CHAPTER VI

BIOACTIVITY OF LUTEIN AND ITS METABOLITES IN VITRO AND IN VIVO

Introduction:


Lutein and zeaxanthin are two of the seven major carotenoids found in human serum, however they are the only carotenoids present in retina and lens (Bone et al 1997). In recent years, there are reports on the oxidation products of lutein in vivo. The stereo isomers of lutein have been synthesized in vitro and studied for their antioxidant and anticarcinogenic properties (Khachik et al 2002). Khachik et al (2006) reported the presence of anhydrolutein and 3’oxolutein in the human eye. Bhosale et al (2007) reported the presence of meso-zeaxanthin in the human eye. There are reports on the biofunctional property of lutein, and other carotenoids and their oxidized molecules. Stratton et al (1993) characterized β-carotene oxidized products formed by interation with azo-compounds. Yeum et al (1995) studied the formation of β-apocarotenals and characterized their antioxidant ability in vitro. Khachik et al


Aim of the present study was to isolate and characterize selected lutein oxidation products from (1) auto-oxidation and UV-oxidation of lutein in vitro to study their antioxidant and antiproliferative property in vitro using Raw 264.7 macrophage cell lines (2) antioxidant and antiproliferative effects of lutein oxidized molecules (crude) generated by azo-mediated oxidation using Hela cells (3) antioxidant property of lutein in mice model.
CHAPTER VI: Bioactivity of lutein and its metabolites

Results:

**Purity of lutein oxidation products by preparatory HPLC**

Lutein oxidized products were purified by semi-preparative HPLC (Chapter 1). Under the conditions, lutein oxidation products were eluted from auto-oxidized lutein within 30 minutes at 450 nm. The peak identity of lutein was confirmed with the UV-Vis spectra of standard lutein. Each fraction has been collected and their respective spectrum and $\lambda_{\text{max}}$ were characterized (Figure 6.1). Among the fragments, two fractions were isolated (Figure 6.1) and referred henceforth as AO1 and AO2 and were used to study antioxidant and antiproliferative property.

Similarly, lutein in phospholipid liposomes exposed to UV-oxidation, was extracted for lutein oxidization products and was analyzed by semi preparative HPLC (Chapter 1). Four fractions were purified (Figure 6.1) and named as UV1, UV2, UV3 and UV4/zeaxanthin fragments. Since, the peak identity and $\lambda_{\text{max}}$ of fragment UV4 is as similar as the UV-Vis spectrum of zeaxanthin, the fraction was not structurally characterized using LC-MS.

Each fraction was concentrated and separately analyzed for impurities if any, spectrum and retention time using analytical HPLC (Figure 6.1). The fragments AO1, AO2 and UV1, UV2, UV3 and UV4/zeaxanthin were further purified by preparative HPLC are concentrated and characterized for their structures (Figure 6.2 and 6.3). The structures, chemical formula and approximate molecular mass of the selected fragments were given in Table 6.1.
Figure 6.1: Preparative HPLC elution profile of lutein oxidation products from auto-oxidation (a) and UV-oxidation (b) and analytical HPLC separation of fragments AO1, AO2 and UV1 to UV4.
Figure 6.2: LC-MS profile, characteristic structure and spectra of the fractions isolated from auto-oxidation of lutein.
Figure 6.3: LC-MS profile, characteristic structure and spectra of the fragments UV1, UV2 and UV3 fractions isolated from UV-oxidation of lutein.
TABLE 6.1: Selected lutein oxidation products used for antioxidant studies, their possible chemical structure, molecular mass and chemical formula.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Mol Wt.</th>
<th>Chemical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein</td>
<td><img src="image" alt="Lutein Structure" /></td>
<td>568.9</td>
<td>C_{40}H_{56}O_{2}</td>
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<tr>
<td>UV1</td>
<td><img src="image" alt="UV1 Structure" /></td>
<td>122.2</td>
<td>C_{9}H_{14}</td>
</tr>
<tr>
<td>UV2</td>
<td><img src="image" alt="UV2 Structure" /></td>
<td>300.5</td>
<td>C_{22}H_{34}O</td>
</tr>
<tr>
<td>UV3</td>
<td><img src="image" alt="UV3 Structure" /></td>
<td>285.5</td>
<td>C_{20}H_{29}O</td>
</tr>
<tr>
<td>UV4</td>
<td><img src="image" alt="UV4 Structure" /></td>
<td>568</td>
<td>C_{40}H_{56}O_{2}</td>
</tr>
<tr>
<td>AO1</td>
<td><img src="image" alt="AO1 Structure" /></td>
<td>366.54</td>
<td>C_{25}H_{34}O</td>
</tr>
<tr>
<td>AO2</td>
<td><img src="image" alt="AO2 Structure" /></td>
<td>417.7</td>
<td>C_{29}H_{39}O_{2}</td>
</tr>
</tbody>
</table>

(3E,5E,7E,9E)-10-(4-hydroxy-2,6,6-trimethyl cyclohex-1-en-1-yl) 4,8-dimethyl deca-3,5,7,9-tetraen-1-ylium

(2E,4E,6E,8E)-9-(4-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)-3,7-dimethylnona-2,4,6,8-tetraen-1-ylium

(2E, 4E, 6E, 8E, 10E, 12E)-13-(2-hydroxy-2,6,6-trimethyl cyclohex-1-en-1-yl) 2,7,11-trimethyl trideca 2,4,6,8,10,12-hexaene-1-one (Apo 8’ Zeaxanthinal)

(2E, 4E, 6E, 8E, 10E, 12E, 14E, 16E)-17-(2,4-hydroxy-6,6-trimethylcyclohex-1-en-1-yl) 2,6,11,15-tetramethylheptadeca-2,4,6,8,10,12,14,16 octaen-1-ylium.
Antioxidant properties of selected lutein oxidation products in vitro:

DPPH radical scavenging activity:

Antioxidant property of UV1, UV2, UV3, UV4/Z, AO1 and AO2 (10µg) were compared with lutein (10µg) and BHT (10µg, positive control). The degree of decrease in the absorbance of DPPH indicates the free-radical scavenging potentials (Figure 6.4). All the lutein oxidized products have shown different levels of antioxidant activity, while UV1, UV2, AO2 and BHT showed 24%, 74%, 62% and 54% higher activity than lutein (Figure 6.4). Interestingly, the radical scavenging activity of UV2 and AO2 was higher (27 and 11%) than BHT, indicating that these fragments can serve as potential antioxidants than lutein and BHT.

![Figure 6.4: DPPH radical scavenging activity of lutein oxidation products/oxidized fractions in comparison with lutein. ND- No activity.](image)

Inhibition of lipid peroxidation assay:

Antioxidant property of UV1, UV2, UV3, UV4/Z, AO1 and AO2 were compared with lutein (10µg) and BHT (10µg, positive control). All the oxidized fractions (10µg) have shown antioxidant activity against lipid peroxides while UV2, UV3, AO1 and BHT have shown 32%, 10%, 10% and 28% higher activity compared to lutein. The fragment AO2 has shown non-significantly higher activity over lutein, whereas UV4 (zeaxanthin) showed 15% lower activity compared to lutein (Figure 6.5). Results indicate that fragment UV2 can serve as potential antioxidant against lipid peroxides than lutein.
Figure 6.5: Inhibition of lipid peroxidation (measured by TBARS method) by lutein oxidation products in comparison with lutein. ND- No activity.

*Hydroxy radical scavenging activity:*

The hydroxyl radical is supposed to be one of the fast initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids. The hydroxyl radical scavenging activity of lutein oxidation products were estimated by generating the hydroxyl radical using an Fe$^{3+}$/ascorbic acid system. The hydroxyl radicals formed by the oxidation react with dimethyl sulfoxide to yield formaldehyde that provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The hydroxyl radical scavenging activity of lutein oxidation products (10µg) in comparison with lutein (10µg) is shown in Figure 6.6. Fragments UV1, UV2, UV4 (zeaxanthin), AO2 and BHT showed a higher antioxidant activity (68%, 61%, 27%, 11% and 66%), while AO1 fraction showed a decreased scavenging activity (11%) over lutein. However, the radical scavenging activity of fragment UV1 is slightly higher (5%) than BHT. Hence, the fragments UV1, UV2 and AO1 can serve as potential antioxidants than lutein against H$_2$O$_2$ radicals.
Figure 6.6: Percent change over control (lutein) in hydroxy radical scavenging activity of lutein oxidized products

Liposome model for antioxidant activity:

In this assay, to evaluate the antioxidant activity, liposome model was used and peroxides were induced using FeCl$_3$ and ascorbic acid. The antioxidative property of the compound depends on decrease in melonaldehyde formed over blank. Results show that capacity of all the fractions (10µg) were higher, compared to lutein (10µg) (Figure 6.7). BHT was used as a positive control. The activity of UV1, UV2 and UV4 were 43%, 55% and 28% higher than lutein respectively, and AO1, AO2 and BHT were higher than lutein by 20%, 41% and 68% respectively demonstrating that lutein oxidation products possess superior antioxidant property than lutein.

