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Current Insights on the Photoprotective Mechanism of the Macular Carotenoids, Lutein and Zeaxanthin: Safety, Efficacy and Bio-Delivery

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ABSTRACT

Ocular health has emerged as one of the major issues of global health concern with a decline in quality of life in an aging population, in particular and rise in the number of associated morbidities and mortalities. One of the chief reasons for vision impairment is oxidative damage inflicted to photoreceptors in rods and cone cells by blue light as well as UV radiation. The scenario has been aggravated by unprecedented rise in screen-time during the COVID and post-COVID era. Lutein and Zeaxanthin are oxygenated carotenoids with proven roles in augmentation of ocular health largely by virtue of their antioxidant properties and protective effects against photobleaching of retinal pigments, age-linked macular degeneration, cataract, and retinitis pigmentosa. These molecules are characterized by their characteristic yellow-orange colored pigmentation and are found in significant amounts in vegetables such as corn, spinach, broccoli, carrots as well as fish and eggs. Unique structural signatures including tetraterpenoid skeleton with extensive conjugation and the presence of hydroxyl groups at the end rings have made these molecules evolutionarily adapted to localize in the membrane of the photoreceptor cells and prevent their free radical induced peroxidation. Apart from the benefits imparted to ocular health, lutein and zeaxanthin are also known to improve cognitive function, cardiovascular physiology, and arrest the development of malignancy. Although abundant in many natural sources, bioavailability of these compounds is low owing to their long aliphatic backbones. Under the circumstances, there has been a concerted effort to develop vegetable oil-based carriers such as lipid nano-emulsions for therapeutic administration of carotenoids. This review presents a comprehensive update of the therapeutic potential of the carotenoids along with the challenges in achieving an optimized delivery tool for maximizing their effectiveness inside the body.

KEY TEACHING POINTS

- Lutein and zeaxanthin are the two most abundant natural xanthophylls (oxygenated carotenoids) with a linear C40 tetraterpene/isoprenoid lycopene-based backbone.
- Presence of extensive conjugation (more than 10 double bonds) enable these molecules to act as accessory light harvesting pigments apart from chlorophyll.
- More importantly, the xanthophylls prevent photobleaching of the pigments and proteins in the Light Harvesting Complex (LHC) by sequestering the excess unutilized blue light and preventing triplet chlorophyll associated formation of Reactive Oxygen Species.
- In human eye, lutein, zeaxanthin along with mesozeaxanthin constitute the three macular pigments forming the so called "yellow spot" of the macula and are implicated in maintaining the redox balance, homeostasis and normal physiology of the eyes.
- However, unlike plants, xanthophylls must be acquired from dietary sources such as colored leafy vegetables and egg yolk.
- Increase in the number of eye diseases in the aging population coupled with insufficient bioavailability of xanthophylls has mandated the industrial production of supplements enriched in xanthophylls.
- The bioavailability and delivery of xanthophylls can be significantly enhanced by suspension in a blend of extra-virgin olive oil and other vegetable oils.

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Introduction

The number of incidences of ophthalmic diseases including cataract, Age-related Macular Degeneration (AMD), glaucoma, and diabetic retinopathy have increased alarmingly over the last few decades leading to gradual and progressive loss of eyesight (1, 2). In addition, the dry eye disease has increased the burden of these debilitating pathologies (3). Various ocular manifestations have also been revealed in young adults as an aftermath of the COVID-19 pandemic partly due to the effect of the virus itself (4) and largely due to the prolonged use of screen time with most of our professional activities being transformed to digital mode (5). Almost all of these traits are complex and multifactorial and diagnosed at a later stage where the damage becomes irreversible (6). Consequently, the management of ocular health has become an intense issue for scientists and health professionals across the globe.

Carotenoids are fat-soluble colored pigments chiefly found in plants and algae which are identified by the bright coloration which they impart to vegetables like pumpkin, carrot, bell-pepper, lettuce, beans, sweet potatoes and many others and cater for a diverse group of functions including acting as precursors of plant hormones, attractants for pollination, antioxidants as well as accessory photosynthetic pigments (7). However, probably the most important physiological function of carotenoids is its role as a photoprotective pigment in the plant. Although the entire band of 400–700 nm is photosynthetically active, the most effective region is above 620 nm. During absorption of the visible spectrum for photosynthesis, the primary photosynthetic pigments are continually exposed to other regions of the spectrum including the UVA and UVB regions. The excess unutilized light mostly belonging to the blue end of the spectrum along with those in the near UV region increases the probability of triplet state transition in chlorophyll which consequently leads to the formation of Reactive Oxygen Species (ROS) damaging both proteins and lipids in the vicinity (8). This results in considerable photobleaching of the chlorophylls leading to their chlorosis or permanent irreversible damage (9). The carotenoids with their conjugated polyene backbone serve as ideal quenchers of the nonproductive radiation and thus safeguard the main photosynthetic pigments from bleaching. In addition, conjugation is conducive to electron delocalization thus stabilizing the ground state of the molecule. Consequently, it becomes equipped to trap free radicals (10) and dampen oxidative stress. Based on this core concept, carotenoids have emerged as effective phytotherapeutics for preserving macular health and protecting the retina from damage by blue light (11). Based on their structure, carotenoids can be classified into the non-oxygenated carotene and oxygen containing xanthophyll. Lutein, zeaxanthin and mesozeaxanthin, together referred to as macular pigments, are integral components of the macula, the so-called "yellow spot" found in the central foveal region of the retina where they provide a filtering effect to cutoff the blue wavelength, stop photobleaching (12), prevent lipid peroxidation (13) and also act as anti-inflammatory agent (14). Xanthophylls are indispensable for ocular health and their daily physiological requirement is met through green leafy vegetables, corn, pumpkins, papayas, spinach, kale and bell-peppers as well as certain fishes like sardine and egg yolk. Apart from higher plants and eggs, considerable amounts of carotenoids are also found in photosynthetic algae (15) and cyanobacteria (16). Since these molecules have a long hydrocarbon chain, the bioavailability of these molecules does not always cater for the body's daily needs, especially if fruits and green vegetables are not present in adequate amounts. In addition to boosting ocular health, the beneficial effects of xanthophylls as anti-inflammatory (17), anticarcinogenic (18) and anti-oxidative (19) agent is also well established. Therefore, nutraceuticals and functional foods fortified with xanthophylls have gained popularity over the last few decades. This review presents an updated insight on the chemistry and biology of lutein, zeaxanthin and mesozeaxanthin with regards to their therapeutic applications; especially alleviation of ocular health as well as the technological constraints in achieving optimized delivery and biosorption.

