Chapter-1
General introduction
General introduction

Lycopene is the compound accountable for the red colour in tomato fruit and is one of the major carotenoid detected in the human blood and tissues. The α-carotene, β-carotene and β-cryptoxanthin are known precursors of vitamin A and well studied (Bendich et al. 1989). The non-pro vitamin A carotenoid such as lycopene has gained special attention, and many studies have correlated its role in reducing the risk of prostate cancers and cardio vascular disease (CVD) (Giovannucci, 2005; Talvas et al. 2010). Considerable efforts have been expended to identify its biological and physiochemical properties. Structurally lycopene consists of open polyene chain lacking the β-ionone ring compared to β-carotene and share a same molecular mass and chemical formula. Even though the metabolism of β-carotene has been extensively studied, the metabolism and biological activities of lycopene is not much detailed. The unique chemical property of lycopene is very sensitive to oxidative modification/ degradation in vivo (Britton, 1995; Siems et al. 1999). Thus, lycopene is more prone to various modifications such as hydrogenation, dehydrogenation, double bond migration, chain shortening or extension, rearrangement, isomerization, oxidation or combinations of these processes under various conditions. The stability studies have shown isomerization and degradation of (all-E)-lycopene, when exposed to light, oxygen, high temperatures and under various exposure conditions (Schierle et al. 1997; Anguelova et al. 2000) (Figure 1.1). Apart from these, lycopene and other carotenoid metabolites may also be formed due to the presence of enzymes monoxygenase, cyclooxygenase, and dioxygenase. The lycopene derivatives (apolycenoids) (Lindshield et al. 2007), are formed by shortening of carbon skeleton from one or both the ends. Understanding the chemical structures of enzymatic/oxidative degradation products of lycopene may help in ascertaining their health benefits, which is yet to be addressed. Studies have claimed that lycopene metabolites are found at physiologically significant levels in serum and tissues in contrast to retinoids (Nikki et al. 2012). Nevertheless, the identification of oxidative metabolites of lycopene in biological fluids is highly critical. The breakdown of lycopene occurs through isomerization followed by oxidation to form epoxides or undergoes eccentric cleavage by carotene-9',10'-monooxygenase (CMO-II) to form apo-lycopenals (Hu et al. 2006; Wang, 2012). The formation of such lycopene
oxidative/cleavage products in vivo remains poorly understood (Caris-Veyrat et al. 2003). However, the characterization of CMO-II enzyme have demonstrated the excentric cleavage of lycopene to form apo-10'-lycopenoids (Hu et al. 2006). There are several reports, support the concept that biological activities of lycopene may be mediated by its oxidative products/cleavage products (Lindshield et al. 2007; Mein et al. 2010; Wang, 2012). Several lycopene metabolites have been identified in vivo and in vitro (Khachik et al. 1997; Kim et al. 2001; Caris-Veyrat et al. 2003; Gajic et al. 2006; Kopec et al. 2010), however their biological activities and mechanism of action of these oxidative products/metabolites in humans and animals needs to be elucidated.

![Lycopene Metabolites](image)

**Figure 1.1.** Formation of different lycopene breakdown products from parent compound.

**Sources of Lycopene**

Lycopene is found in highest concentration in tomatoes and tomato products, such as tomato sauces and ketchup, and in smaller amounts in other fruit such as guava, watermelon, papaya and grapefruit (Clinton, 1998) *(Table 1.1 and Figure 1.2)*. The concentration of lycopene in fresh fruits shows a great variability, depending on environmental conditions, geographic location, climatic situation, species and maturity. The average concentration of lycopene is about 5 to 10 mg per 100 g/fresh weight tomato (Hart et al.1995; Shi et al. 2000). ~ 15 mg lycopene in 100
g/fresh weight tomato fruit has been found for deep red tomato varieties, and yellow species are less rich in lycopene, with a content of only about 0.5 mg per 100 g/fresh weight tomato (Hart et al. 1995). Interest in lycopene has been increasing rapidly with the epidemiologic studies (Giovannucci, 1999) demonstrating lycopene in a lowered risk of certain types of cancer. Studies also revealed that, the primary form of lycopene in tomatoes or tomato based foods exist in all trans-isomer (79-91%) (Clinton et al. 1996), after ingestion, cis isomers constitute more than 50% of the total lycopene in human serum (Stahl et al. 1992). It is unclear whether cis-lycopene isomers are preferentially absorbed in humans, or if metabolic conversion occurs after absorption. Tomatoes also contain a variety of other carotenoids in addition to lycopene. While lycopene comprises approximately 60-64% of the total carotenoids found in tomato products, phytoene, phytofluene, neurosporene and γ-carotene are the next most abundant carotenoids. Carotenoid composition of a ripened tomato consists of δ-carotene (1-2%), γ-carotene (10-11%), β-carotene (1-2%), phytofluene (4-5%), phytoene (10-12%), neurosporene (7-9%), lycopene (60-64%). Tonucci et al. (1995) reported the lycopene content of different processed tomato based foods, such as whole tomatoes, ketchup, spaghetti sauce, tomato paste, tomato puree and tomato sauce (Table 1.2).

**Table 1.1. Lycopene content present in common fruits.**

<table>
<thead>
<tr>
<th>Source</th>
<th>Lycopene content (mg/100 g/fresh wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh tomatoes</td>
<td>0.7–20</td>
</tr>
<tr>
<td>Cooked tomatoes</td>
<td>3.7</td>
</tr>
<tr>
<td>Fresh watermelon</td>
<td>2.3–7.2</td>
</tr>
<tr>
<td>Fresh papaya</td>
<td>2.0–5.3</td>
</tr>
<tr>
<td>Pink guava</td>
<td>5.2–5.5</td>
</tr>
<tr>
<td>Pink grapefruit</td>
<td>0.4–3.4</td>
</tr>
<tr>
<td>Apricot</td>
<td>0.01–0.05</td>
</tr>
</tbody>
</table>

(Clinton et al. 1998; Rao et al. 2007)
Table 1.2. Lycopene content of various tomato products.

<table>
<thead>
<tr>
<th>Source</th>
<th>Lycopene content (mg/100 g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw tomatoes</td>
<td>9.27 ± 1.02</td>
</tr>
<tr>
<td>Ketchup</td>
<td>17.23 ± 2.18</td>
</tr>
<tr>
<td>Spaghetti sauce</td>
<td>15.99 ± 0.90</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>55.45 ± 4.33</td>
</tr>
<tr>
<td>Tomato puree</td>
<td>16.67</td>
</tr>
<tr>
<td>Tomato sauce</td>
<td>17.98 ± 1.47</td>
</tr>
</tbody>
</table>

(Tonucci et al. 1995)

Figure 1.2. Common fruits rich in lycopene content.

Lycopene biosynthetic pathway

Carotenoids are important secondary metabolites synthesized in plants and its parts especially in fruits and vegetables (Khoo et al. 2011). Although, carotenoids are structurally diversified natural pigments, they follow several common features in biosynthesis (Figure 1.3). A number of modifications of the linear back bone by cyclases, hydroxylases, ketolases and other enzymes give rise to varied spectrum of carotenoid diversity. Carotenoids are tetraterpenoids derived from C5 isoprenoid precursor, isopentenyl diphosphate (IPP). The condensation of two molecules of
geranylgeranyl pyrophosphate (C20) is the key step of carotenoid biosynthesis to form a colourless phytoene (C40). In brief, phytoene molecule converts into lycopene via phytofluene, \( \zeta \)-carotene, and neurosporene by phytoene and \( \zeta \)-carotene desaturase. The cyclization of lycopene occurs to yield \( \alpha \)-carotene and \( \beta \)-carotene by lycopene \( \epsilon \)- and \( \beta \)-cyclases is considered to be a crucial branching point in the carotenoids biosynthetic pathway (Figure 1.3).

Figure 1.3. Biosynthesis pathway of lycopene and other carotenoids in plants, microalgae and sea weeds.
Metabolism of lycopene

Absorption of lycopene from dietary sources occurs within the range of 10 to 30% in humans (Stahl et al. 1992). After ingestion, lycopene is taken up by dietary lipid micelles and incorporated into the mucosa of the small intestine. The micelles are packaged into chylomicrons, which are then transported to the liver using the lymph system. Lipoproteins carry the lycopene molecules into the plasma, from where they are distributed to their target organs (Parker, 1996). Maximal concentrations have been found in the testes, prostate, adrenal glands and liver (Rao et al. 1999). The half-life of plasma carotenoids ranges from 12 d for β-carotene, α-carotene, and cryptoxanthin to 12-33 d for lycopene and 33-61 d for zeaxanthin and lutein (Rock et al. 1992). The bioavailability of lycopene in plasma and skin was quantified after a single dose of 14C-lycopene and also to profile the metabolites formed. 14C-lycopene was quantified in plasma, the plasma triacylglycerol rich lipoprotein (TRL) fraction, expired carbon dioxide, urine, and skin. The time to maximum concentration ($t_{\text{max}}$) of total 14C-lycopene in plasma was 6 h, and the elimination half-life ($t_{1/2}$) was 5 d, which were different from the $t_{\text{max}}$ and $t_{1/2}$ of unlabeled lycopene (0.5 and 48 d, respectively). 14C-Lycopene was extensively isomerized after dosing as a 92% all-trans isomer at dosing changed to 50% trans, 38% 5 cis, 1% 9 cis, and 11% other cis isomers after 24 h. A similar pattern of isomerization was seen in plasma TRL fractions (Alastair et al. 2011). Lycopene derived products are formed by free radical oxidation, lipoxygenase activity, phase II detoxification enzymes, or carotenoid cleavage enzymes (Mein et al. 2008). Enzymatic carotenoid metabolism is primarily catalyzed by two carotenoid monooxygenase enzymes, carotene-15,15′-monooxygenase (CMO-I) and carotene-9′10′-monooxygenase (CMO-II). CMO-I centrally cleaves carotenoids, such as β-carotene (Hessel et al. 2007) and CMO-II primarily eccentrically metabolizes non-provitamin A carotenoids such as lycopene (Hu et al. 2006; Ford et al. 2010). The products of CMO-II are short chain aldehydes termed apo-lycopenals. The apo-lycopenals have been identified in vivo at physiologically relevant concentrations. The lycopene enriched diet significantly reduced the expression of CMO-II in renal tissue of F344 rats which was the result of feedback inhibition by lycopene or its metabolites (Zaripheh et al. 2006). Contradictory to this, Hu et al. (2006) demonstrated that, dietary lycopene increased the expression of CMO-II mRNA by 4-fold in the lungs of ferrets. These data suggest that the expression of CMO-II are
tissue specific metabolic and/or oxidative environments that affect lycopene catabolism and/or the possible differential need for lycopene metabolites by specific tissues. The highly oxidative conditions such as lung and tumor tissue may produce or require greater concentrations of lycopene metabolites and therefore inducing CMO-II expression in these tissues. Overall, the effect of carotenoid consumption on the expression of carotenoid cleavage enzymes warrants further investigation (Figure 1.4).