Figure 6.7: Percent change over control (lutein) in antioxidant activity of lutein oxidized products in liposome model.
CHAPTER VI: Bioactivity of lutein and its metabolites

Reducing power

The reducing power of lutein oxidation products (10µg) were compared with that of lutein (10µg) and BHT as shown in Figure 6.8. BHT (10µg) was used as a positive control. Fragments UV1, UV2, AO1, AO2 and BHT have shown higher reducing power over lutein by 43%, 42%, 48%, 46% and 65% respectively, whereas UV3 and UV4 showed a reduced activity by 37% and 23% over lutein respectively. Reducing power of a compound acts as an indicator for electron donor and can react with free radicals to convert them to stable products and terminate chain reactions. Hence, UV1, UV2, AO1 and AO2 are found to be better antioxidants than lutein.

![Figure 6.8: Percent change over control (lutein) in reducing power of lutein oxidized products.](image)

Comparative antioxidant activity of lutein oxidized fragments:

Results (Table 6.2) demonstrate that UV1 and UV2 fragments have higher antioxidant properties when compared to other fragments. UV4 (zeaxanthin) has higher antioxidant property than lutein in lipid peroxidation, hydroxyl radical scavenging and liposome model assay in comparison to lutein. AO1 and AO2 fragments found to be higher antioxidants in liposome model and reducing power assays in comparison to lutein. Further, the antioxidant activity of each fragment depends on its structure and nature of protection and also found to be radical specific.
CHAPTER VI: Bioactivity of lutein and its metabolites

Table 6.2: Comparative antioxidant property of lutein oxidation products

<table>
<thead>
<tr>
<th>Fragments</th>
<th>DPPH</th>
<th>Lipid peroxidation</th>
<th>Hydroxy radical scavenging</th>
<th>Liposome model</th>
<th>Reducing power</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AO2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UV1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UV2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UV3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UV4/Zeaxanthin</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lutein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BHT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

“+” - higher activity compared to lutein
“-” - lower activity compared to lutein

Anti-proliferative ability of lutein and its oxidation products

**MTT Assay**

MTT assay was performed to determine the proliferative status of Raw 264.7 cells (Carcinomatous, Macrophage cells) in the presence and absence of lutein and its oxidation products AO1, AO2, UV1, UV2, UV3 and UV4 respectively. Untreated control cells grew normally and are considered as 100% growth. Treatment with lutein and its oxidation products reduced the cell growth rate to different extent. Fragments UV2 and UV3 reduced the proliferation rate ~ by 2 folds better than lutein. Zeaxanthin (UV4) showed 2.5 folds lesser anti-proliferative effect than lutein. Auto-oxidized fragments of lutein (AO1 and AO2) showed activities similar to that of lutein (Figure 6.9). In comparison, the radical scavenging effect of UV2, UV3 and AO1 using model pro-oxidants and Raw 264.7 cells found to be more efficient than those of other fragments.
CHAPTER VI: Bioactivity of lutein and its metabolites

Figure 6.9: Effect of Lutein and its oxidation products on antiproliferative ability of Raw 264.7 cells. 5x10^4 cells/well; were incubated with the prescribed medium in presence and absence of equal concentration (1.0 µg/1 mL) of samples. Cell viability was measured by MTT assay. Antiproliferative potency was calculated in terms of % inhibition. UV2 offered ~ 80% protection followed by UV3.

**Effect of lutein and its oxidation products on cell morphology**

Cells adhesion plays an important role in tumor cell metastasis and organ invasion. When tumor cells attach to basement membrane and degrade them. Subsequently, cells migrate into underlying stroma. To access whether lutein and its oxidation products are capable of inhibiting cell adhesion, the present study was performed. Since reduction in cell numbers were evident upon treatment with lutein and its oxidation products, results were endorsed by morphological features. Control cells have formed clear monolayer with uniform spread of cells throughout the plate. However, upon treatment with lutein and its oxidation products differential changes were observed in a dose dependent manner. Lutein affected at the adherence at 1.5 µg concentration while UV2 & UV3 affected at 0.5µg concentration itself suggesting the increased toxicity imposed on cancer cells by them than lutein perse. It should also be noted here that although UV2 and UV3 are toxic at equal concentration as revealed by MTT assay, the type of toxicity induced may be different. Cell-cell bridge formation, a typical process of apoptosis was evident by UV3 treatment, while UV2 induced rounding and reduction of cell size, typical of necrosis. Although, both results in cell death the mechanism involved could be different between compounds UV2 and UV3.
This differential activity could be attributed structural difference in UV2 and UV3 by the presence of extra methyl group in UV2 in comparison with UV3. Precise biomolecular analysis is required for enunciation of the same. This observation is confirmed by inhibition of cell adhesion to the plate and confirmed by inhibition of cell adhesion to the plate, cell death/ apoptosis.

Figure 6.10: Effect of lutein and its oxidation products on cell morphology. 5x10^4 cells/well were treated with 0.5 to 1.5 µg concentration of each of samples and examined their effects on morphological features of cells as well as their ability to adhere to the plate. Photographs were captured at 40X.
Effect of apoptosis by Acridine – Orange method

As revealed by antiproliferative properties shown in Figure 6.9 and 6.10, drastic reductions in cell numbers were observed. Figure 6.11 confirms the cell death by lutein and its oxidation products on Raw 264.7 cells by acridine orange and ethidium bromide method. As indicated in Figure 6.11, control cells are normal and flattened with red-ethidium bromide, nucleus at the centre and symmetrically distributed acridine orange stained green cytoplasm. Upon treatment with lutein, cell perturbation was observed as depicted, by pericentric nucleus, cells with no nucleus, in addition to cytoplasmic and nuclear degradation. 1.5µg concentration indeed showed some perturbation in the cells. Zeaxanthin (UV4), which is an isomerized form of lutein affected more since complete degradation of cells were observed at 10 µg concentration itself. Further, it is intriguing to observe total disruption of cells at 0.5 µg concentration of UV2 and UV3, while 1.0 µg of UV1 was required to disrupt the cell. UV1 is the end product with a mass of 122.2 Da, while UV2 & UV3 are intermediates with a mass of 300.5 and 285.5 respectively. UV3 appear to have better effect than UV2, which may be due to its characteristic structure formed by the central cleavage of parent molecule, lutein.
Figure 6.11: Effect of lutein and its oxidation products on cell viability. Raw 264.7 cells were treated with equal concentrations (0.5 to 1.5 µg/ 5x10^4 cells/ well) of lutein and its oxidation products in DMEM medium. At the end of the 72 hr incubation, cells were stained with Acridine and Ethidium bromide. Ethidium bromide stains red for the nucleus and Acridine offers green colour to the cytoplasm.
Effect of nitric oxide (NO) production by lutein & its oxidation products

Nitric oxide (NO) is an important regulator and mediator of numerous processes in the immune system. It mediates macrophage cytotoxicity against tumor cells. Exogenous sources of NO, therefore, constitute a powerful way to supplement NO, when the body cannot generate enough of them. Natural NO stimulators, therefore, are warranted. The current study evaluated the effect of NO production by similar concentrations of lutein and its oxidation products. 5x10⁴ cells /well were incubated with 0.5 to 1.5 µg concentration of test samples. NO release from each well was quantitated as described under materials and methods and release of NO was expressed as µM/mL of the media in each well. UV3 induced NO up to 7 folds than lutein. Values are expressed as µM of NO released/ mL of media. As revealed in Table 6.3, UV3 and UV2 enhanced 6.8 and 4.6 folds higher production of NO than lutein respectively.

Table 6.3: Effect of lutein and its oxidation products on NO release by Raw 264.7 cells.

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Sample</th>
<th>µM of NO released/ mL of the media</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>4.095</td>
</tr>
<tr>
<td>2</td>
<td>Lutein</td>
<td>86.85</td>
</tr>
<tr>
<td>3</td>
<td>UV4/ Z</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>AO1</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>AO2</td>
<td>393.15</td>
</tr>
<tr>
<td>6</td>
<td>UV1</td>
<td>401.60</td>
</tr>
<tr>
<td>7</td>
<td>UV2</td>
<td>401.60</td>
</tr>
<tr>
<td>8</td>
<td>UV3</td>
<td>590.00</td>
</tr>
</tbody>
</table>

ND- Release of NO was not detected.
**Effect of lutein & its oxidation products on proinflammatory cytokine IL-2**

IL-2 is a proinflammatory cytokine released by immune cells including macrophages for immunological activation. Results (Table 6.4) demonstrate that, control Raw 264.7 cells expressed very low levels of IL-2 (0.2 U/5x10^5 cells). However, upon treatment with UV2 and UV3, 1.42 and 1.22 U per 5 x 10^5 cells were observed. Slight reduction in UV3 could be due to enhanced cell death. Since IL-2 is a secretory protein, increased IL-2 levels could be due to triggering of Raw cells towards increased production of IL-2, rather than by just more leaching into the medium due to cell death. However results may be substantiated by working on the effect of these oxidation products on mRNA of IL-2.