The structure, biosynthesis, and dietary source of xanthophylls

Xanthophylls are oxygenated carotenoids with a linear C40 backbone derived from the tetraterpene/isoprenoid lycopene with approximately 9–13 double bonds in trans configuration. Although they are essentially lipophilic in nature, unlike the carotenes, the presence of oxygen atoms in the form of hydroxyl, aldehyde, ketone and epoxy groups at the end of the polyene chain impart some amount of amphiphilicity to these molecules thus enabling them to align in proximity to the membrane phospholipids. The two most abundant xanthophylls present in plants are lutein and zeaxanthin in addition to others including β-cryptoxanthin, capsanthin, astaxanthin, and fucoxanthin. Lutein is chemically a 3*R*,3′*R*,6′*R*-β,ε-carotene-3,3′-diol derived from alpha carotene. In chloroplasts, it comprises half of the total carotenoids. Its structure is optimally suited for the biological role of the molecule. Zeaxanthin (3 R,3′R-β,β-carotene-3,3′-diol) differs from lutein in having two β rings instead of the ε ring with a different location of the double bond (between 4th and 5th carbon instead of 5th and 6th carbon in lutein) which allows for extension of the polyene conjugation to the ring with a resultant bathochromic shift in absorption and enabling zeaxanthin to be a more efficient chromophore than lutein. Zeaxanthin also acts as an efficient photoprotective pigment like lutein; however, it is always present in a lesser amount as compared to lutein for reasons as is explained in the subsequent section of xanthophyll cycle. Although eight stereoisomers are possible for lutein, only the Z (cis)-form (R,R,R) is abundant in nature plausibly because conjugation is not very strong in the other conformations and therefore the other ones have not been selected owing to their primary biological roles. Zeaxanthin, on the other hand, has two chiral centers but shows only 3-stereoisomeric forms (R,R), (S,S) and (R,S-meso).

The biosynthesis of carotenoids in plants occurs chiefly through the mevalonate (MVP) pathway (20) or *via* the alternate deoxyxylulose-5-phosphate (DOXP) or

methylerythritol 4-phosphate (MEP) pathway (21) which synthesizes isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the two isoprenoids, in plant chloroplasts, algae and cyanobacteria which are subsequently channelized for the formation of lycopene, the final precursor of carotenoid synthesis pathway. Alpha carotene, synthesized from lycopene, undergoes hydroxylation at the β and ε positions by two cytochrome P450 enzymes to give lutein (22) whereas zeaxanthin is made from beta-carotene by beta-carotene hydroxylase through the addition of a hydroxyl group (-OH) to carbon 3 and 3′. Mesozeaxanthin, is a stereoisomer of lutein structurally different from the former only with respect to a double bond and is probably synthesized only in the macula of the vertebrate eye (23). Insignificant amounts of mesozeaxanthin are found naturally in turtle fat, fish skin and shrimp cells (7) and therefore their dietary intake is negligible. Apart from lutein-meso zeaxanthin conversion, the entire pathway of carotene biosynthesis is missing in the animal kingdom including human beings and therefore they are completely dependent on chiefly plant based dietary sources such as colored and leafy green vegetables as well as egg yolk (24). The potential of algae as seaweeds as natural sources of xanthophylls like fucoxanthin and astaxanthin have also led to the exploration of species such as *Laminaria japonica, Hizikia fusiformis, Chlorella spp., Rhodophyta spp., or Spirulina spp* as sources of nutraceuticals to boost ocular health (15). In higher vertebrates, beta carotene also acts as a precursor for Vitamin A and its cleavage product, retinal, the most important component of rhodopsin.

The dual role of xanthophylls in photosynthetic plants: the significance of their photoprotective mechanism and the xanthophyll cycle

Xanthophylls are indispensable pigments for protection of the photosynthetic plants from photobleaching. Their integral role is evident in their close association with chlorophyll (Chl) molecules as well as membrane proteins constituting the light harvesting complex (LHCs) (25). Electron transport chains are initiated within the light harvesting complex from the photosynthetic reaction centers which eventually generate reducing equivalents in the form of NADPH used subsequently to fix atmospheric carbon dioxide. However, the LHCs are operative only within an optimal value of light intensity (around 5,000 to 10,000 lux) (26). Lux values significantly higher than this cause substantial photobleaching of chlorophyll whereas low light intensity causes insufficient sensitization of the LHCs thus mandating the recruitment of additional pigments for initiating the light phase. The presence of conjugation in the long aliphatic chain allows the xanthophylls to effectively trap light as additional light harvesting pigments whereas the two aromatic rings form the basis of the protective mechanism of the pigments. Plants which either have mutations in the carotenoid biosynthesis pathway or have been treated with herbicides that are specific inhibitors of the biosynthetic pathway such as norflurazon (desaturase inhibitor) have been especially found to be susceptible to photodamage (27).

The photoprotective properties of the xanthophylls are executed through several mechanisms. Presence of hydroxyl groups at the β- and ε-rings imparts amphiphilic properties to lutein and allows its localization in the thylakoid membrane. Additionally, they also enable the molecule to quench the free radicals from oxygen and chlorophyll thus serving the role of an effective antioxidant. In more severe cases, oxidation of the end rings lead to complete irreversible damage of the xanthophylls, a phenomenon termed as "sacrificial radical scavenging" which provides protection to the primary photosynthetic pigment chlorophyll (28). There are also provisions for extension of the conjugation in the molecule for example by substituting a ketone group to the fourth ring carbon. In addition, they neutralize the harmful effects of singlet oxygen and triplet chlorophyll thus preventing oxidation induced damage to the thylakoid pigments and proteins (29). It is imperative that since the xanthophylls sequester and trap excess unabsorbed radiation during photosynthesis, they compete with the primary photosynthetic pigments or the chlorophylls for trapping the solar radiation. As discussed earlier, zeaxanthin is maximally suited to absorb blue wavelengths due to its extension of conjugation. Therefore, under low light conditions limiting the rate of photosynthesis, zeaxanthin is further converted to violaxanthin (a di-epoxide) *via* the mono-epoxide intermediate antheraxanthin (30). Conversion to epoxide hinders the extension of the conjugation to the ring thus blocking the absorption potential of zeaxanthin and allowing for residual photosynthesis mediated by the primary pigments. During morning hours under bright sunlight, violaxanthin is converted back to zeaxanthin thus restoring its protective role. It was found that the de-epoxidase activity is particularly activated by a drop in pH of the thylakoid membranes due to increase in light absorption beyond simultaneous utilization of energy in the form of ATP (31). The xanthophyll cycle has also been implicated in controlling the photosynthetic yield according to seasonal or spatio-temporal changes in the intensity of sunlight.