Figure 1.4. Schematic illustration of absorption, metabolism, and transport of lycopene.
Lycopene as antioxidants

The reactivity of carotenoids in biological systems depends on their molecular and physical structure, location or site of action within the cells, ability to interact with other antioxidants, concentration and the partial pressure of oxygen (Britton, 1995; Young et al. 2001). Among carotenoids lycopene is known to be the most potent oxygen quenching molecule, and it provides the ability to intervene in reactions initiated by free radicals, like OH\textsuperscript{−} or peroxo radicals (Di Mascio et al. 1989). Due to its polyene structure, providing an electron rich system, lycopene is an eligible target for electrophilic reagents. Thus, it performs an utmost reactivity towards oxygen and free radicals (Krinsky, 1998). Lycopene degradation may result in colour loss when exposed to free radicals or oxidizing agents. This is due to the reaction with free radicals and causes interruption of the polyene chain, in which the conjugated double bond system may either be affected by cleavage or addition to one of the double bonds (Krinsky et al. 2005). The physical quenching rate of lycopene was two times higher than β-carotene and 10 times higher than α-tocopherol. This might be the result of the lowering of the energy level, which provides the ability to approach a triplet energy level. From this stage, the energy transfer from the excited state of oxygen can be adopted easily (Di Mascio et al. 1989). The 5-cis isomer of lycopene was found to be the most stable followed by all-trans, 9-cis, 13-cis, 15-cis, 7-cis, and 11-cis (Chasse et al. 2001). The mechanism of action for lycopene towards the reactive species can be predicted through three possible mechanisms- (1) adduct formation (2) electron transfer to the radical and (3) allylic hydrogen abstraction (El-Agamey et al. 2004). Three possible reactions of lycopene with radical species (R').

1. Adduct formation: Lycopene + R' \rightarrow R-Lycopene\textsuperscript{•}
2. Electron transfer: Lycopene + R' \rightarrow Lycopene\textsuperscript{•+} + R\textsuperscript{−}
3. Allylic H abstraction: Lycopene + R' \rightarrow Lycopene\textsuperscript{•} + RH

Highly reactive oxygen species (singlet oxygen) (\textsuperscript{1}O\textsubscript{2}), are able to oxidize nucleic acids, unsaturated fatty acids or amino acids and can be quenched by lycopene exerting the reaction (Krinsky, 1998)

\[ \textsuperscript{1}O\textsubscript{2} + LYC \rightarrow \textsuperscript{3}O\textsubscript{2} + \textsuperscript{3}LYC \]

\[ \textsuperscript{3}LYC \rightarrow LYC + \text{heat} \]

The exceeding amount of energy, the lycopene molecule gained in this reaction reaching the triplet state, is dispensed through vibrational, as well as rotatory interactions with the solvent, resulting in the release of thermal energy (Krinsky,
Furthermore, lycopene is able to repair vitamin E and C radicals in vivo by conducting the following reaction (Bast et al. 1998)

\[
\text{LYC} + \alpha-\text{Tocopherol} + \text{H}^+ \rightarrow \text{LYC}^+ + \alpha-\text{Tocopherol-H}
\]

Other than its radical reactions, lycopene has also shown to upregulate the antioxidant response element (ARE). Cellular enzymes, like glutathione S-transferase, superoxide dismutase or quinone reductase, are activated by lycopene, resulting in another way of protecting cells against highly reactive oxygen species (Van Breemen et al. 2008). Ben-Dor et al. (2005) reported that lycopene upregulates the ARE in HepG2 and MCF-7 cells through the nuclear factor erythroid 2 related factor 2 (Nrf2) nuclear transcription pathway. Further, Goo et al. (2007) used quantitative proteomics to show that lycopene upregulates the expression of proteins such as epoxide hydrolase 1 (EPHX1), superoxide dismutase-1 (SOD-1), catalase (CAT), and the metal binding protein transferrin (TF) regulated by the ARE in the androgen sensitive human prostate cell line LNCaP. Linniewiel et al. (2009) reported that hydrophilic oxidation products of carotenoids, rather than the intact lipophilic carotenoid molecules, have been found to be responsible for the stimulation of the ARE system, detected in vitro using LNCaP and MCF-7 cells. Oxidized lycopene derivatives, built due to the instability of these molecules, are present in tomatoes, as well as in human serum and tissues. However, these oxidative products have been found to be present in different human tissues only at a rather small amount as compared to the parent compound. The described oxidation is most likely a natural metabolism reaction in tomatoes or can also happen during heat processing of tomato rich foods. Furthermore, in vivo oxidation reactions in humans resulting in the development of lycopene epoxides are demonstrated (Khachik et al. 2002). However, the contribution of these lycopene derivatives as active antioxidant compounds might be insignificant in vivo, due to the predominance of the intact lycopene molecule in most human tissues. Among different carotenoids, lycopene is found to be the most potent antioxidant with the ranking lycopene > \(\alpha\)-tocopherol > \(\alpha\)-carotene > \(\beta\)-cryptoxanthin > zeaxanthin = \(\beta\)-carotene > lutein (David Heber et al. 2002). Rao et al. (1998) conducted a study on 19 healthy human subjects to evaluate the uptake and in vivo antioxidant properties of lycopene, using a randomized, crossover design. The study results indicated that lycopene is readily absorbed from tomato products and may act as an in vivo antioxidant and play an important role in the prevention of cancer (Figure 1.5)
**Lycopene in contrast with other carotenoids**

