carotene, A= absorbance, E=extinction coefficient, L= thickness of cuvettes (path length) =1cm and E of β-carotene =1.25x104 μg/L.

RESULT & DISCUSSION

4.1 Growth Characteristics

Comparison of the difference between the growths in height of the fenugreek plants after every 8 days shows that growth rate of the plants treated with combination of Proline - Tryptophan was the highest followed by Proline and Proline – tyrosine combination. The least growth rate was shown by plant treated with distil water.

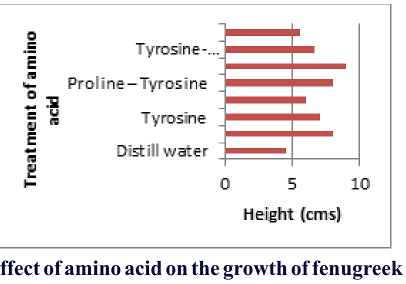


Figure-3: Effect of amino acid on the growth of fenugreek plant.

Table-1: Measurement of fenugreek plant length after amino acid treatment

Sr. No.	Treatment	Height (cms)
1.	Distil water	4.5
2.	Proline	8

3.	Tyrosine	7
4.	Tryptophan	6
5.	Proline – Tyrosine (1:1) combination	8
6.	Proline – Tryptophan (1:1) combination	9
7.	Tyrosine- Tryptophan (1:1) combination	6.6
8.	Proline – Tyrosine- Tryptophan (1:1:1)	5.6

Figure 5. Growth of amino acid treated fenugreek plants

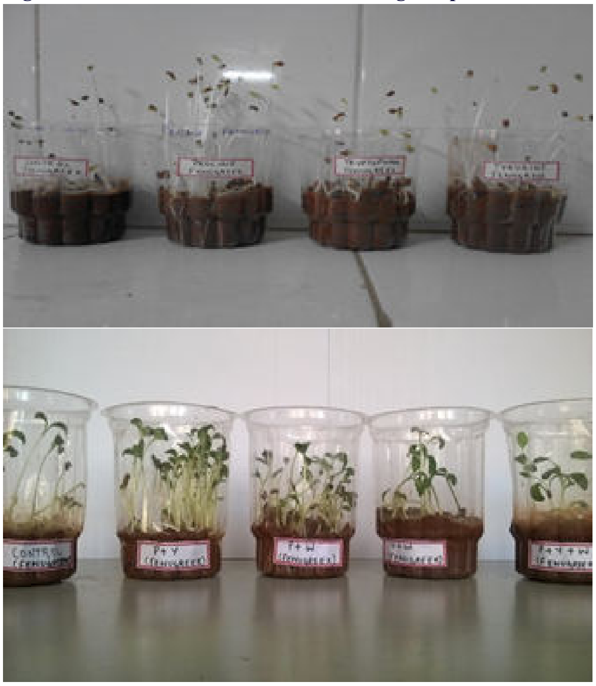


Figure 6. β-carotene extraction of fenugreek plants

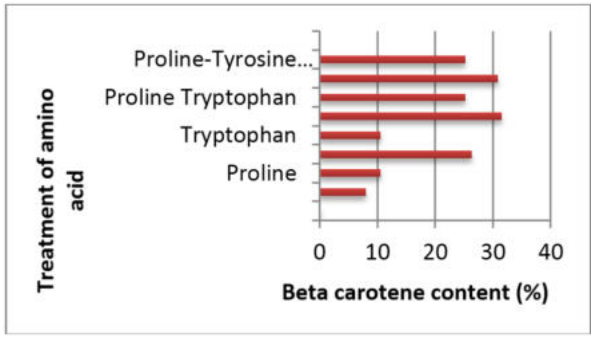
4.2. β-carotene content

The β-carotene content in the fenugreek test plant sample treated with Proline- Tyrosine (31.5%) has shown the highest increase in β-carotene content followed by Tyrosine- Tryptophan (30.9%) and tyrosine (26.51%) in comparison to the distilled water-treated control sample.