Threats to ocular health

Very much like the photosystems, the vertebrate eyes also have light sensitive cells and pigments which are vulnerable to intensity or wavelength inflicted damage (32). Solar radiation is essentially a polychromatic spectrum encompassing the visible (400–800 nm) as well as the UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (200–280 nm) regions (33). UV radiations can cause severe damage to all parts of the eye causing cataract, macular degeneration as well as corneal damage in all age groups but more frequently in elderly people (34). Luckily, the ozone layer protects us from the bulk of the short wave UV-C and a part of UV-B radiation (35). Retinal damage can also result from natural causes such as snow blindness (sunlight reflected from snow) or staring at the sun with bare eyes during an eclipse (36). Photo-oxidation resulting from chronic exposure to radiations of lesser intensity is also an equally potent threat to ocular health. In order for photooxidation to occur, sunlight must first be absorbed by the chromophore present in the

lens and retina of the eye whose composition keeps on changing through the entire life. Chromophores which serve protective roles during the early years of growth are converted to phototoxic molecules with a higher quantum yield (capacity to produce singlet oxygen) (37). For example, adult human lens has an abundance of the yellow chromophore 3-hydroxykynurenine, a metabolite of tryptophan which safeguards the eye both by filtering UV light as well as absorbing blue light and releasing it quickly before it can cause any harm (38). However, slowly thereafter as the body undergoes aging, it is converted to xanthurenic acid by the enzyme kynurenine aminotransferase (39). Unlike kynurenines, absorption of light by xanthurenic acid leads to production of singlet oxygen and other superoxide radicals which elicit oxidative damage to the lens proteins (40, 41). Continuous photooxidation of cellular tryptophan reserve results in a formylated derivative of kynurenine which is also capable of generating singlet oxygen and superoxide (42) subsequently damaging the lens protein. Apart from endogenous chromophores, other externally consumed agents such as drugs or related phytotherapeutics can also absorb light and retain it for considerable time causing damage to the eye. Although underestimated, the shorter wavelength visible spectrum, especially the blue wavelength region between 400 and 500 nm is equally potent in inflicting damage to the eye due to its higher penetrating power. Over the last decade, the menace of the blue light has increased to enormous proportions due to unprecedented rise in the use of LED screens, especially during the long COVID era (43). Scientific studies have established that blue light affects circadian rhythm (44) by inducing secretion of excess melatonin which causes alteration in secretion of cortisol (45); this can adversely affect the body homeostasis resulting in serious ailments including obesity, diabetes, and cancer (46). Particularly, blue light mimics sensitization of retinal ganglion cells (47) which alters the pupil response time; in addition, it induces ROS production in corneal epithelial cells and subsequently activates the pyrin-domain containing-3 (NLRP3)-interleukin (IL)-1β signaling pathway which leads to inflammatory response and concomitant apoptosis of the corneal cells (48). Blue light has also been shown to accelerate AMD. In a study carried out on rabbit retina, application of blue light resulted in scattering of both the outer and inner photoreceptor cells (49). In extreme cases, blue light inflicted death of cone cells in the outer layer of retina results in infiltration of macrophages into the retina and heightened release of pro-inflammatory cytokines causing retinal edema (50) with complete or partial loss of vision. complete loss. Therefore, although overshadowed by UV radiation and its market value, short wavelength visible radiation like blue light poses an equally challenging threat toward eye health.

Major ocular diseases and xanthophylls in their management

The most frequent and significant disease affecting central vision in elderly people is AMD which affects near or far central vision in patients and accounts for close to 10% blindness worldwide (51). It is generally encountered in one of the two forms; the dry AMD occurs due to yellow deposits of lipids and proteins under the retina termed "drusen" accompanied by degeneration of photoreceptors (1) whereas the more threatening wet form is associated with release of exudates arising out of Vascular Endothelial Growth Factor (VEGF) induced choroidal neovascularization (CNV), behind the eye through the retinal pigment epithelium (RPE) (52). This often results in retinal bleeding followed by complete irreversible loss of eyesight. Oxidative damage to the retina inflicted by free radicals is the primary cause of AMD and xanthophylls perform the primary and integral role in continually protecting the macula from free radical induced damage. It has been demonstrated by several studies that the increase in dietary intake of Lutein and Zeaxanthin had a strong positive correlation with decreased risk of AMD, especially at the late stages (53). Additionally, higher incidence of AMD at either early or late stage was associated with decrease in plasma zeaxanthin level (53).

Apart from AMD, other equally significant disorders which can bring about total or partial permanent loss of eyesight include glaucoma, premature retinopathy, diabetic retinopathy and cataract. The term "Glaucoma" is used to describe a group of diseases caused by the damage to the optic nerve, retinal nerve fiber layer and retinal ganglion. The most commonplace reason accounting for the disease is accumulation of fluid from eye exudates due to poor drainage causing gradual increase in ocular pressure without any initial external manifestation. Primary open angle glaucoma (POAG) is the most observed form of the disease (54) caused by rise in Intra Ocular Pressure (IOP). It has been suggested by recent research that besides fluid accumulation, sustained oxidative damage to the retina is also a major factor for development of POAG (55). As already discussed, lutein and zeaxanthin are both excellent quenchers of free radicals and due to their strategic location aligned to the membrane phospholipids, they can effectively protect free radical induced peroxidation of the retinal membrane phospholipids. Therefore, although there has been no conclusive evidence of carotenoid induced regulation of retinal pressure, administration of lutein and zeaxanthin has been found to improve visual acuity by preventing oxidative damage both to the retina and lens (55, 56). Premature retinopathy occurs due to downregulation of the Vascular Endothelial Growth Factor caused by a sudden increase in partial pressure of oxygen in the outside environment as compared to the fetus. Repressed levels of VEGF prevent normal retinal vasculature which in turn leads to abnormal angiogenesis and intravitreal neovascularization, together with accumulation of reactive oxygen species (ROS) (57). Chronic hyperglycemia is a potent elicitor of oxidative stress which causes cellular toxicity (58) *via* by a plethora of signaling pathways including NF-Ķβ, p38 MAPK and stress activated Jun-kinase pathways (59). Diabetic retinopathy is one of such several complications which occurs due to oxidative stress induced damage to retinal protein. The condition entails severe alteration of vasculature with microaneurysms, retinal hemorrhages, discharge of hard exudate