There are more than 750 carotenoids known to be naturally occurring. Carotenoids are pigments with a wide range of spectrum consisting of C40 backbone (Britton et al. 1997). Apo-carotenoids contain less than 40 carbons. The prefix apo is used to identify carotenoids that have been shortened in length by one or more carbons (Britton et al. 2004). Regardless of the number of carbons, all carotenoids possess an isoprenoid backbone (Britton et al. 2004). Fruits and vegetables (Sommerburg et al. 1998; Raju et al. 2007), animal products, marine algae, and certain seaweeds are good sources of carotenoids (Britton et al. 2009) (**Figure 1.6**). Generally, carotenoids are classified as carotenes (carotenoid hydrocarbons) composed of only carbon and hydrogen (β-carotene and lycopene) and xanthophylls (oxygenated carotenoids), which contain an epoxy- (violaxanthin, neoxanthin, fucoxanthin), hydroxy- (lutein and zeaxanthin), keto- (astaxanthin, canthaxanthin) and methoxy- (spirilloxanthin) functional groups. In case of nutritional approach, carotenoids are considered to be an important bioactive compounds, and dietary ingestion is the only source to meet their requirement in humans and animals (Arathi et al. 2015). Several epidemiological and clinical trials have well correlated the consumption of dietary carotenoids with decreased risk of CVD, vitamin A deficiency age-related macular degeneration (AMD) and cancer (Kohlmeier et al. 1995; Diaz et
al. 2003; Bone et al. 2003; Rock, 2009). All carotenoids demonstrate common chemical features, such as distinct conjugated double bond system, a polyisoprenoid structure and an almost bilateral symmetry around the centrical situated double bond, modifications of the main structure, like cyclization of terminal groups and insertion of oxygen functions, lead to different types of carotenoids with varying characteristic colours and antioxidant qualities (Britton, 1995; Agarwal et al. 2000). Among carotenoids, β-carotene, α-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin are the most common carotenoids found in the human diet, blood and tissues (Khachik et al. 1997). The α-carotene, β-carotene and β-cryptoxanthin are known precursors of vitamin A and are well documented (Bendich et al. 1989). β-carotene and lycopene are hydrocarbon carotenoids with the same molecular formula of C_{46}H_{56} and molecular mass 536. Structurally lycopene is acyclic with absence of β-ionone ring, and lacks pro vitamin A activity, which is the reason for its differing biochemistry, as compared to α- and β-carotene (Van Breemen et al. 2008). Lycopene consists of 13 double bonds with two non conjugated, and eleven conjugated double bonds, thereby building a chromatophore (Shi et al. 2000). This extended conjugated polyene chain of lycopene is an electron rich system, susceptible to attack by electrophilic reagents. Therefore, carotenoids like lycopene are unstable and highly reactive towards oxygen and free radicals (Krinsky et al. 2005). This distinctive conjugated polyene structure accounts for the deep red colour with the absorbance maxima at 444, 470 and 502 nm (petroleum ether) and ability to accept energy from various electronically excited species (Miller et al. 1935). Lycopene with lipophilic character is soluble in chloroform, benzene and petroleum ether and practically insoluble in methanol, ethanol and water (Van Breemen et al. 2008). Lycopene is rapidly destroyed by oxidation and by free radicals such as OH and various peroxy radicals. This reactivity of lycopene is the basis for its antioxidant activity in biological systems that might contribute to its efficacy as a chemoprevention agent. In the plant matrix or in solid form, lycopene is relatively stable, but after extraction from the matrix and dissolution in a non-polar organic solvent, lycopene is quite unstable. Fang et al. (2003) evaluated the stability of lycopene in a mobile phase protected from light and air and found that its half-life was ~16 h at 4 °C. Furthermore, lycopene was more than 8-fold less stable than all trans-β-carotene. Therefore, the stability of lycopene must be a consideration during the preparation of samples containing lycopene or during in vitro or in vivo experiments with various
lycopene preparations. The prevalent biological occurring form of lycopene is the all-trans-isomer, which is also the thermodynamically most stable configuration (Clinton, 1998). Indeed, heat, light or several chemical reactions can induce isomerization from the trans-isomer to various mono- or poly-cis forms (Shi et al. 2000). The lycopene forms detected in human serum and tissues range from all-trans to 9-, 13- and 15-cis isomers, whereas the predominant naturally occurring configuration in food is the all-trans form. Other xanthophyll carotenoids such as lutein and zeaxanthin are considered as a vital macular pigments located in central region of retina, acts as filter of high energy blue light, quenches of singlet oxygen, chain breaking antioxidants and involve in the reduction of phototoxic damage to the eye. Lutein’s molecular formula is $C_{40}H_{56}O_2$ and has a molecular weight of 568.88 daltons. Lutein and zeaxanthin have the same chemical formula, the only difference between them is in the location of the double bond in one of the end rings. Zeaxanthin consists of 40 carbon long molecule, with 11 conjugated double bonds. Its molecular formula is $C_{40}H_{56}O_2$ and has a molecular weight of 568.88 daltons. Zeaxanthin shows a better protective ability than lutein because of its greater conjugated, symmetrical structure (Bone et al. 2003). Astaxanthin and fucoxanthin are other promising marine carotenoids with health associated benefits against diabetes, obesity, angiogenesis (Higuera-Ciapara et al. 2006; Sachindra et al. 2007; Woo et al. 2009; Kim et al. 2010). Astaxanthin is a keto-carotenoid with molecular formula $C_{40}H_{52}O_4$ and molecular mass 596.84. It contains not only carbon and hydrogen but also oxygen atoms. Astaxanthin consists of two terminal rings joined by a polyene chain. This molecule has two asymmetric carbons located at the 3, 3’ positions of the β-ionone ring with hydroxyl group (-OH) on either end of the molecule (Ranga Rao et al. 2014). Unlike other carotenoids, fucoxanthin has a unique structure with molecular formula $C_{42}H_{58}O_6$ with a molecular mass 658.9, in which an unusual allenic bond, 9 conjugated double bounds, a 5,6-monoepoxide, and some oxygenic functional groups including hydroxyl, epoxy, carbonyl, and carboxyl moieties are present (Hui Zhang et al. 2015). Although bioavailability and functionality of carotenoids are addressed, however knowledge on metabolomics of carotenoids, their bioefficacy and bioactivity is not detailed.
Non-polar Carotenoids

Potent antioxidant and involved in the reduction of certain cancers and cardiovascular disease (CVD)

Pro-vitamin A carotenoids

Macular pigments, protects against age related macular degeneration

Antioxidant molecule

A potent antioxidant involved in reduction of inflammation and cancer

Antioxidant molecule, with anti-inflammatory, anti-cancer, anti-obese, anti-diabetic, anti-angiogenic and anti-malarial activities

Polar Carotenoids

Figure 1.6. Common carotenoids found in fruits, vegetables, micro algae, seaweeds and their health benefits.

Role of lycopene in implication of cancer prevention

Cancer is a disease initiated by a series of cumulative genetic and epigenetic changes that occur in a normal cell. Cancer development is characterized by three stages initiation, promotion, and progression (Pitot, 1993). Initiation is defined as a rapid and irreversible process that begins with genotoxic DNA damage resulting from exposure to endogenous or exogenous carcinogens. The initiation of chemically
induced tumourigenesis involves carcinogenic metabolic activation and the subsequent covalent modification of the genomic DNA, allowing the activation of oncogenes or the inactivation of tumour suppressor genes. Tumour promotion is a reversible process characterised by the transformation of an initiated preneoplastic cell due to an epigenetic alteration resulting from chronic exposure to tumour promoters (i.e., growth factors, hormones, and UV radiation). The final stage of the neoplastic transformation, progression, involves tumour growth with the potential for invasion and metastasis. Four possible strategies have been described for the reduction of cancer incidence and cancer-related mortality (a) prevention, (b) early diagnosis, (c) improvements on treatment, and (d) improved detection of non-diagnosed cancers (Mukhtar et al. 1999). Of these options, prevention is the most favorable. Between 30 to 40% of cases are reportedly preventable through diet modification, adequate body weight management, and physical activity (Manson, 2003). Chemoprevention aims to stop or to reduce cancer development by intervening in the carcinogenesis process via the administration of natural or synthetic agents. Chemopreventive compounds intended for human use must not elicit significant secondary effects. They must be efficient molecules against multiple molecular targets, and their mechanism's of action should be well defined. Epidemiological studies have suggested that the inclusion of fruits, vegetables, and whole grains in dietary intake might prevent and even reverse the cellular changes associated with carcinogenesis at the initial stages, thus reducing tumour incidence (Giovannucci, 1999). Lycopene is a natural pigment (non-provitamin A carotenoid) synthesised by photosynthetic plants and microorganisms. Lycopene plays an important role in prostate cancer prevention and in other organs, such as the breast, lung, gastrointestinal tract, pancreas, uterine cervix, and the ovaries (Giovannucci, 1999). Although the antioxidant properties of lycopene were thought to be primarily responsible for its biological effects, other mechanisms have also been identified.

**Effect of lycopene on cell cycle arrest**

Cell cycle progression is regulated through the interaction between cyclin-dependent kinases (CDK1, 2, 3, 4, and 6), cyclins (cyclin A, B, D, and E), and inhibitor proteins (p21WAF1 and p27KIP1) (Murray, 2004; Alao, 2007) (Figure 1.7). The coordinated participation of cyclin D/CDK4/6, cyclin E/CDK2, and cyclin
A/CDK2, complexes is required for the G1/S transition and progression through S phase, whereas the cyclin A/CDK1/2 complexes are required for cells to enter mitosis. The activation of the cyclin D/CDK4/6 and cyclin E/CDK2 complexes is essential for the phosphorylation of retinoblastoma (Rb). Rb is constitutively expressed and dephosphorylated, it forms a complex with histone deacetylase-1 and the transcription factor E2F, inhibiting its transcriptional activity. By activating ERK1/2, growth factors might induce an increase in the expression of cyclin D, which binds to and activates CDK4 and CDK6. Rb is phosphorylated by these activated CKDs, inducing the dissociation of histone deacetylase-1 from the complex, allowing for histone acetylation and facilitating the transcription of specific genes. These genes include cyclin E, which binds to and activates CDK2. CDK2 then hyperphosphorylates Rb, releasing the E2F transcription factor, which in turn induces the gene expression necessary for the transition to S phase. Among these genes are DNA polymerase A, dihydrofolate reductases, thymidylate synthase, and cyclins (Sherr, 2000). The activity of cell cycle kinases is often upregulated in cancer due to the overexpression of cyclins and CKDs or to the inactivation of CKD inhibitors (Cristina Trejo-Solís et al. 2013). Deregulated cell cycle is one of the major hallmarks of cancer cells, the cells lose their ability to regulate the cell cycle and control the rate of proliferation. Carotenoids have been shown to inhibit the growth of tumor cells by interfering at different phases of the cell cycle. Carotenoids have been reported to modulate cell cycle arrest by multiple mechanisms in cancer cells. One of the mechanisms by which lycopene reduced the risk of cancer is inhibition of cell cycle progression. Studies demonstrates that lycopene blocks cell cycle in the G0/G1 phase with decreased level of cyclin D, cyclin dependent kinase 4 (CDK4) along with suppressed retinoblastoma (pRB) phosphorylation and increased abundance of p21 and p27 in cancer cells (Amir et al. 1999; Park et al. 2005; Ivanov et al. 2007; Tang et al. 2008). Lycopene elicits these changes by inactivating Ras via the inhibition of the mevalonate pathway and decreased expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA). Lycopene treatment also reduces the farnesylation of Ras, which promotes the cytoplasmic accumulation of Ras and its consequent inactivation. Furthermore, lycopene reduces the Ras dependent activation of the nuclear factor kappa B (NF-kB) transcription factor, which regulates the transcription of prosurvival genes, including cyclin D, Bcl-2, Bcl-XL, cIAP, and c-myb (Palozza et al. 2010). In addition, lycopene was shown to inhibit the growth of MDA-MB-231 cells by blocking cell cycle
progression, inhibiting S-phase kinase associated protein 2 (Skp2), and increasing p27 levels (Chang et al. 2011). Breast cancer cells treated with lycopene also exhibited an arrest of the cell cycle in the G0/G1 and S phases, which was confirmed by the accumulation of cells in the G0/G1 phase and decreased number of cells in the G2/M phase (Nahum et al. 2001; Teodoro et al. 2012; Takeshima et al. 2014). The molecular mechanisms underlying cell cycle arrest and antiproliferative activity in breast cancer by lycopene were in association with down-regulated expression of cyclin D1/D3 and pRB protein with subsequent increase of p21 protein, and altered expression of cell cycle proteins (Nahum et al. 2001; Teodoro et al. 2012; Takeshima et al. 2014; Uppala et al. 2013). In A549 nonsmall lung carcinoma cells, lycopene derived metabolites, such as apo-10'-lycopenic acid, can induce cell cycle arrest at the G1/S, transition. This cell cycle arrest is associated with a decrease in cyclin E, an increase in the cyclin dependent kinase inhibitors p21 and p27, and the transcriptional induction of the retinoic acid receptor β (RAR β) tumour suppressor gene. (Lian et al. 2007). Studies also reported that lycopene and its metabolite apo-12'-lycopenal, significantly suppressed the proliferation of prostate cancer cells through alteration in the normal cell cycle at different phases depending on the time of exposure (Ford et al. 2011; Soares et al. 2013).