Table 6.4: IL-2 Production by Raw 264.7 cells by lutein and its oxidation products.

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Sample</th>
<th>Concentration of IL-2 (Units/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>Lutein</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>UV4/Z</td>
<td>0.95</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>8</td>
<td>UV3</td>
<td>1.22</td>
</tr>
</tbody>
</table>

**Influence of lutein oxidized products on cytotoxicity and antioxidant status of Hela cells**

The aim of this study was to explore the peroxyl radical induced oxidation products of lutein, for possible cancer-preventive action in comparison with lutein against oxidative stress, cytotoxicity and induction of apoptosis in Human Cervical Carcinoma Cells (HeLa). Lutein in liposomes was mixed with 2,2’-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) (5 mM) prepared in water was added to initiate the radical generation and oxidation of lutein and incubated at 37 °C, at dark for 3 hr with regular shaking (100 rpm/min) in a water bath. Further oxidation
of lutein was prevented by adding 100 µL of 0.1% BHT (w/v) in ethanol. Liposome with no added AAPH treated similarly was considered as control. Extraction of lutein and its oxidation products were processed immediately. Liposome without AAPH was kept in dark at room temperature (28 ± 2 °C) was considered as control. Lutein oxidized products were extracted and analysed for antiproliferative effect in comparison with lutein and BHT (positive control).

Lutein and its oxidized products extracted from AAPH treated samples show the free radical scavenging activity measured by DPPH assay. In addition, the scavenging activity of those extracts augmented with the increase in their concentrations from 5-10 µg/mL. Free radical scavenging activity of oxidized lutein was higher by 32.7% (IC₅₀, 2.64 µg) than lutein 44.1% (IC₅₀, 5.28 µg), while in case of BHT, activity was decreased by 30% (IC₅₀, 5.41 µg) (Figure 6.12a).

As described in materials and methods section, Hela cells were grown and experimented for cell viability and antiproliferative effect. To evaluate the effect of lutein and its oxidation products on the viability of cells, cells were seeded at a density of 5X10⁴ cells per well containing 100 µL of culture medium. The effect of lutein and oxidized lutein on cell toxicity showed that viability of oxidized lutein treated cells was lower by 22.4, 53.6 and 6% when compared to lutein, control (no lutein) and BHT treated culture (Figure 6.12b), respectively. Similarly, in BHT treated culture, the cell viability was lower by 70% and 6% compared to control and oxidized lutein whereas, in case of lutein, 44.1% higher cell viability was observed than BHT (Figure 6.12) respectively. Results of glutathione levels were 8.71, 6.51, 4.46, 3.72 µmol/mg proteins in control, lutein, oxidized lutein and BHT treated cultures, respectively (Figure 6.12c). Glutathione level in cells treated with oxidized lutein was lower by 48.8 and 31.5% compared to control and lutein treated cells, respectively. Similarly, the influence of oxidized lutein on the lipid peroxidation is shown in Figure 6.12d, where the MDA level in oxidized lutein was lower by 41.2 and 20.9% than control and lutein in culture cells. Whereas, the MDA level in oxidized lutein was 19% higher than BHT treated cells. The influence of oxidized lutein in comparison with lutein and control on the HeLa cells are shown in Figure 6.14. Results show the typical apoptotic cells in oxidized lutein than lutein and control treated cells, respectively. The cells were photographed with a microscope showing typical apoptotic cells.
Figure 6.12: Free radical scavenging activity (a), cells viability (b), glutathione level (c) and MDA levels (d) after incubation of cells either with lutein or Azo-oxidized lutein (OXL) or BHT. Values are mean ± SD (n=5). Values not sharing a common letter are significantly different (p < 0.05) between the samples.
CHAPTER VI: Bioactivity of lutein and its metabolites

Figure 6.13: Influence of lutein and oxidized lutein on viability of HeLa cells cultured for 48 h. Cells were seeded and cultured for 24 hr, and then treated either with no lutein (control) (a) or lutein (20 µmol/L) (b), or oxidized lutein (OXL) (c) or BHT (d). After culturing for 24 h, cells were fixed using 4% formaldehyde. Arrows indicate typical apoptotic cells. The cells were photographed with a microscope (100 X).

Bioactivity of lutein and its oxidation products in vivo:

Repeated dose study: Plasma and liver antioxidant molecules and lipid peroxidation

In this study, the effect of feeding lutein with specific lipids on the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione transferase (GST), glutathione reductase (GR), and levels of reduced glutathione (GSH), lipid peroxidation (measured in terms of MDA formed) was measured in plasma and tissues of mice.

As described in materials and methods (Chapter I), lutein solubilized in glyco- (GL), phospho- (PL), neutral (NL) lipids and wheat germ oil (WGO) or control micelles were gavaged to mice for 2 weeks. Mice not fed lutein micelles was
considered as baseline. Results on the activity of antioxidant enzymes in plasma and liver of mice are given in Table 6.5. GPx and CAT activities and levels of GSH and melanaldehyde (MDA) were measured in plasma. The activities of GPx and CAT increased by 52% and 48% respectively, while there was no significant change in MDA and GSH levels in control group over baseline values.

It is evident that lutein gavages with GL, PL, NL and WGO resulted in altered levels of antioxidant defence enzymes. The activity of GPx in plasma was increased by 51% in case of GL group, while the effect was non-significant in PL, NL and WGO groups over control group (Figure 6.14). CAT activity increased by 16%, 12% and 44% in GL, PL and WGO groups whereas the activity decreased by 16% in NL group over control group. In GL, PL, NL and WGO fed groups, GPx (69%, 58%, 54% and 52%) and CAT (56%, 54%, 40% and 71%) activities were significantly increased over baseline group. The plasma GSH levels in GL fed group was increased by 33% over control whereas the increase was non-significant in PL, NL and WGO groups.

The comparative results show that the plasma GPx, CAT and GSH levels in GL fed group increased over NL (32%, 27% and 28%) and PL (33%, 4% and 35%) groups. In WGO fed group, the plasma GPx activity and GSH levels were lowered by 35% and 17% respectively whereas the CAT activity increased by 33% over GL group. In case of NL group, the CAT activity and GSH levels were decreased (51% and 15%) over WGO fed group.

The activity of GPx, CAT, GST, GR and GSH levels in liver were higher (15%, 16%, 45%, 12% and 30%) but MDA levels was lowered by 10% in control group over baseline groups. The liver SOD and GR activities were increased in GL (52%, 32%), PL (50%, 20%), NL (16%, 7%) and WGO (55%, 35%) groups over control group. Results show that no significant change was seen in the GPx activity in GL, PL, NL and WGO fed groups when compared to control group. In PL group, GST activity increased by 26% while in GL and NL fed groups its activity decreased by 28% and 71% and no difference was observed in WGO group compared to control group. Liver GSH levels increased by 18%, 22% and 18% in GL, NL and WGO fed groups respectively over control group. Comparative results show that in liver, GPx, SOD, GST, GR activities in GL group were higher by 22.3%, 43%, 25% and 26% respectively over NL group. Liver GPx, GR, GSH levels in GL fed group was also
higher by 16%, 14% and 14% respectively over PL fed group. Similarly, in WGO fed group, GPx, SOD, GST, GR and GSH levels were higher by 14%, 47%, 22%, 30% and 16.3% respectively over NL fed group.

Figure 6.14: Percent change over control in antioxidant profile (a) GPx activity in plasma and liver (b) SOD and GR activities in liver (c) GSH levels in plasma and liver (d) MDA levels in plasma and liver of glycolipid (GL), phospholipid (PL), neutral lipid (NL) or wheat germ oil (WGO) lipid groups. (GPx- Glutathione peroxidase; SOD- Superoxide dismutase; GR- glutathione reductase; GSH- Reduced glutathione; MDA- Melanoldehyde)
Table 6.5: Activity of antioxidant enzymes/ molecules in plasma and liver of mice after administration of lutein solubilized in either GL, PL, NL, WGO lipid or control micelles for 2 weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>GL</th>
<th>PL</th>
<th>NL</th>
<th>WGO</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><strong>PLASMA</strong></em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (µmol/min/mg protein)</td>
<td>9.3 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.5 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.7 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.8 ±1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.4 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase (µmol/min/mg protein)</td>
<td>37 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione reduced (µmol/dL)</td>
<td>47 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.5 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Melanoldehyde (nmol/mg protein)</td>
<td>15.1 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.5 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.9 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><em><strong>LIVER</strong></em></td>
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<tr>
<td>Glutathione peroxidase (µmol/min/mg protein)</td>
<td>4.8 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.45 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.11 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.77 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Superoxide dismutase (U/min/mg protein)</td>
<td>12 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.3 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione transferase (µmol/min/mg protein)</td>
<td>3.6 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8 ± 1.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.86 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.1 ± 0.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.7 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione reductase (µmol/min/mg protein)</td>
<td>3.9 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione reduced (µmol/g)</td>
<td>84 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Melanoldehyde (nmol/mg protein)</td>
<td>29 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Value at each row not sharing a common letter are significantly different (p<0.05) between groups determined by ANOVA with Tukey’s test. GL- glycolipid; PL- phospholipid; NL- neutral lipid; WGO- wheat germ oil
Overall, the results show that the activity of antioxidant molecules in the GL group was higher than other groups which may be due to higher bioavailability of lutein. Further, the lower levels of MDA in these groups over control groups, further supports the higher levels of lutein in these groups over control.