from the eye, blockage in capillary circulation and subsequent macular edema. The chance of development of the disease is significantly higher in case of chronic Type 1 diabetes than Type 2 diabetes (60). A third significant threat to ocular health is posed by cataract or clouding of the retina caused by misfolding and aggregation of the lens protein crystallin which slowly accumulates on the retina causing blurring of vision (61). The interior side of the retina is lined by a single layer of epithelial cells. Eventually, these cells are differentiated into the fiber cells which orient as thin layers on the retina. To maintain the transparency of the retina, the turnover of crystallin molecules is kept at minimum and confined only at the outermost layer termed as the lens cortex. However, the small rate of turnover cannot account for maintenance of the retinal clarity as the person grows old. This results in aggregation of the old crystallin molecules which plausibly also initiates misfolding of the newer protein molecules, thus initiating cataract or opacity of the retina (62).

Significance of xanthophylls in human retina: Dietary source, route of uptake and function

Much like their roles in plants, oxygenated carotenoids or xanthophylls serve integral roles in maintaining the redox balance, homeostasis, and normal physiology of vertebrate eyes. However, carotenoid biosynthesis pathways are missing in animals and therefore they must be procured from diet. Lutein, zeaxanthin and meso zeaxanthin are the major xanthophylls of the human retina which play integral roles in protection of the retina from blue-light induced damage and photo-oxidation of key membrane proteins as well as in improvement of visual acuity. Together, they are known as the macular pigments (63) and represent the best possible combination of carotenoid pigments for the human eye for prevention of Age-related Macular Degeneration (AMD) by offering synergistic protection to the neurosensory retina (55). Dark leafy vegetables, although rich in lutein (15–47 mole percentage), contain very little or no zeaxanthin. Out of the vegetables, maize has the highest amount of lutein (60%) and orange pepper possesses the highest amount of zeaxanthin (37%) (64). Other fruits and vegetables enriched in considerable amounts of both the xanthophylls include the more colorful ones such as grapes, orange as well as kiwi fruit and kale, spinach, lettuce and einkorn, khorasan, squash and durum wheat (11). Apart from fruits and vegetables, egg yolk was also found to contain substantial amounts of lutein and zeaxanthin almost similar to that of maize.The two major xanthophylls, lutein and zeaxanthin are both lipid soluble due to their long aliphatic chain; consequently, fat is essential to ensure their absorption and distribution to various body tissues *via* the bloodstream. Before absorption by the cells of the intestinal lining through the scavenger receptor class B Type 1 (SR-B1) (65), lutein esters must be hydrolyzed by GI tract enzymes such as cholesterol esterases. Consequently, they are incorporated into chylomicrons and released into the lymph. Apart from the retina of the eye, the liver hepatocytes and skin adipocytes are the other destinations of xanthophylls within the body One intrinsic advantage regarding dietary intake of xanthophylls stems from their considerable stability at elevated temperatures (66); therefore, pretreatment with heat as well as simple cooking facilitates bioavailability of these molecules from plant materials without sacrificing their biological attributes.

Inside the eye, the retinal membranes around Henle's fibers and the membranes of the photoreceptors are the chief sites of localization of lutein and zeaxanthin. Presence of more than ten alternate double bonds along the tetraterpene backbone makes this molecule ideally suited to quench singlet oxygen generated during oxidative stress, moreover, the hydroxyl groups impart partial polar property to the molecule so that they can be aligned along with the lipid bilayer of the photoreceptor membranes and protect the membranes from lipid peroxidation. It has been demonstrated that processing of light stimulus takes place much faster and more accurately through the fovea of the retina which has a higher concentration of cone cells as compared to the visual periphery. Therefore, much like the thylakoid, zeaxanthin concentrations are significantly (about 2.5 times) higher in the fovea or the central portion of the eye which receives the highest concentration of light and its gradient fades toward the periphery. On the contrary, lutein has higher concentration in the peripheral segment of the retina providing continual protection against the damaging effects of free radicals especially in the rod outer segments. Meso-zeaxanthin has the highest anti-oxidative potential amongst the three and has the highest density at the macular epicenter (67). Each of the two carotenoids is retinally uptaken by separate binding proteins. The human Glutathione S transferase 1 protein (GSTP1 isoform) has been identified as the candidate protein responsible for binding zeaxanthin in the macula (68). Lutein is found in many tissues, but the relative amount of this xanthophyll in the retina is the highest among tissues. A lutein-binding protein was recently isolated from the human peripheral retina and shown to interact with antibodies against a protein belonging to the steroidogenic acute regulatory domain (StARD) family (69). Tubulin has been reported to be the third xanthophyll-binding protein (70). The third xanthophyll-binding protein is tubulin present in high concentrations in the axons of photoreceptor cells (Henle fibers). Apart from the retina, these macular pigments are also present in the stratum corneum or the outermost epidermal layer of the skin as well as in the liver adipose tissue (71). Owing to its wider distribution, the daily need of lutein is approximately 10 mg as compared to that of 2 mg of zeaxanthin (72).

In addition to their integral role in prevention of blue light and UV induced photo-oxidation of the retinal pigments, lutein and zeaxanthin also impart the ability to avoid chromatic aberration and increase the ability to discern visual stimuli based on the wavelength, a phenomenon termed as contrast sensitivity (CS) (73). It is a frequent problem of the eye to discriminate between widely differing frequencies. Lutein and Zeaxanthin cut out the blue light from the spectrum to minimize the glare, luminance and chromatic aberration thus effectively increasing contrast sensitivity (Figure 1).