These results suggest that lycopene blocks cell cycle progression from G1 to S phase, predominantly by reducing the levels of cyclin D and E and subsequently by inactivating CDK2 and 4 and decreasing the hyperphosphorylation of Rb. Furthermore, lycopene increases the expression of CDK inhibitors including p21 and p27, as well as the tumour suppressor gene p53, and decreases the expression of Skp2. Lycopene can also block growth factor mediated antiapoptotic signals by inhibiting the binding of growth factors to their receptors or by inhibiting downstream components of the serine/threonine kinase also known as protein kinase B (PI3K-AKT) pathway. AKT phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β). Thus by inhibiting GSK3β, a growth factor might promote the dephosphorylation and stabilisation of cyclin D and c-myc. Cyclin D facilitates S-phase entry, and c-myc stimulates cell proliferation and survival. AKT phosphorylates and inactivates the cyclin dependent kinase inhibitors p21 and p27. An increase in the expression of these inhibitors could in turn inhibit the activity of the CDK 4/6/cyclin D and CDK2/cyclin E complexes and reduce the phosphorylation of Rb. Reduced phosphorylation or hypophosphorylation of Rb leads to the inactivation of the E2F
transcription factor and the suppression of the S-phase cyclin A. An additional target of AKT is Mouse double minute 2 homolog (Mdm2), which mediates the ubiquitination and degradation of p53, which plays a key role in the induction of cell cycle arrest in response to a variety of genotoxic stresses and to the activation of oncogenes such as c-myc, thereby preventing the propagation of abnormal cells.

![Cyclin expression cycle at checkpoints.](image)

**Figure 1.7.** Cyclin expression cycle at checkpoints.

**Effect of lycopene on apoptosis**

There is a link between the regulation of the cell cycle and apoptosis: cell cycle deregulation is one of the most potent triggers for apoptosis. Specifically, the deregulation of most components of the cell cycle machinery, including Rb, E2F, p21, p27, cyclin D, or CDK1, might be involved in the apoptotic process (Green et al. 2004). Apoptosis is regulated by a complex network of pro- and anti-apoptotic proteins it can be induced by either extrinsic or intrinsic pathways (Figure 1.8).

**Extrinsic pathway**

The extrinsic signaling pathways are mediated by transmembrane death receptors such as Tumor necrosis factor receptor (TNFR), Fas and TNF-related apoptosis-inducing ligand receptor (TRAIL-R), that are members of the tumor necrosis factor (TNF) receptor gene super family (Locksley et al. 2001). These receptors possess a cysteine-rich extracellular domain and an intracellular domain of about 80 amino acids called the 'death domain' that transmits the death signal to the intracellular signaling pathways (Ashkenazi et al. 1998). The sequence of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and
TNF-/TNFR1 models. Once Fas ligand binds to Fas receptor, the receptor undergoes trimerization and the intracellular death domain of the receptor recruits adaptor proteins like Fas associated protein with death domain (FADD). FADD then associates with initiator caspases such as procaspase-8 and procaspase-10, forming the death inducing signaling complex (DISC) (Kischkel et al. 1995). Procaspses are auto-catalytically activated at the DISC. Once caspase-8 is activated, it activates caspase-3 and the execution phase of apoptosis is triggered. Extrinsic pathway can be regulated by an inhibitor protein called FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) which can bind to FADD and caspase-8, rendering them ineffective (Kataoka et al. 1998).

Intrinsic pathway

The intrinsic pathway of apoptosis is initiated by intracellular stress signals such as growth signal withdrawal, DNA damage or oxidative stress. All of these stimuli cause changes in the inner mitochondrial membrane that results in the opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins into the cytosol (Saelens et al. 2004). The first group consists of cytochrome c, Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) and the serine protease (HtrA2/Omi). Cytochrome c binds to Apaf-1, procaspase-9 and adeno nucleotide triphosphate, forming a supramolecular complex termed "apoptosome" that activates procaspase-9 through autocatalysis (Chinnaiyan, 1999). Caspase-9 then activates the executioner caspase-3. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting inhibitors of apoptosis proteins (IAP) activity (Schimmer, 2004). The release of second group of pro-apoptotic proteins, apoptosis inducing factor (AIF), endonuclease G and caspase activated DNases (CAD), is a late event that occurs after the cell has committed to die. AIF translocates to the nucleus and causes DNA fragmentation into 50-300 kb pieces and condensation of peripheral nuclear chromatin (Joza et al. 2001). AIF and endonuclease G both functions in a caspase independent manner. CAD is subsequently released from the mitochondria and translocates to the nucleus where, after cleavage by caspase-3, it leads to oligonucleosomal DNA fragmentation and a more pronounced and advanced chromatin condensation (Enari et al. 1998). Mitochondrial membrane integrity is
controlled by the Bcl-2 family of proteins which include pro-apoptotic or anti-apoptotic proteins (Cory et al. 2002). To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-X, Bcl-XI, Bcl-XS, Bcl-W, BAG and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik and Blk. Bax and Bak are thought to induce permeabilisation by forming pores upon oligomerization. The pro-apoptotic BH3-only family members (such as Bid, Bim, Bad, Noxa and Puma) activate Bax and/or Bak. Two models have been proposed for this activation; several studies suggest that they do so through binding of anti-apoptotic Bcl-2 proteins, thereby relieving the inhibitory function of these anti-apoptotic proteins. Others propose that a subset of BH3-only proteins can directly bind and activate Bax and/or Bak. A few possible mechanisms have been studied but none have been proven definitively. Mitochondrial damage in the Fas pathway of apoptosis is mediated by the caspase-8 cleavage of Bid (Li et al. 1998; Esposti, 2002). This is one example of the “cross-talk” between the death-receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Igney et al. 2002).

Carotenoids have been reported to possess chemopreventive activity by reducing the cancer incidence in humans through apoptosis (Gosselau et al. 2004). Among carotenoids, one of the ongoing research candidates for the prevention of cancer is lycopene. It is the most abundant carotenoid present in tomatoes which has been reported to possess effective anti-cancer properties including apoptosis-inducing effects (Rao et al. 2007). Lycopene mediates apoptosis via death receptors and intrinsic pathway. Tang et al. (2009) reported that lycopene and EPA (eicosapentaenoic acid) synergistically inhibit the activation of protein kinase B (AKT) and mammalian target of rapamycin (mTOR), enhancing the accumulation of Bax and Fas ligand and blocking the survival of HT-29 human colon cancer cells. Moreover, in combination with S-allyl cysteine, lycopene significantly blocks the in vivo development of gastric cancer by inducing apoptosis via reduced expression of Bcl-2, increased expression of Bax and Bim, and increased activation of caspases 8 and 3 (Velmurugan et al. 2005). The metabolite (E, E)-4-methyl-8-oxo-2, 4, 6-nonatrienal (MON), which is obtained from the autooxidation of lycopene, induces apoptosis in HL-60 human promyelocytic leukaemia cells as evidenced by morphological changes including chromatin condensation and DNA fragmentation, both of which are associated with decreased Bcl-2 and Bcl-XL expression and the activation of caspases 8 and 9. MON was suggested to induce apoptosis via the
mitochondrial and death receptor pathways, as it might induce the cytosolic release of cyt c by decreasing the expression of Bcl-2 and Bcl-XL. The enhanced activation of caspase 8 induced by MON might be mediated by death receptors, either by direct binding or by induction of Fas ligand expression (Zhang et al. 2003). In the LNCaP cell line, lycopene at physiologically attainable concentrations induces mitochondrial apoptosis in a dose-dependent manner by reducing mitochondrial membrane potential and inducing cyt c release into the cytosol (Hantz et al. 2005). Recently, combination treatment with lycopene and genistein has been demonstrated to significantly reduce the development of a chemically induced breast cancer in vivo. This suppression was associated with decreased Bcl-2 expression, increased Bax expression, and the activation of caspases 3 and 9 (Sahin et al. 2011). Furthermore, Wang et al. (2007) reported that lycopene can induce apoptosis in PC-3 cells by altering the cell cycle distribution, decreasing cyclin D and Bcl-2 expression, and increasing Bax expression. Similarly, Palozza et al. (2007) demonstrated that tomato products that contain lycopene can inhibit the growth of colon adenocarcinoma cells by decreasing the expression of cyclin D and the anti-apoptotic proteins Bcl-2 and Bcl-XL. They also showed that lycopene induces apoptosis in immortalised fibroblasts by promoting cell cycle arrest via decreased cyclin D levels and reduced AKT and Bad phosphorylation (Palozza et al. 2005). Combining lycopene with docetaxel has been shown to induce p53 in LNCaP cells (Palozza et al. 2010) and might synergistically decrease survivin expression levels in vitro and in vivo (Tang et al. 2011). Another study suggested that apoptosis induction by lycopene in prostate cancer cells is associated with increased expression of Bax and decreased expression of Bcl-2 gene (Soares et al. 2013). It induced apoptosis in breast cancer cells by up-regulating proapoptotic protein, Bax and by increasing poly-ADP-ribose polymerase (PARP) cleavage (Gloria et al. 2014; Takeshima et al. 2014). Teodoro et al. (2012) observed a two to four fold increase in apoptosis when colon cancer cells were exposed to lycopene. In lung cancer cells, a remarkable increase in the percentage of apoptotic cells was observed following lycopene (10 μM) treatment for 24 h.