**Dietary study: Antioxidant molecules and lipid peroxidation:**

Results on the activity of antioxidant enzymes in plasma and liver of mice on dietary feeding of lutein (one month) with in lipids or FGL are given in Table 6.6. In comparison to baseline group, the activities of CAT (53%), SOD (34%) and GSH (21%) were increased significantly on lutein feeding (control group), while no significant change was seen in GPx activity and MDA levels compared to baseline values. Results (Figure 6.15) demonstrate that plasma GPx activity was higher by 18%, 23% in GL and WGO fed groups and no significant increase was observed in PL group over control group. Plasma CAT and SOD activities were increased in GL (20%, 52%), PL (28%, 14%) and WGO (9%, 20%) groups over control group. Similarly, the plasma GSH levels increased by 39% and 42% over PL and WGO fed groups. As in case of purified lutein fed group, FGL and FGL+ WGO fed groups, the GPx (5%, 58%) and SOD (40% and 5;0%) activites elevated than control group, while no significant change was observed in CAT activity.

On comparision, results (Table 6.6 and Figure 6.15) indicate that GPx activity and GSH levels were higher in FGL+ WGO over PL (43%, 44%), GL (36%, 10%), WGO (31%, 40%) and FGL (40%, 18%) groups respectively. Increase in GPx activity in GL, WGO and FGL+ WGO in comparision with other groups may be due to the higher glycolipid along with lutein levels in GL and WGO fed groups.

In liver, apart from the antioxidant enzymes those were measured in plasma, GST activity and protein carbonyls levels were also measured. Protein carbonyls also act as secondary indicator for oxidative stress in vivo. In control, on dietary feeding of lutein, the SOD activity was increased by 24%, while GPx, CAT and GST activities did not alter significantly over baseline group. No significant change was observed in case of liver GPx, SOD and CAT activities over control in GL and PL fed groups. Comparitively, GPx and CAT activities were higher in PL group over GL group by 18% and 10% respectively. The GSH levels increased non significantly in GL group by 10% over WGO fed group (Figure 3.22). Liver SOD and CAT activities increased
in FGL (58%, 53%) and FGL+WGO (39%, 32%) groups over control group. In FGL+WGO fed group, the GPx activity was higher by 23% while SOD, CAT, GST activities were lower by 32%, 30% and 38% over FGL fed group.

Results on the plasma MDA levels show that its level was significantly lower in GL (54%), WGO (22%) and FGL+WGO (8%) while it was non-significantly higher in PL and FGL group over control group. The comparative data on MDA level in plasma demonstrate that MDA level was lower by 57% and 25% in GL over PL group. Addition of WGO along with FGL resulted in decreased lipid peroxidation and improved antioxidant activity, which may be due to alteration in saturated: unsaturated fatty acid ratio. Liver MDA and protein carbonyl levels were decreased in GL (44%, 7%), PL (25%, 30%) and WGO (25%, 21%) groups compared to control.

Further, in vivo results indicate that activity of antioxidant enzymes increased with increase in bioavailability of lutein. As shown in repeated dose studies, the bioactivity of lutein with respect to lowering lipid peroxidation is higher in GL fed group compared to NL and PL fed groups. Further, lutein from fenugreek leaf powder enhances the antioxidant property of lutein compared to purified lutein. It is also evident that wheat germ oil in diet along with fenugreek leaf powder impoves bioactivity as shown by the activities of antioxidant enzymes in plasma and liver. The improved lutein bioactivity may be due to the presence of lutein oxidation products in glycolipid group in comparison to control group. The present results suggest that lutein acts as an antioxidant and antiproliferative agent.
Table 6.6: Activity of antioxidant enzymes/ molecules in plasma and liver of mice after one month dietary feeding of lutein mixed with no added lipids (control), GL, PL, WGO, FGL and FGL+WGO.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>GL</th>
<th>PL</th>
<th>WGO</th>
<th>FGL</th>
<th>FGL + WGO</th>
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<tbody>
<tr>
<td><strong>PLASMA</strong></td>
<td></td>
<td></td>
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<tr>
<td>Glutathione peroxidase (µmol/min/mg protein)</td>
<td>2.3 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase (µmol/min/mg protein)</td>
<td>32 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Glutathione reduced (µmol/dL)</td>
<td>3.3 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.5 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Superoxide dismutase (U/min/mg protein)</td>
<td>6.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.2 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.1 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.5 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.5 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Melanoldehyde (nmol/dL)</td>
<td>55 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51 ± 1.8&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td><strong>LIVER</strong></td>
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<tr>
<td>Glutathione peroxidase (µmol/min/mg protein)</td>
<td>7.6 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.8 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Superoxide dismutase (U/min/mg protein)</td>
<td>58 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95 ± 6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase (µmol/min/mg protein)</td>
<td>9.4 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione transferase (µmol/min/mg protein)</td>
<td>5.1 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.6 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.5 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione reduced (µmol/g)</td>
<td>120 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>134 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>140 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>121 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>141 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>161 ± 5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Melanoldehyde (nmol/g)</td>
<td>7.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.7 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.3 ± 0.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein carbonyls (nmol/mg protein)</td>
<td>1.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD, (n=6 mice/group). Value in rows not sharing a common letter are significantly different (p<0.05) between groups determined by ANOVA with Tukey’s test. GL- glycolipid; PL- phospholipid; NL- neutral lipid; WGO- wheat germ oil ; FGL- fenugreek leaf powder.
Figure 6.15: Percent change over control in glutathione peroxidase activity (a), reduced glutathione, GSH (b), and MDA (c) in plasma and liver of mice fed purified lutein with no added lipids (control), GL, PL, WGO or FGL or FGL+WGO groups.
CHAPTER V: Bioactivity of lutein and its metabolites

Discussion:

The oxidative oxidation products of carotenoids are reported to involve in various biological functions like signal molecules and as photo sensitizer in vision (Palozza et al 2004). Thus, it is worth evaluating the biological effects of carotenoid oxidation products in comparison with intact carotenoids. Lakshminarayana et al (2010) reported that lutein oxidation products originated from by photo-oxidation exhibited higher antioxidant and cytotoxic properties than lutein. The oxidized forms of lutein may be highly reactive, since oxidation results in radical ions, which can combine with similar reactive oxidative species that could lead to higher antioxidant effect.

Khachik et al (1992) used semipreparative HPLC for separation of carotenoids from tomato with a flow rate of 1 mL/min. Breithraupt et al (2002) separated lutein monoesters using semi preparative HPLC with petroleum ether: acetone (85:15 v/v). The concentration of each eluent has been calculated on the basis of € value of lutein in methanol (Breithraupt et al 2002). Further, the higher antioxidant effect of lutein oxidised products as evidenced in UV1, UV2, UV3 and AO2 may be due to free electron rich polyene chain over lutein. This electron rich chain may act in scavenging free radical ion species. Dissimilarly, Wang et al (1999) reported that apocarotenoids formed from β-carotene induced cytochrome P-450 that destroys retinoic acid. In the present study, lutein oxidation products are protective in nature and in particular lutein oxidation products formed on reaction with AAPH mainly compose Apo-zeaxanthinal, is protective in nature and decreases cancer cell viability. Further in support, Siems et al (2000) reported the effect of carotenoid oxidation products on inhibition of Na+K+ATPase activity. Aust et al (2003) and Zhang et al (2003) isolated and characterised lycopene oxidation products are reported to increase gap-junctional communication with oxidised metabolite in comparision with lycopene. This may be true in this study that antioxidant property of oxidized lutein was higher and correlates with free radical scavenging activity (Figure 6.11). Lakshminarayana et al (2010) reported that oxidized products of lutein are chemically more reactive, the cells treated with oxidised lutein were considered to play important role in cancer cell cytotoxicity. The authors reported that oxidation products might be formed by reacting with reactive oxygen species. A polar oxidation product of β-carotene, 5, 8-endoperoxy-2, 3- didhydro- β-apocarotene-13-one was reported to inhibit cell growth and cholesterol synthesis in MCF-7 mammary cancer cells (Hu et al 1998).
Furthermore, 4-oxo-retinoic acid, an oxidation product of canthaxanthin activated RAR β-gene promoter and enhanced gap junctional communications (Hanush et al 1995). Similarly, the present results showed an increased apoptosis in oxidised lutein treated cells than lutein. Apoptosis could be due to increased oxidative status of cancer cells as indicated by higher level of accumulation of MDA and depleted glutathione levels. It is intriguing to point out here that since normal cells contains higher levels of glutathione; oxidised lutein may not cause toxicity to them (Lai et al 2001). Imbalance in the redox system in cell appears to be responsible for cytotoxicity of oxidized lutein in HeLa cells. This also justifies the differential effects of such compounds on normal cells where they can be cytoprotective. Further, under the experimental conditions used in this study, the concentration of lutein and lutein oxidation product formed are unlikely to affect normal cells. Oxidized lutein may have antioxidant and cytotoxic properties therefore can contribute potentially to anticancer property. Antioxidant property of oxidized lutein evidenced in this study is not related to inhibitory effect of the compound on cancer cell growth, which may be due to the effect on signalling pathway-involving apoptosis.