Figure 9 Increase in β-carotene content percentage in amino acid treated fenugreek plants

Table 3: Concentration of β-carotene on the amino acid treated fenugreek plants.

Sr. No.	Treatment	Concentration (μg/l)
1.	Distil water	0.8
2.	Proline	1.04
3.	Tyrosine	1.52
4.	Tryptophan	1.04
5.	Proline – Tyrosine (1:1) combination	3.28
6.	Proline – Tryptophan (1:1) combination	1.76
7.	Tyrosine- Tryptophan (1:1) combination	3.04
8.	Proline – Tyrosine- Tryptophan (1:1:1)	1.76



A significant difference was observed in the growth of fenugreek and mustard plants, 8 days after the treatment with Proline, Tyrosine, Tryptophan, Proline-Tyrosine, Proline-Tryptophan, Tyrosine - Tryptophan, and distilled water.

Treatment of amino acids like Proline, Tyrosine, Tryptophan, and their combination has shown a significant increase in the growth of the plant and β-carotene concentration, and the combination of any two amino acids was more successful then combining all the three amino acid. Therefore the growth and β-carotene concentration in different fruits and vegetables can be increased by using different combinations of Proline, Tyrosine, and Tryptophan.

CONCLUSION

Amino acids, the building block of proteins play a very significant role in growth and enhancement of beta carotene content. Combination of two amino acid had given greater yield of beta carotene content as compared to the combination of all three amino acids in fenugreek plants.

Therefore, it is necessary to determine the role of amino acid in increasing the beta carotene content at genetic level so as to use this simple yet an effective technique for enhancing the Beta-Carotene content in plant.

Moreover, amino acid supplementation can also be used for enhancing the beta carotene content of staple crops and can serve as a tool of enhancing the agronomic quality of various crops by stimulating vegetative growth and yield of essential nutrients.

REFERENCES:

1. Christopher I. Cazzonelli, Australian Research Council Centre of Excellence in Plant Energy Biology, College of Medicine, Biology and Environment, Research School of Biology, The Australian National University, Building 134, Linnaeus Way, Canberra, ACT 0200,2011.
2. Akhtar S, Ahmed A, Randhawa MA, Atukorala S, Arlappa N, Ismail T et al. Prevalence of Vitamin A Deficiency in South Asia: Causes, Outcomes, and Possible Remedies. J Health Population Nutrition. 2013; 31: 413-23.
3. K.N. Chidambara Murthy, 'Production of β-carotene from cultured Dunaliella sp. And evaluation of biological'. University of Mysore, Department of Biotechnology, 2005.
4. Syed Imtiaz Ali Shah, Shujaat Ali Shah, Partab Rai, Shahid Jamal Siddiqui, Safdar Ali Abbasi, Shabir Ahmad Bhutto, Pak J Ophthalmology 2015, Vol. 31 No. 3.
5. Rosenfeld L. Vitamine--vitamin. The early years of discovery. Clinical Chemistry. 1997; 43: 680-5.
6. Gerster H. Vitamin A-Functions, dietary requirements and safety in humans. International Journal of Vitamin & Nutrition. 1997; 67: 71-90.
7. World Health Organization (WHO). Global prevalence of vitamin A deficiency in populations at risk 1995-2005. WHO Global Database on Vitamin A Deficiency. Geneva: WHO; 2009.
8. Sommer A, Tarwotjo I, Hussaini G, Susanto D. Increased mortality in children with mild vitamin A deficiency. Lancet. 1983; 2(8350): 585-8.
9. Cohen N, Rahman H, Sprague J, Jalil MA, Leemhuis de Regt E, Mitra M. Prevalence and determinants of nutritional blindness in Bangladeshi children. World Health Stat Q.