These studies have suggested that lycopene induce apoptosis by decreasing the expression of Bcl-2, Bcl-XL, and survivin, by increasing the expression of the proapoptotic proteins Bax, Bad, and Bim and Fas ligand and by increasing the activation of caspases 8, 9, and 3. Lycopene can also block growth factor mediated antiapoptotic signals by directly inhibiting the binding of growth factors to their
receptors or by inhibiting the downstream PI3K-AKTpathway. Lycopene can elicit AKT induced cell cycle arrest and apoptosis through inactivation of p53 via Mdm2.

Figure 1.8. Intrinsic and extrinsic pathway of apoptosis.
Influence of lycopene in gap junction communication

Gap junctions are tiny aqueous channels which connect the cytoplasms of contiguous cells, enabling cells to share directly small metabolites by the process called gap junction intercellular communication (GJIC) or cell coupling. GJIC has a role in maintaining tissue homeostasis or its variant morphostasis, which is largely achieved by regulating the balance between cell gain and cell loss (Figure 1.9). There is a large body of evidence that GJIC are implicated in the regulation of cell growth, mostly by negative control of cell proliferation (Loewenstein, 1992). Correspondingly, disorders of GJIC are strongly associated with aberrant cell growth diseases, including cancer (Yamasaki, 1990). Due to their frequent functional alteration in tumors, the genes of gap junction proteins - Connexins (Cx) have been classified as tumor suppressors (Chipman, 1995; Yamasaki et al. 1996).

In cancer, an increase in cell number is often a result of suppressed cell loss (apoptosis) rather than increased cell proliferation (McDonnell, 1993). In view of this, and the ubiquitous role of cell coupling in the maintenance of tissue homeostasis, its implication in regulation of apoptosis was postulated. Bertram et al. (1991) demonstrated a significant correlation between the ability of diverse carotenoids to inhibit chemically induced neoplastic transformation and their ability to stimulate junctional communication in 10T1/2 cells via increased levels of connexin 43 (Zhang et al. 1991). It has previously been shown that increased levels of connexin 43 are associated with increased gap-junctional communication (Zhang et al. 1992). Moreover, Zhang et al. (2001) demonstrated that overexpression of connexin 43 is associated with suppressed proliferation of human osteosarcoma cells through inhibition of cell cycle transition from the G1 to the S phase. Sies et al. (1997) suggested that stimulation of gap junctional communication to be one of the biochemical mechanisms underlying the cancer preventive activity of carotenoids. The stimulatory activity is associated with structural properties of the carotenoids. It appears that the presence of a six membered ring substituent at the end of the conjugated system of double bonds is required for the effects, five membered ring carotenoids are less active. There is increasing evidence that oxidation products of carotenoids, especially retinoic acid analogs, significantly contribute to this biological property. Teicher et al. (1999) tested the biological activities of cantaxanthin metabolites and demonstrated that 4-oxo-retinoic acid, known to stimulate GJC and to activate the RAR-β2 receptor. The effects of 4-oxo-retinoic acid were comparable to
those of retinoic acid. Their data suggest that the major biological effects of canthaxanthin on retinoid signaling pathways are related to activities mediated by the products of the central cleavage. Stahl et al. (2000) evaluated the effect of lycopene and its possible oxidation product, acyclo-retinoic acid on GJC on stabilization of connexin 43 mRNA, and on the transactivation of the RAR-β2-promoter in vitro (human fetal skin fibroblasts). Lycopene was effective at a lower concentration, whereas higher amounts of acyclo-retinoic acid were needed for comparable stimulation. This data demonstrate that acyclo-retinoic acid is much less active than retinoic acid with respect to GJC and retinoid-related signaling. Aust et al. (2003) reported a stimulatory effect of a lycopene oxidation product (2,7,11-trimethyl-tetradecahexaene-1,14-dial) on GJC in rat liver epithelial WB-F344 cells. Fornelli et al. (2007) evaluated the inhibitory effects of lycopene on MCF-7 cell growth and suggest that lycopene is involved in the modulation of the gap junction intercellular communication.

![Figure 1.9. Gap junctions providing channels of cell-to-cell communication.](image-url)

**Lycopene and cancer overview**

Cancer is a global burden of disease in the decades to come and it is one of the leading causes of death in both developed and developing countries including India. Cancer rate is increasing as a result of adoption of cancer-associated lifestyles including smoking, physical inactivity, and food habits. Based on the GLOBOCAN 2012 estimates, there were 14.1 million new cancer cases, 8.2 million cancer deaths
and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide. 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions. Lung, stomach, liver, colon and breast cancer causes most cancer deaths in each year. Except lung cancer is preceded by prostate cancer as the most frequent cancer among males in developed countries (Parker et al. 1997). Prostate cancer is the fourth most common cancer in both sexes combined and the second most common cancer in men. An estimated 1.1 million men worldwide were diagnosed with prostate cancer in 2012, accounting for 15% of the cancers diagnosed in men, with almost 70% of the cases (759,000) occurring in more developed regions. Likewise, Breast cancer is the second most common cancer in the world and the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). It is the most common cancer in women both in more and less developed regions with slightly more cases in less developed (883,000 cases) than in more developed (794,000) regions. The World Cancer Report documents that cancer rates are set to increase at an alarming rate globally and projected the cancer rates could increase by 50% new cases for the year 2020 (ICMR report, 2004; WHO report, 2009). A substantial proportion of the worldwide burden of cancer could be prevented through the application of existing cancer control knowledge and by implementing various health awareness programs and early detection and treatment, as well as a healthier dietary intake (Jemal et al. 2011).

Considerable interest and research efforts will be expended to uncover the potential roles of carotenoids in human health and disease. While early studies focused on pro-vitamin A carotenoids, more recent research have focused on the potential roles in health and disease of the non-provitamin A carotenoids, such as lycopene. Although, evidence from epidemiological and animal studies supports a potential role of lycopene (Giovannucci et al. 2002; Canene-Adams et al. 2005). Further the biochemical mechanisms behind such beneficial effects have not been well defined. Furthermore carotenoid oxidative products can possess either more or less activity than the parent compound or entirely independent functions are yet to be resolved.
Lycopene and Prostate cancer

Lycopene products are well tolerated and meet the requirements of the US food and drug administration and generally recognized as safe (GRAS). Prostate cancer is a common illness for aging males and correlated with dietary regulation. It was also reported that frequent intake of lycopene reduces the risk of prostate cancer (Giovannucci et al. 2002). Supplementation of lycopene at 30 mg/day was found to reduce the level of serum prostate specific antigen (PSA) in Afro-Caribbean men (Bunker et al. 2007). A study conducted at memorial Sloan-Kettering Cancer Center determined an inverse relationship between the plasma carotenoids such as lycopene, lutein, zeaxanthin and β-cryptoxanthin, and prostate cancer (Lu et al. 2001). In a nested case-control study, lycopene significantly reduced the risk of prostate cancer, but the effect was observed only in older participants who did not have a family history of prostate cancer, suggesting that lycopene activity is effective against sporadic cancer (Wu et al. 2004). A study suggested that intake of retinol, α-carotene, and β-carotene reduces the risk of prostate cancer (Schuurman et al. 2002). Recent studies have shown that prostate cancer patients are particularly attracted by complementary or alternative medicine products (Bishop et al. 2011). The prevalence of using complementary and alternative therapeutic methods among patients with prostate cancer has been shown to be around 30% (Bishop et al. 2011), and approximately 25% use herbs, minerals, vitamins and antioxidants, such as green tea, selenium, vitamin E or lycopene (Chan et al. 2005). Several studies have shown the chemopreventive effect of lycopene against chemically induced prostate cancer in rats (Imaida et al. 2001; Guttenplan et al. 2001; Boileau et al. 2003). Tang et al. (2005) analysed the effect of natural lycopene on the growth of androgen-independent human DU145 prostate cancer cells after subcutaneous injection (1 X 10^7 cells/100 μL Matrigel™) into the flanks of 4–6 week old male BALB/c nude mice. The lycopene-formulation used consisted of > 95% pure lycopene with 6% lycopene oleoresin extracted from tomatoes. After tumour cell injection, the mice were gavaged five days per week with different dosages of lycopene for eight weeks. The authors showed a decrease in tumour growth by 55.6% and 75.8% in mice treated with 100 mg and 300 mg lycopene. Tang et al. (2011) investigated the effect of lycopene and docetaxel on the survival and growth rate of xenografted tumours in nude mice by injecting DU145 prostate cancer cells. Docetaxel plus lycopene led to a significantly higher tumour regression and increase in survival when compared to docetaxel alone. The lycopene
was able to enhance the antitumor capacity of docetaxel, even when it was given at a suboptimal dosing. However, lycopene alone had no effects on tumour regression or survival. Jinyao et al. (2013) carried out a meta-analysis studies and suggested that tomato may play a modest role in the prevention of prostate cancer. Further research would be needed to determine the type and quantity of tomato products regarding their potential in preventing prostate cancer. Ping et al. (2015) determined the role of dietary concentration of lycopene and correlated with the reduction of prostate cancer, they used the meta-analysis data to elucidate the efficiency of lycopene. Further they concluded based on the linear and nonlinear dose response relations with data from categories of lycopene consumption/circulating concentrations and concluded that that higher lycopene consumption was linearly associated with a reduced risk of prostate cancer. In vivo studies strongly postulated the role of carotenoids on inhibition of cancer development. However, the efficiency of lycopene largely depends on kind of animals, age and mode of treatment. Further, lycopene formulations vary from synthetic lycopene to purified naturally derived lycopene and non-purified tomato powder or mixtures of different substances. Several other critical issues must to be considered, like dosage, form and bioavailability of the active component and the timing of the treatment.