Although carotenoids have been reported as potent anticancer agents, their applications were hindered due to the pro-oxidant effect particularly β-carotene (Khachik et al 1995; Britton 1995). Individual lutein oxidation products identified in this study by LC-MS are given in Figure 6.2 and 6.3, which cause apoptosis induction against cells, are currently underway. Oxidized lutein was found to reduce the cancer cells viability through induction of apoptosis. These products have shown higher effect against cell proliferation and was found in the order of oxidized lutein > lutein. In agreement with earlier reports (Lakshminarayana et al 2010), the present investigation strongly suggests that oxidized lutein may affect proliferation of cancer cells efficiently.

The activity of antioxidant molecules was elevated in rats fed with oils containing unsaturated lipids compared to rats fed with coconut oil (Reena and Lokesh, 2010). The present results are in correlation with the above study that WGO rich in linoleic acid has higher antioxidant property when compared to NL group, may be due to higher level of PUFA content and presence of polar lipids. Polar lipids increase lutein bioavailability and also stabilize membrane lipids thereby alleviating the levels of antioxidant enzymes, and decreasing MDA and protein thiol formation.

204
Sindhu et al (2010) reported that lutein significantly enhances the antioxidant enzyme system in blood and liver tissue. He et al (2011) demonstrated that oral administration of lutein significantly suppressed the MDA production and increased the GSH levels in the eyes in mice. The authors also suggested that lutein can improve antioxidant activities at gene level in eye diseases. O’Connor and O’Brien (1998) demonstrated the protective effects of lutein on UVA induced oxidative stress by reducing MDA and increasing the antioxidant defence mechanisms. The antioxidant activity of lutein is higher than the baseline values which is in correlation with above reports, while the increase in antioxidant activity increased with the repeated gavage or dietary supplementation of lutein with GL and PL. However, WGO also enhanced antioxidant defence enzymes, which may be due to the synergistic effect of polar lipids present in it. In dietary study, the antioxidant activity in FGL group was higher when compared to WGO and FGL+WGO indicating that whole leafy greens act in enhancing bioavailability thereby enhancing antioxidant activity than supplements (van het Hoff et al 1999).

Detailed characterization and apoptotic properties of individual lutein oxidized molecules at molecular level and their relation to biological function *in vivo* deserves future studies.
GENERAL DISCUSSION, SUMMARY, CONCLUSION AND SIGNIFICANCE OF THE STUDY

Discussion

Having eye problems due to lutein deficiency isn’t foregone conclusion. There are steps that help ensure that our eyes stay healthy. Proper nutrition has been shown to help eyes healthy and fight off the impact of degenerative diseases and age related eye problems such as macular degeneration. Studies have shown that lutein and zeaxanthin play a vital role in eye health. These xanthophyll carotenoids are found in leafy green vegetables, fruits and vegetables. On consumption, these carotenoids specifically get concentrated in the macula of the eye. The macula is responsible for vision of the eye. Thus, people experiencing macular degeneration which is often age related. Nutritional and medical studies have shown that lutein and zeaxanthin are of the key carotenoids heading for maintaining good eye health and helping decrease the risk of developing macular degeneration. Lutein protects the eye against oxidative stress (He et al 2011). Studies found that an increase in lutein in macula lowers the risk of eye diseases (Arnal et al 2009, Johnson 2002). The antioxidant property of lutein may have protective effects against light/ prooxidant induced damages.

Although lutein has the potential to prevent various degenerative diseases, studies relevant to the real sources in which lutein content is higher in plants of Indian origin and its bioavailability are scanty. Further, the bioavailability of carotenoids, in general, lutein in specific, is lower than that of other lipid soluble components such as β-carotene, α-tocopherol and triacylglycerides (Richelle et al 2004, Reboul et al 2007). The major cause of low bioavailability is the poor solubility of lutein in digestive fluid (Yonekura and Nagao, 2007). At first, lutein must be released from the food matrix, the matrix interferes with the release of lutein (Mamatha and Baskaran, 2011). Lutien is poorly released from raw vegetables due to solid structure of cell walls, but processing and heat treatment accelerate the release of lutein by destroying the structure (Mamatha et al 2011). The released lutein must be well solubilized in the gastrointestinal fluid. However, its dispersion is greatly limited in digestive tract due to the high hydrophobicity of C_{40} isoprenoid carbon skelton. In this step, dietary lipids facilitate the carotenoid dispersion, followed by lipids helps the process of
emulsification and micellarization where lipolytic enzyme like lipase and bile salt/fluids plays a major role and finally lutein is solubilized in the mixed micelles (Nara and Nagao 2011). These mixed micelles loaded with lutein are taken up by the intestinal epithelial cells.

The above listed literature on the digestion and absorption physiology of lutein clearly demonstrate that work relevant to the role of newer lipids like glycolipid or wheat germ oil on the bioavailability and metabolism of lutein is not available. Moreover, scientific data on the natural produce that contain higher levels of lutein in India is also not studied in detail. Hence, in the present investigation, it was investigated (a) various natural produce for lutein and zeaxanthin (b) effect of dietary lipids on bioavailability of lutein and its metabolism/oxidation in vitro and in vivo and finally (c) the biological activity of lutein and its oxidation products.

In this study, green leafy vegetables (n=7), vegetables (n=21), spices (n=31), fruits (n=15) and vegetable oils (n=12) were analyzed for quantification of carotenoids using HPLC, LC-MS and OCC techniques. The available procedures (Kimura et al 2002, Burns et al 2003, Aman et al 2004) as discussed in Chapter 3, have reported a longer separation time (23, 30 and 17 min), whereas in the present study, 99% pure lutein is isolated using HPLC with at a run time of 4 min.

The present study reveal that among GLVs screened for carotenoid composition, in general, and lutein and zeaxanthin in specific, sheep’s sorrel, prostate amaranth and fenugreek leaves could be recommended for supplementation of food as they were found to contain higher levels (270, 180, 140 mg/100g dry wt.) of lutein and zeaxanthin. The level of total xanthophylls separated in leafy vegetables covered in the present study is about 5-10 times greater than that of the hydrocarbon carotenes. Values for xanthophylls reported in present study for spinach is about 4-6 times greater than those reported by Khachik et al (1986) . In vegetables, the present study shows that pumpkin has the highest level of lutein (10,620 µg/100g edible portion). Murkovic et al (2002) reported that lutein content of pumpkin varies from 0.14-17 mg/ 100g edible portion and they stated that the variation may be due to variety and place of cultivation. In India, there are many varieties of brinjal out of which, we have studied four varieties (round and black, round, purple and spotted, long and green and long and purple) and found that the long and green variety has highest level of lutein (1.80 mg/ 100g edible portion), which is 46% higher than the reported value (960µg) in lyophilized brinjal by Ben-Amotz & Fisher (1998). Further, changes in lutein
concentration during the maturation differ depending on the vegetable; in some cases an increase in lutein concentration has been reported, whereas in other cases, a decrease has also been reported (Calvo, 2005).

Among the spices used in this study, coriander, spearmint, curry leaves, red chilli and mustard seeds contain highest level of lutein (2 to 16 fold) than mace, anise seeds, onion, fenugreek seeds and carum in which the range was 0.62 to 0.84 mg/100 g dry wt. The levels of lutein (1.21 mg/100g dry wt.) in mustard seeds are higher when compared to mustard oil (0.78 mg/100g of oil) (Aruna et al 2009). The present results reveal that the level of β-carotene in turmeric and mace was below the detectable limit (0.568 ng) while Bhaskarachary et al (2008) reported β-carotene levels (µg/100g food stuff) as 60 (turmeric) and 2170 (mace). Data from this study show that kiwi fruit has the highest level of lutein (0.45 mg), whereas USDA (2003) and O’Neill et al (2001) have reported 0.12 mg and 0.14 mg/ 100g edible portion respectively. Among the vegetable oils studied, mustard and palm oil have highest levels of lutein compared to other vegetable oils, which may be due to deodorizing and processing of vegetable oils. Calvo (2005) reviewed that lutein concentration is higher in green vegetables than in yellowish white vegetables. The present study is in correlation with the above study that green vegetables have higher lutein content than light colored ones like onions. Moreover, changes in photosynthetic activity during maturation influences changes in lutein concentration. This may be true in carotenoids composition of unripened tomato (Murkovic et al 2000). Hornero-Mendez and Minquez-mosquera (1994) found that concentration of lutein decreases in peppers with increase in maturity; the same may be true in case of green chilli which decreases with increase in maturity. Numerous factors can influence lutein and other carotenoids concentration in natural produce (Calvo 2005). The data generated on the composition of carotenoids in the present study could be helpful to suggest better sources of lutein and zeaxanthin as a part of the daily meal to consumers, to overcome health disorders like AMD and cataract.