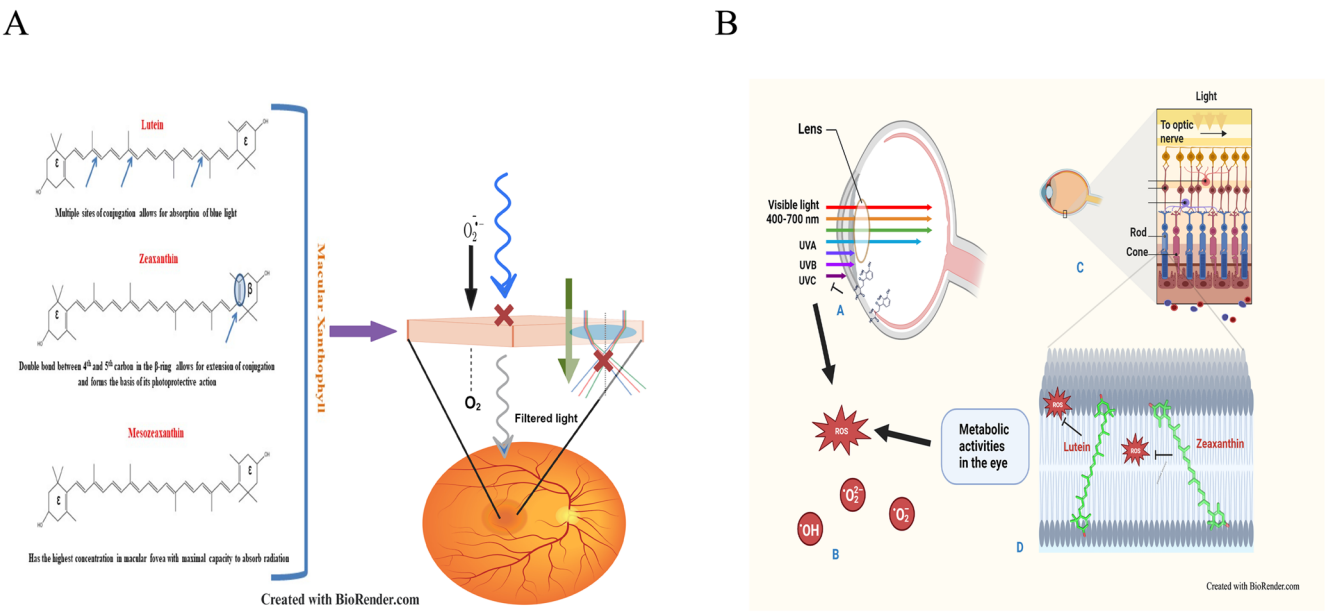

Figure 1. Structure of the three macular pigments and the chemical basis of their photoprotective action. (A) Lutein is chemically a 3 R,3′R,6′R-β,ε-carotene-3,3′-diol derived from alpha carotene whereas the precursor of zeaxanthin (3 R,3′R-β,β-carotene-3,3′-diol) is beta carotene. Zeaxanthin has a double bond between 4th and 5th carbon atoms instead of 5th and 6th which extends conjugation and makes zeaxanthin a more efficient chromophore. Mesozeaxanthin is synthesized by isomerization of lutein inside macula only and is an equally efficient photoprotective chromophore as zeaxanthin. The three macular pigments together ensure elimination of harmful blue light, sequestration of singlet oxygen and minimization of chromatic aberration. (B) Mechanism of bio-protective action of lutein and zeaxanthin: Although the UV light is filtered from entering the eye by the eye carotenoid 3 hydroxykynurenine (A), it can still give rise to Reactive Oxygen Species (ROS) both from the photooxidation events as well as the intrinsic metabolic activities going on inside the eye (B) causing elicitation of inflammatory response. The retina containing the photosensitive rod cells and cone cells as well as the eye lens are susceptible to the effect of free radicals and resultant inflammatory response causing irreversible partial or complete blindness over a period of time (C) The macular carotenoids, lutein and zeaxanthin can align themselves in the membrane of the rod cells due to their partial amphiphilic character, presence of several conjugated double bonds in these molecules help to quench the free radicals and thus protect the rod and cone cells membranes from peroxidation and other injuries (D).

Other bioprotective roles of xanthophylls

Apart from their indispensable role in photoprotection of the eye, xanthophylls also serve a plethora of bioprotective functions owing to their unique structural features. A significant amount of esterified xanthophylls are also found in the human skin implicating their roles in protection of the skin from harmful UV radiation (74) and consequent inflammatory response causing the so called sunburn or apoptotic destruction of skin keratocytes (75). Dietary lutein and zeaxanthin were also found to prevent UV mediated swelling of skin and uncontrolled division of skin cells (76). Since oxidative stress and inflammation are closely linked to carcinogenic development, astaxanthin, β-cryptoxanthin and fucoxanthin, in addition to lutein and zeaxanthin, have shown promising anticancer activities in animal models (77). Presence of two keto groups forms the basis of antioxidant action of astaxanthin. The molecule has been shown to arrest carcinoma in a hamster buccal pouch carcinogenesis model through inhibition of nuclear factor kappa B (NF-κB) and Wnt/β-catenin signaling pathways with parallel activation of nuclear factor-erythroid 2 related factor 2 (Nrf2) transcription (78). Gap junction communication between normal and cancer cells is a major governing factor behind initiation of metastasis (79). Astaxanthins increase the number of gap junctions thus effectively blocking the progression of cancer. Xanthophylls also indirectly prevent development of carcinoma by facilitating regulation of the cell cycle (80) and initiation of apoptosis (81).