There are several in vitro reports, shown the anti-proliferative effect of lycopene to inhibit proliferation of several types of cancer, including prostate, breast, endometrium, lung, and leukemia. Amir et al. (1999) demonstrated the effect of lycopene treatment resulted in reduction of HL-60 cell growth in a dose dependent manner. This effect was accompanied by inhibition of cell cycle progression in the G0/G1 phase. Further, they examined the synergistic effect of lycopene at lower concentration compared to lycopene treated alone with cytotoxic higher concentrations with phyto compound (1,25-dihydroxyvitamin D3). Such synergistic anti-proliferative and differentiating effects of lycopene and other compounds found in the diet and in plasma may suggest the inclusion of the carotenoid in the diet as a cancer preventive measure. Kim et al. (2002) measured the effect of lycopene on the proliferation of LNCaP human prostate cancer cells in culture. They observed the inhibition of cell proliferation in a dose dependent manner. Based on the results, they believed that the inhibition of cell proliferation may be due to the antioxidant activity. Hwang et al. (2004) demonstrated that lycopene at phisiological concentration (5 μM) accumulated the number of cells in the G2/M phase of the cell cycle (13% to 28%)
with decreased S-phase cells (45% to 29%). Hantz et al. (2005) studied the effect of lycopene at physiologically attainable concentrations on apoptosis, cellular proliferation, and necrosis in LNCaP human prostate cancer cells. They observed lycopene did not alter cellular proliferation compared to control groups. They showed decreased mitochondrial function (61%-83%) with increasing concentrations of lycopene. Further, they also demonstrated higher concentrations of lycopene significantly reduced mitochondrial transmembrane potential, induced the release of mitochondrial cytochrome c, and increased annexin V binding, confirming induction of apoptosis. Ivanov et al. (2007) investigated the molecular mechanism underlying anti-cancer activity of lycopene based products in androgen-responsive (LNCaP) and androgen-independent (PC-3) cells. They demonstrated that lycopene exposure suppresses the phosphatidylinositol 3-kinase-dependent proliferation and survival signalling in androgen-responsive LNCaP and androgen-independent PC-3 cells suggesting that the molecular mechanisms for the cytostatic and cytotoxic actions of lycopene involve induction of G0/G1 cell cycle arrest. Kanagaraj et al. (2007) examined the effect PC-3 cells treated with lycopene and showed a significant decrease in cell proliferation by upregulation of IGFBP-3. Lycopene-induced DNA fragmentation was observed after 48 h treatment, which was not attained at 24 h treated culture. There was a significant decrease in IGF-IR expression in cells treated with lycopene and IGF-I. These data suggested components of the IGF may act as a positive regulator of lycopene-induced apoptosis. Teodoro et al. (2012) determined the effect of lycopene on anti-proliferation of eight different human cancer cell lines. They demonstrated a significant decrease in cell number in three cancer cells lines (HT-29, T84 and MCF-7) compared to other cancer cells after 48 h treatment with lycopene. Lycopene promoted cell cycle arrest and apoptosis after 96 h, as compared to controls. They also suggested that lycopene may alter cell cycle regulatory proteins depending on the type of cancer and the dose of lycopene. Soares et al. (2013) evaluated the influence of lycopene on cell viability, cell cycle, and apoptosis of human prostate cancer cells and benign prostate hyperplastic cells. They observed a decreased cancer cells after treatment with lycopene. Further they demonstrated decreased percentage of cells in G0/G1 phase and increased in S and G2/M phases with altered gene expression of Bax and Bel-2. However, there is no effect in benign prostate hyperplasia cells. Rafi et al. (2013) examined the effect of lycopene on proliferation, survival, and biomarker gene expression in prostate cancer cells in
culture. Cell proliferation assay showed that lycopene induces a biphasic effect on PC-3 cells with a modest increase in proliferation at 1-5 μM, no change at 10-25 μM and a decrease at 50-100 μM doses in culture. Interestingly, combination of lycopene with temozolomide induced anti-proliferative effect on PC-3 cells. Lycopene also augmented the anti-proliferative effect of peroxisome proliferator activated receptor gamma (PPARγ) agonists, but not doxorubicin or taxol, in prostate cancer. Lycopene combination with chemotherapeutic agents and PPARγ agonists, induced cell cycle arrest with significant increase in cell death by apoptosis and necrosis on prostate cancer. In addition, combination of chemo drugs with lycopene alters the expression of growth and apoptosis associated biomarkers.

Lycopene and Breast cancer

Breast cancer is the second leading cause of cancer deaths in women today and the most common cancer among women (Greenlee et al. 2001). Several studies correlated the dietary intake of fruit, vegetable, and antioxidant micronutrient is associated with a reduction in breast cancer incidence. There has been a growing interest in the tomato carotenoid, lycopene, as a cancer preventive agent. Epidemiological studies have suggested that lycopene decreases the risk of several types of human malignancies, such as breast (Zhang et al. 1997). Sato et al. (2002) investigate the association between serum and plasma concentrations of retinol, retinyl palmitate, α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, total-carotenoids, α-tocopherol, and γ-tocopherol with subsequent development of breast cancer. A study was conducted among female residents of Washington County, Maryland, were matched on age, race, menopausal status, and date of blood donation, and the analyses were stratified by cohort participation. Median concentrations of β-carotene, lycopene, and total carotene were significantly lower in cases compared with controls. The results suggested that carotenoids may protect against the development of breast cancer. In contrast, Sesso et al. (2005) concluded that neither higher dietary nor plasma lycopene levels were associated with a reduced risk of breast cancer in middle-aged and older women. Another study in postmenopausal women reported an inverse association of breast cancer risk with dietary intake of carotenoids (α-carotene, β-carotene, and lycopene) (Cui et al. 2008). Levy et al. (1995) demonstrated that lycopene inhibits the growth of mammary, endometrial, lung cancer. This inhibition was associated with a delay in cell cycle progression from
G1 to S phase. Karas et al. (2000) determined the mitogenic activity of insulin-like growth factor 1 (IGF-I) in mammary cancer cells can be reduced by the dietary carotenoid lycopene. Further they confirmed that the inhibitory effects of lycopene on MCF-7 cell growth by reduced IGF-I stimulation of tyrosine phosphorylation of insulin receptor substrate 1 and binding capacity of the activator protein 1 (AP-1) transcription complex. These effects were not associated with changes in the number or affinity of insulin-like growth factor 1 (IGF-I) receptors, but with an increase in membrane-associated IGF-binding proteins, which were previously shown in different cancer cells to negatively regulate IGF-I receptor activation. The inhibitory effect of lycopene on IGF signaling was associated with suppression of IGF-stimulated cell cycle progression of serum-starved, synchronized cells. Nahum et al. (2001) reported reduction in cyclin D1 level is the key mechanism for lycopene and atRA inhibitory action on insulin-like growth factors (IGF-I)-induced cell cycle progression. Human breast and endometrial cancer cells were synchronized in G0/G1 phase by serum deprivation followed by stimulation with IGF-I. Cell treatment with lycopene and atRA inhibited IGF-I-stimulated cell cycle progression from G1 to S phase and decreased retinoblastoma protein phosphorylation. Wang et al. (2007) tested the effect of lycopene on the proliferation and apoptosis of the estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines. Lycopene inhibited the growth and DNA synthesis of both cells in a dose-dependent pattern. The cell were accumulated in G0/G1 phase and decreased in the S and G2/M phase. Lycopene inhibits the growth of ER-positive MCF-7 cells through the inhibition of the cell cycle progression where as inhibition of ER-negative MDA-MB-231 cells is associated with the G1 phase cell cycle arrest and induction of apoptosis. Hence they concluded that the effect of lycopene was more prominent in MDA-MB-231 than MCF-7 cells. Min-Soo et al. (2010) investigated the inhibitory effect of lycopene on invasive and migratory phenotypes of two highly aggressive breast cancer cell lines, H-Ras-transformed MCF10A human breast epithelial cells (H-Ras MCF10A) and MDA-MB-231 human breast cancer cells. They found that lycopene significantly inhibited invasion, migration and proliferation of H-Ras MCF10A and MDA-MB-231 cells. Further, they also showed lycopene activates extracellular signal-regulated kinases (ERKs) and AKT pathway in anti-proliferative and/or anti-invasive/migratory effects in these cells. Gloria et al. (2014) determined the capacity of lycopene and β-carotene to inhibit cell proliferation, arrest the cell cycle in different phases, and
increase apoptosis. Their findings showed that carotenoids are potential agents for biological interference with cancer. Takeshima et al. (2012) screened the influence of lycopene on three different cancer cells (ER/PR positive MCF-7, HER2-positive SK-BR-3 and triple-negative MDA-MB-468) to demonstrate the molecular mechanism. Lycopene treatment induced cell cycle arrest at the G0/G1 phase at physiologically achievable concentrations found in human plasma. The greatest growth inhibition was observed in MDA-MB-468, with demonstrable cleavage of PARP. Lycopene induced activation of ERK1/2, with concomitant cyclin D1 suppression and p21 upregulation in these three cell lines. Further, in MDA-MB-468 cells, lycopene inhibited the phosphorylation of AKT and mTOR, with upregulation of pro-apoptotic Bax without affecting anti-apoptotic Bcl-XL. They concluded that the anticancer activity of lycopene was predominant in MDA-MB-468 (triple negative breast cancer).