Human studies have shown supplementing diet with lutein and zeaxanthin can raise macular pigment density, but that both serum and macular pigment responses are quite variable (Neuringer et al 2004). Improving the bioavailability of lutein is important since lutein is not synthesized de novo, and depend on dietary intake of lutein rich vegetables. Zaripheh and Erdman (2002) and Tyssandier et al (2003)
opined that very small transfer of carotenoids from vegetable matrices to micelles which explain poor bioavailability of lutein. In the present study it was found that dietary fat i.e., glycolipids and wheat germ oil plays an important role in absorption of lutein and improve lutein micellarization in vitro. Deming and Erdman (2005) and Van het Hoff et al (2000) reported that dietary fat plays a major role in carotenoids absorption. Lutein being lipophilic their absorption follows that of lipid. Roodenberg et al (2000), Brown et al (2004), Unlu et al (2005), Lakshminarayana et al (2005) reported that addition of full fat spreads, avocado oil, full fat dressings, olive oil increases lutein bioavailability in humans and rats. Lakshminarayana et al (2009), Nidhi and Baskaran (2011) reported that dietary vegetable oils like olive oil enhances lutein absorption by modulating fatty acid profile in rats and increasing micellarization in vitro.

In the present study, glycolipid was used as lutein delivery vehicle to improve lutein bioavailability, since it modulates the process of micellarization, activity of intestinal lipase and plasma triglycerides response. Earlier studies (Sugawara et al 2001, Baskaran et al 2003, Yonekura et al 2006, Lakshminaryana et al 2006, Raju et al 2006, Raju and Baskaran, 2009) reported that phospholipids with long chain triglycerides increase lutein and \( \beta \)-carotene absorption in Caco-2 cells and rats. Despite the importance of phospholipids as effective emulsifiers, little is known with regard to their influence on lutein bioavailability. In view of biofunctional properties of glycolipids (Sugawara and Miyazawa 2000, Murakami et al 2003), recent research on glycolipids as potential drug delivery agents (Falco ner and Toth, 2007, Fricker et al 2010) for lipophilic vitamins.

Micellarization is an essential step in the process of absorption of carotenoids. In the present study, results on the percent micellarization in vitro demonstrate that glycolipids increases lutein micellarization by 67% over control, which may be due to higher activity of lipase that acted on the hydrolysis of lipids resulting in smaller particle size of GL micelles. Garett et al (1999) and Nidhi and Baskaran (2011) reported a higher micellarization with vegetable oils (Olive oil) than chicken and Ham. The micellarization of lutein is higher in glycolipids compared to phospholipid and neutral lipid indicating that the fatty acid composition of lipids may have a role. Further, the AFM studies suggest that micelle size in case of glycolipid is smaller in size compared to phospholipid and neutral lipid. The decrease in micelle size has
attributed to increased micellarization and increased bioavailability as seen in vitro and in vivo studies. Ulrich-Bott and Wiegandt (1984) have reported that glycolipid micelles are oblate shape and 60 A° in size, whereas the results of the present study show that glycolipid micelles are round in shape and 3.43 µm in size. Laksminarayana et al (unpublished data) reported that phospholipid micelles are globular in shape and 13.5 µm in size, whereas in the present study, the size measured was 5.78 µm. In the present study, size of GL (17 fold) and PL (10 fold) micelles are smaller than NL micelles. Hollander and Ruble (1978) and Raju et al (2006) reported that linoleic acid micelles larger in size decrease β-carotene absorption in rat model compared to oleic acid micelles. The size of the glycolipid micelles was smaller which may be due to its lipid components like DGDG (bilayer lipid) and MGDG (non bilayer lipid). Presence of MGDG may decrease the micelle size, thereby help in greater incorporation of lutein micelles, into the intestinal membrane when compared to phospholipid (bilayer phosphotidylcholine). Non bilayer glycolipid inserted into bilayer may modify biomembrane which assemble into hexagonal phase as they have conical shape with small polar head group and bulky acyl moiety (Goss et al 2005) which may favour lutein bioavailability. The result of the present study show that the mean plasma response of lutein was 6 fold higher for WGO fed group followed by GL (3 fold), PL (2.7 fold) and NL (2 fold) fed groups than control group. The lower micelle size may also be attributed as the reason for higher lutein bioavailability in single dose studies in case of GL group compared to PL and NL fed groups.

Results from single dose studies demonstrate that AUC values of lutein for 8 h is 6 fold higher in WGO lipid group compared to control. Ahuja et al (2005) and Laksminarayana et al (2006) reported an increased plasma response of lycopene and lutein after inclusion of olive oil to diet as compared to corn oil or groundnut oil. They also affirmed that the rate of intestinal uptake of those carotenoids depend upon their hydrophobicity of fatty acids in which carotenoids are solubilized and delivered. Goss et al (2005) suggested that the presence of higher levels of unsaturated fatty acids as found in the the wheat germ oil used in this study may be due to synergistic effect of polar lipids, may favour lutein bioavailability. In repeated dose studies, there is no significant increase in plasma lutein levels in phospholipid and glycolipid fed groups, while the eye and liver lutein levels were higher in GL fed group compared to PL fed group. Raju and Baskaran (2009) also reported that the mean plasma response
of lutein is lower, but the accumulation of lutein is higher in liver of rats fed on mixed phospholipid micelles. The increase in lutein accumulation in glycolipid group indicates that glycolipid may act as a potent facilitator in increasing lutein bioavailability compared to phospholipid and neutral lipids.

In dietary studies, on feeding mice with purified lutein with GL, PL and WGO, lutein levels in plasma and liver increased in the order of WGO > GL > PL > control, indicating the higher absorption of lutein in the WGO fed group, whereas the accumulation of lutein in eyes is higher in GL fed group compared to other groups. Further, the results demonstrated a higher bioavailability and efficiency of lutein in fenugreek leaf powder (FGL) fed group compared to purified lutein fed group. Moreover, the bioavailability of lutein is significantly higher in group fed on FGL+ WGO compared to purified lutein fed group, indicating the importance of WGO as lipid source and fenugreek leaf powder as lutein source as reported by Lakshminarayana et al (2007). Van Het Hof et al (2000) reported that lutein bioavailability is increased on feeding natural sources like leafy greens and vegetables than lutein supplements.

James et al (1997), Zhi et al (1996) reported that ingestion of diet with lipase inhibitor may suppress fat and β-carotene absorption. The present results also suggest an important role of lipase in lutein bioavailability in mice. Tyssandier et al (2001) reported that lipase plays a major role in absorption of xanthophyll carotenoids like lutein. Deming and Erdman (2005) reported that the presence of dietary fat in the duodenum triggers the release of bile acids from the gall bladder and regulates the level of lipase. The correlation between the plasma lutein response and intestinal lipase activity, as observed in present study, further supports the role of complex lipids in enhancing lipase activity and plasma lutein levels. Chitchummoonchokchai and Failla (2006) also reported that micellarization of zeaxanthin in vitro is increased on addition of lipase.

Results of the present study show that on feeding lutein, the plasma TG level proportionally increased with that of plasma lutein indicating that TG acts as a carrier for lutien. Further, the present results are in agreement with the studies of Baskaran et al (2003) and Lakshminarayana et al (2009, 2006), where they have also reported an elevated levels of TG on feeding gavage or dietary lutien. Feeding lutein with glyco-, phospho- and neutral lipids increased the liver TG level too. However, in plasma, the
TG levels are higher in neutral lipid group which may be due to higher level of TG in the neutral lipids. Borel et al (1998) and Odeberg et al (2003) reported that incorporation of β-carotene and astaxanthin into chylomicrons in human subjects was lower with emulsions containing medium-chain than long-chain triglycerides and suggested that medium-chain triglycerides are mainly transported by portal blood and do not favor the lymphatic transport of lipoproteins. The higher level of plasma TG reported in this study after repeated gavage and dietary feeding of lutein support the above mechanism. Lakshminarayana et al (2007) reported that feeding lutein with oleic acid rich micelles improved lutein bioavailability over linoleic acid rich sunflower oil in rats, whereas in the present study, linoleic acid rich WGO and glycolipid improved lutein bioavailability.

The knowledge on enzymatic and non-enzymatic oxidative defence mechanisms will serve as a guiding principle for establishing the most effective nutrition support to ensure the biological safety (Fang et al 2002). Antioxidants (GSH) and antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidases) exert synergistic actions in scavenging free radicals (Fang et al 2002). The role of lutein as an antioxidant was reinforced by the results of several in vivo tests in human and rats (Sindhu et al 2010). The activity of antioxidant enzymes studied was found to be higher with increased lutein bioavailability. As shown in repeated and dietary lutein feeding studies, the activity of antioxidant enzymes higher in GL and WGO fed groups when compared to PL and NL fed groups that may be due to an increased bioavailability of lutien, higher PUFA content and being polar in nature of the lipids fed. Reena and Lokesh (2010) reported increased levels of antioxidant enzymes when rats were fed with lipid rich in unsaturated lipids compared to saturated lipids. Lakshminarayana et al (2009) reported increased levels of antioxidant enzymes in rats of oleic acid fed group compared to linoleic acid fed groups.