Since xanthophylls protect cells from oxidative stress and inflammation, they are also effective to combat cardiovascular diseases (CVD). Administration of lutein was found to significantly decrease the chances of coronary artery disease and stroke by lowering the levels of IL-6, IL-1β, and TNF (82). Moreover, lutein exposure of peripheral blood mononuclear cells from CVD affected patients dampened the secretion of pro-inflammatory cytokines. The results cumulatively hint at the prospects of these xanthophylls as therapeutics for treating CVDs. Xanthophylls are also known to prevent bone degeneration and thus arrest osteoporosis by inducing death of osteoclasts (83). The antiosteoporotic activity of xanthophylls has also been reported in menopausal women (84). However, this effect on osteoclasts seems to be unique to β-cryptoxanthin and has not been reported till date in other xanthophylls. In addition, significant leads toward managing obesity and nonalcoholic fatty liver disease (NAFLD) have also been obtained from the use of β-cryptoxanthin (85). Chronic NAFLD may lead to severe inflammation and injury to hepatocytes which eventually turns into steatohepatitis leading to liver cirrhosis or hepatocellular carcinoma (86). β-Cryptoxanthin by the virtue of its potential to arrest lipid peroxidation and modulate macrophage formation can effectively block steatosis. The use of lutein and zeaxanthin is increasingly becoming popular for alleviation of cognitive damage. Several placebo-controlled studies have indicated that a combination of these two xanthophylls improved cognitive performance, memory

retention, verbal fluency and curbed the symptoms of dementia (87, 88). Conversely, in patients with Alzheimer's disease or milder forms of dementia, lutein and zeaxanthin levels have been found to be very low (89).

Bioavailability of xanthophylls

To exert their physiological effects, carotenoids must be absorbed and transported by the bloodstream. The strong lipophilic nature of these molecules mandates the deployment of high-density lipoprotein (HDL) as the chief delivery agent for their transportation to the retina (90). Because of the hydroxyl groups, lutein and zeaxanthin have partially amphiphilic characters as compared to the completely hydrophobic carotenes (α-, β-carotene, and lycopene). Therefore, in order to ensure optimal biosorption, a strong understanding of both the *in vivo* chemistry and biology of these molecules are required. The chief factors that determine the absorption of the essential macular pigments from food are (i) the form (raw/processed/cooked) and texture (hard/soft) of the food (ii) the content of dietary fat and dietary fibers in the food which mediates easy release of the carotenoids and (iii) the chemical nature of the carotenoids themselves. As stated previously, due to the higher thermostability of the carotenoids, cooked food might have a better release efficiency of these molecules as compared to raw food (91). However, it must also be reinstated here that during industrial production of these carotenoids, use of higher temperature may cause gradual degradation of both lutein and zeaxanthin into low molecular weight compounds thus significantly reducing their shelf lives (92). Fruits provide a better bioavailability of xanthophylls (close to 100%) as compared to green vegetables (about 19–38%) of similar carotenoid content owing to their better transfer to micelle (93). This seems intriguing because most of the xanthophylls from fruits are esterified and before efficient absorption into chylomicrons, these must be de-esterified (94). The micellization or effective transfer of xanthophylls from the food matrix to the chylomicrons determines the bioaccessibility and corresponding health augmentation from a particular source of dietary xanthophyll. An effective test to assess micellization involves *in vitro* simulated digestion of the food source with gastric juice followed by centrifugation and transfer of the supernatant present in the carotenoids to artificial micelles (95). In healthy individuals, about 25% of lutein, zeaxanthin and meso-zeaxanthin are concentrated in the macula as a yellow spot; while lutein and zeaxanthin of are also found in serum, meso-zeaxanthin conversion is strictly restricted in the retina only (96). Mesozeaxanthin is the strongest of the three macular pigments in terms of anti-oxidative potential owing to the extension of conjugation in the ring from the aliphatic chain. In spite of around forty carotenoids found in the human serum, the retina contains only lutein, zeaxanthin and mesozeaxanthin with a highest concentration of about 1 mM at the foveal center which is about thrice the concentration of these pigments found in other locations of the body. In the macular fovea, the concentration of these pigments is the highest which falls off toward the periphery. The maintenance of this much high concentration of these selective macular pigments mandates the deployment of a large number of lipoprotein carriers including low-density lipoprotein (LDL) accounting for 55% of the cargo, high-density lipoprotein (HDL), accounting for 33% of the cargo and very low density lipoprotein (VLDL) carrying the remaining 10–19% of the pigments. In a colon carcinoma Caco2 cell line, administration of Tween 40 resulted in increased uptake of the macular pigments (97). These data indicate that the serum lipid profile is one of the major governing factors behind bioaccessibility of xanthophylls. The essentiality of lipids in the diet for transportation of xanthophylls particularly to the retina has been highlighted by several studies. In a Wisconsin Hypoalpha Mutant Chicken with HDL deficiency, the retinal concentration of these pigments didn't pick up in spite of feeding them with a high lutein diet; however, its concentration in other tissues including the serum increased significantly (98). Although egg yolk is a rich source of xanthophylls and can potentially impart a better bioavailability of the pigments into the macula owing to the associated high lipid content, consumption of yolk is associated with risk of CVDs. Consumption of three eggs per day for a period of 12 wk resulted in elevated lutein and zeaxanthin levels (21% and 48%) in a study carried out on 20 adults (99). It is also possible that apart from micelles, concentration of other xanthophyll binding circulating proteins such as albumin, tubulin and lactoglobulin also play important roles in affecting the retinal localization of the pigments (100).

The global market of xanthophylls

The versatile therapeutic potential as well as fast growing application in cosmetic industry as a substitute of synthetic colors has witnessed an unprecedented growth in the global market value of carotenoids with a current market size of USD 1.5 billion and an estimated rise to USD 2.1 billion by 2030. The demand of xanthophylls has also increased proportionally with luteins only accounting for 23% of all carotenoids (101). Marigold flowers of the genus Tagetes are the currently exploited raw materials for industrial production of lutein (102, 103). Microalgal biomass promises to provide abundant low-cost raw material for the industrial production of a plethora of therapeutics, nutraceuticals, food and feed supplements and other value-added products owing to high growth rate and correspondingly vast production potential without significant use of land and water resources. Production and extraction of carotenoids from microalgae has been no exception in this regard as these marine organisms have been found to be prolific producers of a battery of carotenoids including astaxanthin, beta-carotene, lutein, fucoxanthin and zeaxanthin (104). Carotenoids from microalgae have one potential advantage as compared to those from plants on being produced and secreted as free de-esterified forms. However, there are a few technological limitations. Also, the adoption of Salt water is known to be highly damaging for photosynthetic pigments and results in oxidative