**Biotransformation of lycopene**

Even though more than 750 carotenoids are identified and predicted in the natural source, only a major notable carotenoids (β-carotene, lycopene, lutein, astaxanthin, fucoxanthin, canthaxanthin) have been studied. Presently, carotenoids research is mainly focused on the dietary carotenoids and linked to biological functions. Since, carotenoids are unstable molecules, it undergoes various modifications (discussed elsewhere) and it is challenging to identify and analyze such biotransformed carotenoids or its metabolites. β-carotene metabolites such as retinol, retinal, and retinoic acid are studied extensively due to its significance in human health. This creates an awareness to study the metabolism of other carotenoids and their bio-functions. The carotenoid metabolites are involved in differential gene expression, cell to cell communication and cell differentiation. Furthermore, it is interesting to address the molecular interaction of carotenoids metabolites/oxidation products with free radicals, protein, nucleic acids and lipids at cellular levels. Studies also shows that carotenoids oxidative products may be involved in oxidative stress and act as pro-oxidant (Siems et al. 2002). The identification of oxidative metabolites is considered to be more important before addressing their biological activity.

Eventhough the metabolism of β-carotene is studied extensively, but biological activities of lycopene is not detailed. Lycopene eccentrically cleaves to form apo-10-carotenoids by BCMO-II enzyme. Cleavage of cis-lycopene by BC0-II may occur either 9, 10 or 9', 10' double bond to produce apo-10'-lycopenal, which
can be oxidized to apo-10'-lycopenoic acid or reduced to apo-10'-lycopenol (Figure 1.11). Lycopene metabolites, including apo-6'-, apo-8'-, apo-10'-, apo-12'-, and apo-14'-lycopenal were detected in the plasma of humans (Kopec et al. 2010). However, the identification of cleaved lycopene metabolites in vivo is challenging. Previously, Khachik et al. (1997) identified a group of lycopene oxidative products, 2, 6-cyclolycopene-1,5-diol in human serum and tissues. In animals several metabolites are detected in lung tissue of lycopene-supplemented ferrets. The intermediate primary cleavage product apo-10'-lycopenal could be either reduced apo-10-lycopenol or oxidized to apo-10'-lycopenoic acid. The conversion of apo-10'-lycopenal into apo-10'-lycopenoic acid in the presence of NAD$^+$, and to both apo-10'-lycopenoic acid and apo-10'-lycopenol in the presence of NADH (Hu et al. 2006). Gajic et al. (2006) reported apo-8'- and apo-12'-lycopenal as well as other unidentified polar metabolites of lycopene in the liver of rats when supplemented lycopene-rich diet. Reynaud et al. (2011) indicated that apo-10'- and apo-14'-lycopenoic acid have a remarkable ability to up-regulate BCO-II expression. These results shown that lycopene converted to apo-10'-lycopenoids in mammalian tissues both in vitro and in vivo. These observations raise the question whether lycopene metabolites play an important biological functions related to human health. The molecular structures of lycopene and their metabolites are shown in Figure 1.10.
Figure 1.10. The molecular structures of lycopene and their metabolites.
**Figure 1.11.** Proposed metabolic pathway of lycopene found in human plasma and tissues.

Cleavage of cis-lycopene by BCO-II may occur at either the 9,10 or 9′,10′ double bond to produce apo-10′-lycopenal, which can be oxidized to apo-10′-lycopenoic acid or reduced to apo-10′-lycopenol.
Analysis of carotenoids and their isomers, cleavage products/oxidation products or metabolites in food and biological samples.

HPLC with C18 and C30 stationary phases either with reverse phase or normal phase are used extensively for the separation of a diverse group of carotenoids in various natural sources including food and biological samples (Gokmen et al. 2002; Su et al. 2002). In general, the separation and resolution of carotenoids is better with gradient than isocratic solvents system. However, these methods require higher analysis time and solvent consumption. Further, these conditions and stationary phase were successfully employed in the analysis of carotenoids and their isomers or related derived products in biological fluids and tissues samples (Epler et al. 1993; Bone et al. 2000). Others have attempted and identified several types of carotenoids and their metabolites/oxidative products by employing gas chromatography and mass spectrometry. Stratton et al. (1993) determined β-carotene oxidation products as β-ionone, β-apo-l4'-carotenal, β-apo-10'-carotenal, β-apo-8'-carotenal, and β-carotene 5, 8-endoperoxide by using RP-HPLC and GC-MS. Wyss et al. (1997) developed an HPLC method with automated column switching for the simultaneous determination of endogenous levels of 13-cis-retinoic acid, all-trans-retinoic acid and their 4-oxo metabolites in human and animals tissue samples. Khachik et al. (1997) separated, identified, quantified, and compared 34 carotenoids, including 13 geometrical isomers and 8 metabolites in breast milk and serum of lactating mothers by HPLC-PDA-MS. Wolz et al. (1999) investigated the astaxanthin metabolites in primary cultures of rat hepatocytes by GC-MS. Siems et al. (2000) demonstrated the oxidation products of β-carotene by using capillary gas-liquid chromatography and HPLC. The developed method was linear in the range of 0.3-100 ng/mL with a lower quantification limit. Kim et al. (2001) isolated and analyzed auto-oxidation products of lycopene by GC-MS. Subsequently, Bernstein et al. (2001) identified and quantified the dietary lutein and their geometrical (E/Z) isomers and related metabolites by HPLC in tissues of the human eye. Dutcher et al. (2001) identified carotenoid stereoisomers in spinach and human retina samples by using HPLC on-line coupled to mass spectrometry and nuclear magnetic resonance spectroscopy. Aust et al. (2003) has reported lycopene oxidative product 2,7,11-trimethyltetradecahexaene-1,14-dial by using GC-MS. Sommerburg et al. (2003) separated and identified 5,6-epoxi-β-ionone, ionone, β-cyclocitrinal, β-ionone, dihydroactinidiolide, and 4-oxo-β-
ionone as major cleavage products of β-carotene mediated by hypochlorous acid using GC-MS.

Simultaneously from the late 2000s, spectroscopic and mass spectrometric techniques have been used for qualitative and quantitative analysis for structurally different carotenoids and their derived products. The carotenoid analysis is done by using different ionization modes such as matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI). These methods are employed successfully to target the mass with better ionization and high resolution for the identification of broad range of natural organic molecules including carotenoids (Fraser et al. 2007; Crupi et al. 2010; Rivera et al. 2014). Further, this application is extended to explore several metabolites and oxidative products of carotenoids in rodents and human biological samples (Bhosale et al. 2007; Lakshminarayana et al. 2008; Sangeetha et al. 2009). Initially, van Breemen (1998) developed an APCI-LC-MS method and analysed all-trans-retinol and all-trans-retinyl palmitate with the lower limit of detection (LOD) of 34 fmolcs/µL and 36 fmolcs/µL (on-column) and limit of quantitation (LOQ) was 500 fmolcs/µl and 250 fmolcs/µl (on-column) respectively. Later, Zhu et al. (2006) developed LC/APCI-MS a negative ion mode for the measurement of labeled and unlabeled β-carotene in human serum and feces to demonstrate bioefficacy of orally administered β-carotene, the limit of detection of 0.25 pmol (on injection of 20 µL of 0.0125 µM β-carotene), and LOQ was 1.0 p mol (on the injection of 20 µL of 0.050 µM β-carotene) with the linear range of 1.1 to 2179 p moles on-column. Further, they believed that wide linear range with low LOD and LOQ facilitated sensitive and selective analysis of pro-vitamin A carotenoids. Fraser et al. (2007) used MALDI/TOF-MS to detect and quantify plant carotenoids, and other metabolites (m/z) in complex biological systems. Schäffer et al. (2010) validated RP-HPLC-DAD method for simultaneous quantification of retinol, retinyl esters, tocopherols, and selected carotenoids in the lung, liver, and plasma of various animal samples. Wurtinger et al. (2012) demonstrated influence of, analytical parameters like constituents of mobile phase, including the modifiers added (acids, bases, dopants, metals, and salts) and other experimental conditions (collision energy, flow collision gas, temperatures, etc.) on the ionization of the analytes (e.g., ESI, APCI, APPI, FAB). Consequently, Giuffrida et al. (2012) for the first time analyzed and identified 52 carotenoids among the various cultivars of capsicum using HPLC-
DAD-APCI-MS. Further, Rivera et al. (2011) studied the effect of ionization of carotenoids and oxygenated carotenoids using ESI, APCI, and APPI, and reported that 12 of the 16 carotenoids exhibited strongest signal strength with APCI.