The lipid profile in plasma and liver after lutein gavages and dietary feeding studies in the present study, suggest that diet or micelles with lower the unsaturated: saturated fatty acid ratio improve the lutein bioavailability and increase antioxidant enzyme activity. Niranjan and Krishnakantha (2001) reported that increased saturated: unsaturated fatty acid ratio in the diet leads to increased lipid peroxidation which affect the membrane fluidity on feeding saturated fat rich ghee. This is in agreement
with the present study that feeding dietary lipids (glyco-, phospho and WGO) decrease the saturated: unsaturated fatty acid content in plasma and liver, which in turn may influence the lipid peroxides and antioxidant enzyme activity. O’Connor and O’Brien (1998), Heagel et al (2000), Laksminarayana et al (2009), He et al (2011) reported a decrease in lipid peroxidation and antioxidant defence mechanisms on feeding lutein in humans and mice. Gruszeski and Strazlka (2005) reported that lutein and zeaxanthin act as “molecular rivets” and decrease membrane susceptibility to oxidative degradation due to unsaturated fatty acids. The authors suggested that lutein decreases lipid peroxide levels, thereby increase the lutein bioavailability. The present study suggests that polar lipids strengthen lipid membrane, which may be efficient in decreasing oxidative stress compared to neutral lipid. Mataix et al (1998) reported that oleic acid decreases lipid peroxidation compared to linoleic acid, which is in disagreement with the present study. Parker (1996) and Dixon et al (1994) suggested that carotenoids affect the expression of endogenous antioxidant enzymes, which is in correlation with the present study that increase in plasma lutein response and activity of endogenous antioxidant enzymes.

Oxidative degradation was used for many years in classical chemistry that led to the elucidation of carotenoid structures (Karrer and Jucker, 1950). In the present study oxidative degradation was used to study the reaction mechanism and to characterize the metabolites/ oxidation products and to compare them with that formed in vivo. Packer et al (1981), Krinsky et al (1989), Truscott (1996), Britton (1995) studied the oxidation of β-carotene by free radical, pulse radiolysis and photosensitizer reactions and reported the bleaching of β-carotene with time. These studies were in agreement with the present study that lutein on reaction with free radicals bleaches and degrade with time as shown in photo- and UV- oxidation. Krinsky and Deneke (1982) reported that carotenoids bleach on exposure to sunlight, which may be due to breakdown of conjugated polyene chain and degradation of carotenoids chromophore, which is observed in the present results of photo - oxidation and the formation of breakdown fragments C and D. The absorption of blue light by endo- and exogenous photosensitizers generates electronically excited reactive species (Schalch and Dayhaw-Barker 1999), which are efficiently absorbed by lutein. Jughans et al (2001) reported that lutein plays an important role in blue light filtration than other carotenoids, indicating that lutein effectively absorbs and prevents the excited
reactive species of blue light to affect the eyes, which was in correlation with the present study that lutien protects the eyes from harmful photo- and UV- oxidation.

Handelman et al (1991), Stratton et al (1993), Woodall et al (1997) used azo-compounds as peroxy radical generator, and reported that carotenoids degrade on exposure to peroxy radicals. Krinsky and Yeum (2003) studied Azo mediated oxidation of β-carotene and reported the formation of apocarotenals and endoperoxides, which was in agreement with the present study that lutein on reaction with peroxy radical generators, like azo-compounds resulted in the formation of apocarotenals and epoxides. Stratton et al (1993) reported the formation of β-apocarotenal on reaction of β-carotene with oxygen and proposed a possible attack of oxygen at 7,8-,9,10-, 11,12-, 13,14- double bond positions. A similar type of oxygen attack is observed with lutein in the present study which resulted in the formation of apo carotenals in auto- and azo mediated oxidation. Similarly, Yamuchi et al (1998) used azo-compound for auto oxidation of β-carotene and reported the formation of epoxides. The authors also reported that epoxide formation is slow without addition of azo compounds. Mortensen and Skibsted (1997), Krinsky and Yeum (2003) reported that addition of radicals to xanthophylls form epoxides. Oxidation studies of β-carotene and the formation of oxidation products of β-carotene were studied, but the studies regarding lutien oxidation products were scarce, which makes the present study unique. Lakshminarayana et al (2008) studied the oxidation products of lutein formed due to photo-oxidation and suggested the protective role of lutein in rat eye. Henry et al (1998) reported that degradation constant of lutein in safflower oil and revealed that degradation constant of lutein is lesser when compared to lycopene and β-carotene. This is the first study regarding lutein degradation kinetics with different types of radicals. Results on degradation kinetics in the present study demonstrate that degradation of lutein is higher in photooxidation followed by azo- and UV- mediated oxidation.

Studies suggest that complete characterization of lutein and their metabolites in retina may help in understanding the functional properties of lutein (Khachik et al 1996). Khachik et al (1992, 1995 and 1997) isolated and separated 3’epilutein, 3’ oxolutein and anhydrolutien from human and monkey eyes and serum. In the present study, 3’- epilutein (in HPLC) and anhydrolutein (LC-MS) was observed in intestine and also in the eyes of mice fed lutein indicating their circulation from intestine to eyes via circulation. Khachik et al (1997) also pointed that these metabolites or
oxidation products are formed as a result of dehydration of lutien under acidic conditions. This may be true in the formation of anhydrolutien and dehydrolutien in intestine. Lakshminarayana et al (2008) reported the formation of lutein oxidative/degraded products formed by photo-oxidation and in plasma, liver and eyes of rats. The authors proposed that the metabolites/oxidized products formed in vivo in eyes may be due to photo oxidation and that in the plasma, liver may be due to the peroxy radical attack. This observation is in agreement with the present study that lutein metabolites formed in vitro in photo- and UV- are similar to that of the eyes and metabolites formed in vivo, in liver and plasma are similar to that of azo mediated and auto oxidation mechanisms. Khachik et al (1995) proposed that dehydration products like anhydrolutien can be partially synthesized in vitro. Bone and Landrum (2001), Bernstein et al (2001) and Bhosale and Bernstein (2005) identified the presence of meso-zeaxanthin and its isolation in the eye and serum. Khachik et al (1997) and Khachik et al (2006) proposed the reaction mechanism for the formation of 3’ epilutein in the eyes and serum. As reported in this study, stereo isomers of lutein and zeaxanthin have also been identified in vivo in human eyes and plasma (Bone et al 1992, Khachik et al 1992). Khachik et al (2006) suggested that presence of lutein metabolites in human ocular tissues may be attributed to photo induced or enzymatic transformations of these carotenoids by a process unique to eyes or alternatively, these metabolites may be simply transported and accumulated in ocular tissues via circulatory system. The present study further supports the above mechanism. Nara and Nagao (2011) studied the oxidative transformations of lutein in mammals and reported the presence of anhydrolutien, 3’ epilutein and epoxy lutein from plasma of lutein fed rats. Wolz et al (1999) studied the astaxanthin metabolism in rat hepatocytes and reported that astaxanthin is cleaved at 9 and 9’ position in rat hepatocytes by β-carotene oxygenases, which may be one of the reason for formation of β-apo caroteanls in liver and plasma in mice. Mein et al (2011) reported in vitro expression of β-carotene oxygenases, which act on lutein thereby cleavage of lutein molecule to form apo-carotenals.

Dietary carotenoids and their metabolites/oxidation products have been implicated in the prevention of cancer, cardiovascular diseases and AMD (Khachik et al 2006). The higher antioxidant potency of lutein metabolites has been proposed by Khachik et al (2006) and Lakshminarayana et al (2008, 2010). The present study is in
correlation with the reports of Aust et al (2003) and Zhang et al (2003) who suggested that lycopene metabolites have more antioxidant nature in comparison to the lycopene and increase gap-junctional communications. Salgo et al (1999), Wang et al (1999), Siems et al (2000), Alija et al (2010) studied the negative effect of β-carotene oxidized products (apocarotenals) on DNA damage, Cytochrome P 450, Na+ K+ ATPase activity. Studies on antioxidant properties of individual metabolites of lutein suggested that UV3 and UV2 has higher antioxidant activity, which may be due to the presence of hydroxyl group at C-3 position. Santocono et al (2006), McNulty et al (2007) reported that zeaxanthin protects the DNA efficiently when compared to lutein and other carotenoids, which is in correspondence in the present study that zeaxanthin acts as a better antioxidant in comparison to lutein, which may be due to the presence of longer conjugated diene chain in zeaxanthin than lutein. As suggested by Lakshminaryana et al (2010) that lutein photo-oxidized products decreases cancer cell viability and lipid peroxidation, in the present study, cancer cell viability is decreased in comparison to lutein. The present study indicates the importance of lutein metabolites/oxidation products over lutein. However, further studies regarding individual lutein metabolites are warranted.