1985; 38: 317-30.
10. Nair H, Arya G, Vidnapathiranad J, Tripathi S, Talukder SH, Srivastava V. Improving neonatal health in South-East Asia. Public Health. 2012; 126: 223-6.
11. Gilbert C. The eye signs of vitamin A deficiency. Community Eye Health. 2013; 26.
12. Underwood B A. Vitamin A Deficiency Disorders: International Efforts to Control A Preventable "Pox". J Nutr. 2004; 134: 231S-6S.
13. Omayma A. Eldahshan1 and Abdel Nasser B. Singab1*
14. Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.
15. Mervat Sh. Sadak and Hisham A. M. Mostafa , Botany Dept; Division of Agricultural and Biology, National Research Centre, Dokki, Giza, Egypt, 33 El Bohouth st P.O.12622 C. Gopalan, B. V. Rama Sastri & S. C. Balasubramanian. Nutritive value of Indian foods, 10-11.
16. George Ansstas, 'Vitamin A deficiency'. University School of Medicine, Washington, Jun 11, 2014.
17. Clare Gilbert, 'Vitamin A Deficiency Disorder in Children', 2013.
18. Ajit Sinha, MD; Jost B. Jonas, MD; Maithili Kulkarni, MD; Vinay Nangia, MD. 'Vitamin A Deficiency in School children in Urban Central India: The Central India Children Eye Study', August 2011, Vol 129, No. 8.
19. Umesh Kapil & H.P.S. Sachdev. 'Massive dose vitamin A programme in India - Need fora targeted approach', All India Institute of Medical Sciences & Sitaram Bhartia Institute of Science &Research, New Delhi, India. Indian J Med Res 138, September 2013, pp 411-417.
20. Mustapha, Y. and Babura, S.R. "Determination of carbohydrate and β- carotene content of some vegetable consumed in Kano metropolis, Nigeria" Department of Biological Sciences, Bayero University, P.M.B. 3011, Kano, Correspondence author.
21. Lichtenthaler HK, Wellburn AR., 2003. Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical Society Transactions, 11, 591–592.
22. Peter Weber, MD, PhD Professor of Nutrition Corporate Scientist for Human Nutrition & Health DSM Nutritional Products Ltd. Kaiseraugst, Switzerland.
23. Alfred Sommer, Dean, School of Hygiene and Public Health Johns Hopkins University Baltimore, MD, USA.
24. Groff JL, Gropper SS, Hunt SM. Advanced Nutrition and Human Metabolism. New York: West Publishing Co. 1995. 575 pp.
25. Olson JA. Vitamin A. In Ziegler EE, Filer LJ. Present Knowledge in Nutrition. Washington (DC): ILSI Press. 1996. P 109-19. © 1997. USANA, Inc. All rights reserved. 4 BETAC-8/97.
26. Handelman GJ. Carotenoids as scavengers of active oxygen species. In Cadenas E, Packer L (Eds). Handbook of Antioxidants. New York: Marcel Dekker, Inc. 1996. P 259-314.
27. National Research Council. Recommended Dietary Allowances. Washington (DC): National Academy Press. 1989. 284 pp.
28. Swanson JE, Parker RS. Biological effects of carotenoids in humans. In Cadenas E, Packer L (Eds). Handbook of Antioxidants. New York: Marcel Dekker, Inc. 1996. P 337-70.
29. Khachik F. Carotenoids in fruits, vegetables, and human blood and their role in nutritional prevention of cancer, heart disease, and cataract. Nutrient Composition Laboratory, BHNRC, ARS, USDA. 2005.
30. Ali H. Jasim Basheer A. Wassan M. College of Agriculture University of al-qasim College of Science University of Babylon.
31. T. Ul Hassan1, A. Bano, Department of Plant Sciences Quiad-i-Azam University, Islamabad 45320, Pakistan.
32. Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia Gombak, 53100 Kuala Lumpur, Malaysia.
33. Ministry of Marine Affairs and Fisheries, Republic of Indonesia, KS. Tubun Petamburan VI, 10260, Central Jakarta, Indonesia.
34. Center for Agro-Based Industry, Ministry of Industry Affairs, Republic of Indonesia, Ir. H. Juanda 11, 16122, Bogor, West Java, Indonesia. Accepted 5 December, 2011.
35. Erich G. Annual Report, The Genetics and Biochemistry of Floral Pigments Plant Biology 2006; 57:761–80