degradation of the carotenoids. Growth in seawater, therefore, decreases the net biomass and consequently production of carotenoids. Culturing in freshwater is one possible route to circumvent this problem but taking into account the scarcity of freshwater resources, this again imposes a bottleneck in microalgal carotenoid production. However, a recent study has revealed that regulated salt stress can actually increase the production of carotenoids (105). Other similar studies have highlighted the role of various additives such as Titanium oxide (as a photocatalyst) (106), gamma amino butyric acid (107), sodium chloride (108), etc. in boosting production xanthophyll under salt stress. Apart from microalgae, certain species of filamentous fungi as well as yeasts have also shown the potential to serve as good production hosts. For example, *Blakeslea trispora*, a filamentous fungus, has been extensively studied for the production of β-carotene and lycopene (109). Yeasts along with bacteria have also been reported to be efficient producers of Zeaxanthin (110). Bacterial species chiefly belonging to the genera *Flavobacterium* and *Paracoccus*, have been found to produce substantial amounts of these macular pigments (110). Introduction of TCA cycle intermediates also boosted the metabolism and simultaneous production of zeaxanthin (111). Alongside, metabolic engineering of key pathways has also aided in higher production of xanthophyll in heterologous yeasts as well as bacteria (112).

Evaluation of efficacy and safety of edible oils as vehicles for transport and sustained release of xanthophylls

Xanthophylls have been traditionally extracted in industrial scale using organic solvents such as hexane or ethanol with subsequent filtration. During extraction of pigments from maize, a protein termed zein co-separates in the ethanol fraction along with the xanthophylls (113) which is subsequently removed by ultrafiltration. Xanthophyll is then separated from the solvent ethanol employing nanofiltration (114). However, use of organic solvents are not free from environmental hazards and pose serious manufacturing leftover concerns. In order to maximize solubility and attain a sustained release of xanthophylls in the target tissue, the prospects of using edible oil from corn, rapeseed, canola and soybean for extraction of the xanthophylls have been explored extensively; edible oil-based carriers are eco-friendly and generally free of contaminants. Additionally, they prevent oxidative damage and slow down degradation by shielding them from exposure to oxygen. In a study on extraction of xanthophylls from dried pumpkin pulp, highest yield was obtained by using 10 times weight by volume of canola oil (115). Another group achieved a promising yield of carotenoids from dried carrot using an optimized mixture of soybean oil and olive oil (116).

However, higher viscosity often becomes a significant challenge for their subsequent extraction even at elevated temperatures. Use of ultrasound-assisted extraction (UAE) technology has been able to circumvent this bottleneck considerably (114). Other green extraction technologies for optimizing yield coupled with downstream inclusion into micelles such as accelerated solvent extraction, microwave assisted extraction, pulsed electric field assisted extraction, supercritical fluid extraction and enzyme assisted extraction have been reviewed extensively by Mikšovsky et al. (117).

Over the last few decades, several toxic side effects have surfaced regarding the use of vegetable oil for extraction and formulation of the industrially manufactured carotenoids intended for human consumption. A preliminary microarray analysis carried out on a stroke-prone spontaneously hypertensive rat model indicated that administration of both canola oil and soybean oil significantly altered the steroid hormone metabolism and brought about reduction in testosterone levels in the rat, with the effect of canola oil surpassing that of soybean (118). Both soybean and canola oil was found to cause atrophy of the seminiferous tubules and hypertrophy of the Leydig cells in pig models; corresponding DNA microarray analysis revealed downregulation of gonadotrophin in the pituitary gland (119). However, there was no directly available evidence of vegetable oil associated-cytotoxicity on the retinal cells themselves. In a separate study conducted on retinal epithelial cells, all four vegetable oils including olive oil, corn oil, argan oil and camelina oil increased the membrane fluidity (120). Therefore, although there have been few concerns regarding the side effects and associated toxicity regarding the use of vegetable oil as the primary medium for emulsification of the carotenoid formulation, they have been mostly insignificant and probably only of concern on consumption at a higher concentration.

Ensuring stability of carotenoids in commercial nutraceutical formulations

Several pieces of evidence have cited that it is not possible to obtain the two integral macular pigments, lutein and zeaxanthin, in optimal ratio from diet (121, 122). Both bioaccessibility (total amount of absorbable pigments after digestion) as well as bioavailability (amount entering circulation for affecting physiological changes) of the pigments are largely dependent on the type and nature of the matrix (123). As an additional challenge, the carotenoid contents as indicated on the product labels of commercially sold preparations of lutein and zeaxanthin most often do not tally well with the laboratory analysis of the sold product (124) plausibly owing to the degradation of these pigments in shelf. Therefore, the development of nutraceutical formulations incorporating the goodness of the macular pigments in optimal amounts as well as ensuring their stability and shelf life have been the major challenges of the nutraceutical industry. Several factors influence the level of both bioaccessibility and bioavailability including food matrix composition, processing level, and interaction with other dietary compounds (123). The two chief challenges limiting the shelf life of these products have been spontaneous oxidation of the carotenoids as well as solubility issues (122). The accelerated degradation of these carotenoids, especially commercially manufactured lutein at above optimal temperatures during

storage also presents a serious bottleneck toward achieving a stable formulation. Both lutein and zeaxanthin contain several conjugations in trans conformations which are pivotal for the chromophoric property of these molecules. However, thermal stress causes partial conversion of these double bonds to cis conformation thus seriously affecting their photophysical and photochemical properties (92, 125).