**Summary**

- Among the agricultural produce studied, lutein + zeaxanthin levels were higher in sheep sorrel, prostate amaranth, fenugreek leaves, yellow pumpkin, brinjal, green chilli, curry leaves, spearmint, coriander leaves, red chilli, mustard, bay leaves, kiwi fruit, guava, watermelon, mustard oil and palm oil. This data on carotenoid composition could help in suggesting better source of lutein for food supplementation.
- The analytical methods (OCC, HPLC, TLC and LC-MS) developed for separation and quantification of carotenoids were simple, accurate and reliable to obtain lutein with 99% purity.
- The OCC and TLC methods developed for isolation, separation and purification of wheat germ oil and glycolipid (99%) from wheat germ is novel.
- WGO and GL improve percent micellarable lutein in vitro and lutein levels in plasma, liver and eye in mice indicating the beneficial effect of GL and WGO.
• The influential effect of GL and PL may be due to smaller micellar size than the NL lipids. The size of micelles with different lipids is inversely correlated with plasma level of lutein indicating that the size of micelles and nature of lipids may play a critical role in lutein absorption.

• Lutein bioavailability from the fenugreek leaves is higher than purified lutein in mice indicates that lutein from complex food matrices and natural sources are more bioavailable than from purified lutein supplements.

• Presence of complex polar lipids like GL and PL increase intestinal lipase activity thereby influence the lutein micellarization and intestinal uptake.

• Feeding lutein with lipids decreases plasma and tissue lipid peroxidation, in the order of GL < WGO < PL < NL and increase the antioxidant defence enzymes like GPx, CAT and SOD. The present findings demonstrate for the first time the application of glycolipids and wheat germ oil as lipid vesicle to aid lutein bioavailability.

• Lutein degradation rate constants indicate that lutein decomposes faster in photo-oxidation followed by azo-mediated oxidation, UV- and auto-oxidation.

• Breakage of polyene chain, demethylated products of lutein formed on photo- and UV- oxidation and the similar kind of molecules, detected in the eyes indicate the photo-protective nature of lutein in eyes.

• Lutein on reaction with peroxy radicals mediated by azo-compounds and auto-oxidation, accepts the oxygen thereby results in the formation of epoxides and apocarotenals. These oxidation metabolites/ products are also evidenced in liver and plasma, indicating the peroxyl radical scavenging activity of lutein in vivo. This is the first finding detailing the oxidation pathway of lutein under different pro-oxidant attack in vitro and in vivo, which are the important findings of the thesis.

• Lutein degrades at low pH resulting in formation of breakdown products, while it oxidizes at alkaline pH which results in generation of apocarotenals and epoxy carotenoids. The critical pH range wherein lutein is stable is 6.5 to 7.

• The standardized procedure for extraction and purification of lutein metabolite/ oxidation products from UV- and auto-oxidized lutein are novel and new.
• The antioxidant properties of lutein oxidized products/metabolites formed by UV- and auto-oxidation were higher than lutein (M+H\(^+\)), while the antiproliferative properties were higher in fragments UV2 (M+H\(^+\)-C\(_{19}\)H\(_{27}\)O) and UV3 (M+H\(^3/2\)) as evidenced by morphological characteristics of Raw 264.7 macrophage cancer cell lines.

• The anti-inflammatory property (NO and IL-2 release) of fragments UV3 and UV2 are higher when compared to lutein, as evidenced by cancer cell line (Raw 264.7 macrophage cells) studies.

• The Azo-mediated oxidized lutein found to lower lipid peroxidation, HeLa cells viability and glutathione levels than lutein, indicating the antiproliferative properties of oxidized lutein.

• Finally, it is to state that lutein oxidation fragments possess significantly higher antioxidant and anti proliferative properties than lutein.

Conclusion

In conclusion, this study shows that leafy greens like sheep sorrel, prostrate amaranth, fenugreek are rich in lutein and β-carotene and among vegetables, lutein level is highest in pumpkin, followed by brinjal and chilli respectively. The spices (curry leaves, spearmint, coriander, red chilli, mustard, bay leaves) and fruits (kiwi, guava, watermelon) analysed were rich in lutein. Among vegetable oils analyzed, lutein level is highest in mustard and palm oils, whereas sunflower, olive, almond, rice bran and corn oils do not contain lutein, which may be due to the removal of carotenoids during the process of refining. The data generated on the composition of lutein and zeaxanthin in agri/ horticultural produce will help in choosing the right source or their combination that can provide therapeutic/ nutritional benefits. Awareness with respect to carotenoid content in natural sources may go long a way toward preventing, not only vitamin A deficiency and AMD-related disorders, but also may protect against chronic degenerative diseases, such as cancer and cardiovascular disorders and hence will be highly beneficial to the populace. Further, this is the first study on Indian spices, fruits and vegetable oils with regard to macular pigments, lutein and zeaxanthin.

The influence of lipids on lutein bioavailability in vitro and in mice was in the order of WGO > GL > PL > NL. Lutein bioavailability was higher for GL and PL
than NL lipids, but the effect was lower than WGO. This study suggests that ingestion of lutein with glycolipids or phospholipids is best for maintaining eye health, which is of high potential interest to prevent or treat AMD. Further, the choice of carrier lipid and its fatty acid profile is critical to achieve the above mentioned health benefits. The relative bioavailability of lutein could be improved with WGO and glycolipid in turn that may help in positively modulating the tissue fatty acid profile and antioxidant molecules. This finding may imply a new insight for the dietary recommendations of lutein with WGO and glycolipid for improved lutein bioavailability.

The present study shows that lutein is degraded faster in photo-oxidation compared to azo-mediated, UV- and auto-oxidation. Degradation of lutein in the body indicates that lutein is involved in various photochemical reactions (in eyes) and as an antioxidant in various peroxyl radical mediated reactions (plasma and liver). Based on the results, this study proposes metabolic pathways for the conversion of dietary lutein to its oxidation products. This study emphasizes the essentiality of maintaining dietary lutein status to function as an antioxidant in the body and photoprotective agent in eyes, because it is easily degraded. Further research is needed to identify lutein metabolites in detail in biological samples to evaluate their possible biological significance with reference to AMD. Further, it gives information on demethylated and dehydrated oxidation products of lutein formed in the intestine that may be transported to and accumulated in the retina via the circulatory system. But in-depth studies on the proteins in either lutein or its oxidized molecules binds and transported to target tissues are warranted. This is the first study on lutein oxidation/degradation kinetics with different radical species, and comparison of them with that of metabolites formed in vivo.

The oxidized lutein may be highly reactive, since oxidation by azo-mediated oxidation results in peroxyl radical ions, which can react with conjugated polyene chain of lutein that could lead to higher antioxidant effect. The higher antioxidant property of oxidized lutein than lutein correlates with free radical scavenging activity and cytotoxic effects on Raw 264.7 macrophage cells and HeLa cells. The oxidation products/metabolites formed in UV- and auto-oxidation indicate a higher antioxidant, anti proliferative and anti inflammatory properties when compared to lutein. Further, in depth knowledge on the biofunctional properties with respect to their role in
combating AMD are warranted. This is the first study on lutein oxidation products/metabolites isolation and their characterisation with respect to antioxidant and antiproliferative properties. Purification or synthesis of lutein oxidation products like UV2, UV3 and AO2 may have potential application in the areas of nutrition and pharmaceuticals.

**Significance of the study**

In the present study, an analytical procedure for carotenoid quantification and purification by OCC, HPLC from natural produce were standardized and found to be simple, compared to available procedures. The present findings may help to suggest better dietary sources of lutein and lipids as a part of the daily meal to consumers, to overcome AMD and cataract, which affords valuable information to the field of nutrition. The results suggest that dietary lipids rich in glycolipids help in improved lutein bioavailability by modulating the micelle formation and intestinal lipase activity are new findings of this study. Further, findings also provide a dietary approach to improve lutein bioavailability with glycolipids and wheat germ oil. The lutein oxidation products characterized from *in vitro* studies and similar components identification in plasma, intestine, liver and eyes of mice, evidencing the reaction mechanism of lutein *in vivo* are noteworthy in the field of nutritional biochemistry. Finally, the mechanism of action of lutein oxidation products with pro-oxidants and cancer cell lines at molecular level demonstrates their antiproliferative properties, merits further application of such molecules for maintaining the health of the eye with respect to AMD and cancer.

**Proposed future line of research**

Studies on the effect of polar lipids and their influence in modulating intestinal lipase activity, proteins involved in lutein binding and transport needs further investigation. Further, the metabolites/oxidation products formed *in vitro* and *in vivo*, needs further studies on mechanism of their action at molecular level. In depth knowledge on the formation of metabolites, their transportation to various target organs like macula lutea, corpus lutea, etc., are warranted. Antiproliferative properties and anti-inflammatory properties need further studies at genetic (mRNA) level to confirm their protective role against AMD and cancer.