Two different approaches of matrix selection and packaging are in vogue for industrial manufacturing of lutein and zeaxanthin; (a) a powdered mixture of the carotenoids with filler which minimizes air space but cannot completely evacuate air and (b) an oil filled deaerated soft gel capsule packed under nitrogen blanketing to ensure complete absence of air. The oil filled soft gel based matrix has gained significantly more popularity owing to its capability to completely prevent any kind of oxidative damage to the carotenoids as well as preserve their original physicochemical properties. Conventional stabilization approaches include spray drying and microencapsulation using malto-dextrin, arabic gum and modified starch, however the procedure is complex and not cost effective. In order to protect the pigments from spontaneous oxidation, it is advisable to mix with antioxidants. Advent of new dispersion technologies have aided the development of several new emulsion techniques including liposomes, oil-in-water emulsion, microencapsulation and nano-emulsion, microencapsulation, and liposomes which can ensure a much higher bioavailability and prevent oxidation induced degradation of the carotenoids (126). A new formulation of dietary lutein and xanthophyll made from dried marigold flowers developed in our laboratory effectively caters for both the stability issue as well as the bioavailability by incorporating extra virgin olive oil (with 67% of oleic acid, 10% linoleic acid and 62% oleic acid) and Brassica napus L. (canola/rapeseed) oil having 62% oleic acid and approx. 20% linoleic acid (Patent applied) (Figure 2). Virgin olive oil has additional bioprotective roles. Oleocanthal, a phenolic derived from virgin olive oil, possesses significant anti-inflammatory, anti-oxidative, anti-microbial and neuroprotective activity (127). In addition, the formulation has also been fortified with quercetagetin extracted from defatted marigold flowers to enhance the shelf life and stability of the formulation as well as to act as additional safeguard against oxidative damage (128). The said formulation has a shelf life of about 36 months at 25○C temperature and stability of about 6 months at about 40 °C temperature. More studies are currently underway to investigate long term safety and stability of the formulation.

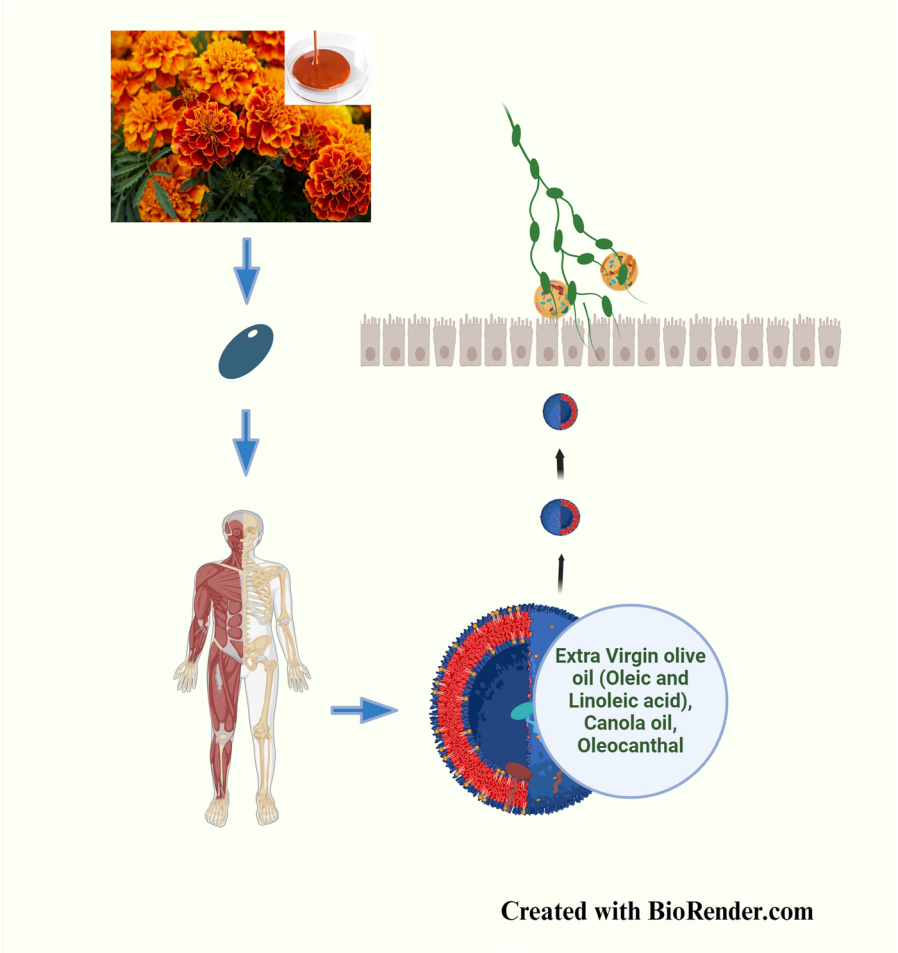

Figure 2. Biodelivery of macular carotenoids: Due to the presence of long chain aliphatics, lutein and zeaxanthin pigments must be packaged into a compatible vegetable oil-based liposome for ensuring optimal delivery and bioavailability. After being transported through the blood vessels, these liposomes are taken up by intestinal cells by inclusion into chylomicrons and subsequent delivery into the lymphatics. A new commercially viable formulation of these pigments made from marigold petals uses a mixture of extra virgin olive oil and canola oil and quercettagetin (antioxidant) to achieve a shelf life of 36 months at 25 °C.

Conclusion

Xanthophylls have emerged as one of the most sought-after pigments of therapeutic or other commercial value. Nature has gifted an abundance of these macular pigments in the form of fresh fruits and vegetables; however, as the human civilization has slowly metamorphosed from dependance of physical labor to complete reliance on brain and vision, the extra workload on eyes coupled with erratic dietary habits has contributed to the growth of macular degeneration to unscrupulous proportions over the last few decades raising it to the status of a lifestyle disorder. The unique metabolic journey of these pigments from the food through the bloodstream, right to the retinal fovea, ferried by the chylomicrons gives a telltale description of their structure, biochemical attributes as well as physiological functions. The integral role played by these pigments in averting phototoxicity is probably sufficient to earn them the status of paramount importance; however, over the recent years, other equally important physiological roles as well as therapeutic applications have surfaced which have given the much-needed shot in the arm to the xanthophyll industry. Extraction and bioavailability have been the two major bottlenecks in ensuring optimal delivery and distribution of the xanthophylls into the retina. Extraction has been facilitated by new generation green technologies to reduce the environmental burden. In order to avert the bioavailability issue, novel technologies such as cyclodextrin based carotenoid inclusion complexes (129), encapsulation within nanocages (130), nanodispersions (131) and nanoemulsions (132) have evolved over the last decade. Riding on these state-of-the-art extraction technologies and delivery agents, carotenoid-based nutraceutical industries are soaring high and promise to deliver the right blend of retinal pigments to ensure photographic vision thwarting the aging process. However, as a note of caution for ensuring holistic health, we must reassess the side effects and long term toxicities of each of the components which goes into the carotenoid formulation without getting too biassed only toward visual acuity.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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