Furthermore, MS/MS is considered to be an efficient method for carotenoids identification by the use of transitions through precursor and daughter ions. This approach is also suitable for the determination of carotenoids with the same molecular mass such as structural isomers. Prasain et al. (2005) developed a sensitive and specific ESI-MS/MS method using MRM for the detection of carotenoid oximes (zeaxanthin oxidation products) in a human eye sample. Further, this method provides invaluable information on the characterization and quantification of carotenoids and their oxidized products formed during in vitro and in vivo studies. Crupi et al. (2010) investigated carotenoids in a typical wine grape variety by using RP-HPLC-DAD-MS (ESI) method. Due to an unusual ionization process and their mass spectra of carotenoids comprised both protonated molecules and molecular ion radicals. Further, they were subjected for the selective collision-induced dissociation (CID) to differentiate structural and geometrical isomers such as lutein isomers (zeaxanthin, 9Z and 9′Z-lutein and a cis-isomer of β-carotene (9Z- β-carotene), 5, 6-epoxy xanthophylls, violaxanthin, (9′Z)-neoxanthin, lutein-5,6-epoxide and 5,8-epoxy xanthophylls diastereoisomers (neochrome, auroxanthin, luteoxanthin, flavoxanthin, chrysanthemaxanthin). Kopec et al. (2010) performed HPLC-MS/MS using APCI′ve mode to separate and detect the apo-6′, apo-8′, apo-10′, apo-12′, apo-14′, and apo-15′-lycopenal products formed by in vitro oxidation reaction. The quantitative analysis of carotenoids and other fat soluble compounds performed simultaneously on a C30 column and detected by APCI-MS/MS with operation of selected reaction monitoring (SRM) mode. Also, this method calibrated to shown a less variability in intra- and inter-day precision analysis. Recently, ultra-high performance liquid chromatography (UPLC/UHPLC) has attracted much attention due to faster analysis and higher sensitivity with 2 μm particle size stationary phases, and thereby increasing column efficiency, decreasing band broadening, and increasing resolution (Arathi et al. 2015). Granado-Lorencio et al. (2010) assessed the suitability of UHPLC for the simultaneous determination of biomarkers of vitamins including vitamin A (retinol, retinyl esters) and major carotenoids in human serum. This method allowed a better resolution for carotenes and xanthophylls isomers provides better sensitivity and reproducibility in peak area and retention time than the HPLC, with mean RSDs.
Rivera et al. (2011) analyzed various carotenoids by UHPLC-MS/MS detection. Further, they compared three different ionization techniques (ESI, APCI, and APPI) to ionize the carotenoids and concluded that APCI has a powerful technique to ionize carotenoids. They also used dopants (acetone, toluene, anisole, and chlorobenzene) that allowed the enhancement of the carotenoid signals strength up to 178-fold. Delpino-Rius et al. (2014) analyzed simultaneously epoxy-carotenoids, hydroxyl-carotenoids and carotenes in fresh homemade and industrially processed fruit products by UPLC. They identified 27 carotenoids eluted within 17 minutes, furthermore this method allowed to differentiate the carotenoid profiles, and 5, 6- to 5, 8-epoxycarotenoids. Separation of carotenoids on UHPLC columns illustrate less analysis time compared to HPLC C30 column however, the separation is better in C30 column. Hence, there is a requirement for appropriate UHPLC column for rapid and sensitive analysis of carotenoids with better separation. Li et al. (2015) developed a quick and simple ultra-high performance supercritical fluid chromatography-photodiode array detector (UHPSFC-PDA) method and validated the determination of carotenoids in dietary supplements using Acquity UPC2HSS column by gradient elution with carbon dioxide and solvent system. We have developed a rapid method for analysis of lycopene isomers and their fragmentation pattern with (collision induced dissociation) CID and demonstrated the importance of ion mobility to distinguish carotenoids geometrical isomers using UPLC-MS/MS (Arathi et al. 2014). Raphael et al. (2016) identified canthaxanthin oxidation products and compared the similarity of β-carotene like oxidation products by using both LC–MS and GC–MS chromatograms.

Apart from these, Orbitrap MS a high-resolution mass spectrometry was also exploited for carotenoid analysis to generate mass spectra with a resolving power up to 100,000 at full-width half-maximum and mass accuracies within two parts per million (ppm). Due to its high mass resolution and exact mass screening detectors probable molecular formulae of the ions and fragments were elucidated (Kind et al. 2006). In continuation, Van Meulebroek et al. (2014) developed a full-scan high-resolution Orbitrap-MS method enabling the metabolomic screening for carotenoids in tomato fruit tissue. The validation demonstrated the excellent performance in terms of linearity, repeatability, and higher range of mean corrected recovery. Additionally, a well-established detection technique, i.e., MS/MS and ultraviolet-visible spectroscopy photodiode array, indicated superior performance of high-resolution
Orbitrap-MS (with limits of detection ranging from 1.0 to 3.8 pg µL⁻¹). Contemporarily, 2D-LC and multidimensional chromatography have emerged as a tool for carotenoid analysis. This provides an excellent separation and resolution for analysis of complex matrices. In this regard, Cacciola et al. (2012) developed and applied a comprehensive normal-phase×reversed-phase liquid chromatography (NP-LC X RP-LC) system for analysis of the intact carotenoid composition of red chili peppers, with photodiode array and mass spectrometry detection. A total of 33 compounds were separated into 10 different chemical classes in the two-dimensional space and identified by accurate IT-TOF (ion trap-time of flight) MS detection. Apart from these, the robust technique LC-NMR offers 1-D and 2-D NMR spectra for the components separated by HPLC. Recently, LC-NMR is used because of improved sensitivity due to higher magnetic fields. Further, NMR provides information about conformational geometry for structural elucidation. LC-NMR is established as a method of analyzing major carotenoids and metabolites in food and biological samples (Tode et al. 2009). The NMR studies on carotenoids metabolites are scanty due to quantitative limitations. Therefore, LC-MS-APCI studies are widely used in the characterization of carotenoids metabolites or oxidative cleavage products. The approach of LC-MS techniques and ionization modes for characterization of carotenoids and their metabolites are shown in Figure 1.12.

Even with the advancement in hyphenated analytical techniques, inconsistency of results may arise due to several pre-chromatographic (samples or tissues, the nature of carotenoids, sample preparation, incomplete extraction, solvent incompatibility, isomerization/oxidation, physical losses of carotenoids/metabolites and its accountability) and post-chromatographic (low recovery, less stability, inaccurate method validation, co-elution, unavailability of standards, selection of improper mode of ionization, carotenoid/metabolites with same molecular mass) errors in the analysis of carotenoids and their metabolites (Arathi et al. 2015). The qualitative and quantitative analyses of carotenoids need a proper method validation as per the ICH (International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) and IUPAC (International Union of Pure and Applied Chemistry) guidelines. The method validation mainly comprises of precision, specificity, LOD, LOQ, linearity, sensitivity, range, and robustness. Carotenoid standard curves prepared for subsequent quantification by HPLC-PDA. The amounts of carotenoids calculated based on the regression equations. The LOD and LOQ need
to be calculated for each standard on the basis of the signal-to-noise ratio (3:1 for LOD and 10:1 for LOQ). LOD is the amount that resulted in a peak with a height three times that of the baseline noise respectively and the LOQ determined as lowest injected amount which could be quantifiable reproducibility (relative standard deviations-RSD ≤ 5%). The precision evaluated by the relative coefficient of variation (%). The inter- and intra-day RSD for retention times of individual carotenoid need to be considered for standard concentration to check the reproducibility of the method. The accuracy of the extraction method assessed by determining recovery of carotenoids with a mean value respectively.

Figure 1.12. Advanced hyphenated techniques for the characterization of carotenoids and their metabolites.

Bio-functionality of carotenoid metabolites

β-Carotene is one of the most potent vitamin A precursor among other provitamin A carotenoids (α-carotene, β-cryptoxanthin and γ-carotene). Chemically vitamin A refers to all isoprenoid compounds that possess the biological activity of all-trans-retinol. The parent structure of most retinoids contains a substituted β-ionone ring with a side chain of three isoprenoid units linked at the six position of the β-ionone ring. Retinol plays an important role in vision, differentiation and proliferation of a wide range of epithelial cells, bone growth, reproduction and embryonic development. β-carotene can be converted to retinal, retinoic acid and other active forms of the vitamin A family. Retinoic acids serve as ligands for nuclear retinoic
acid receptors (RARs), namely RXR and RAR, mediate vitamin A dependent activities. Several studies have reported the biological properties of β-apocarotenoids. Zile et al. (1987) demonstrated that retinoyl β-D-glucuronide (a metabolite of vitamin A) could arrest HL-60 cell proliferation and induces differentiation into mature granulocytes, it may act by itself or by being hydrolyzed to retinoic acid. Others have reported that β-carotene cleavage products, such as β-apo-12′-carotenoid acid inhibit the proliferation of HL-60 cells and β-apo-14′-carotenoid acid stimulates the differentiation of U937 leukemia cells (Suzuki et al. 1995; Winum et al.1997). Hu et al. (1998) characterized a polar oxidation product of β-carotene 5,8-endoperox-2,3-dihydro-β-apocarotene-13-one, and demonstrated the inhibitory action on cell growth and cholesterol synthesis in MCF-7 cells. Kawada et al. (2000) suggested that the inhibitory action of adipocyte differentiation by carotenoids and retinoids exhibited through the RAR up-regulation and the suppression of PPARγ. Tibaduiza et al. (2002) tested the effect of synthetic excentric cleavage products of β-carotene such as β-apocarotenoids acids, including β-apo-8′-, β-apo-10′-, β-apo-12′-, and β-apo-14′-carotenoid acid on the growth of the MCF-7, Hs578T, and MDA-MB-231 human breast cancer cells. Further, they observed β-apo-14′ and β-apo-12′-carotenoid acid significantly inhibited MCF-7 growth, and only β-apo-14′-carotenoid acid inhibited Hs578T growth. None of these treatments inhibited the growth of MDA-MB-231 cells. Also, authors concluded that apocarotenoids acids exhibit anti-proliferative effects by down-regulation of cell cycle regulatory proteins and inhibition of AP-1 transcriptional activity. Rühl et al. (2005) also reported that β-carotene is involved in activator of the human pregnane receptor (PXR) a ligand-activated transcription factors involved in xenobiotic detoxification in the liver. Ziouzenkova et al. (2007) demonstrated that β-apo-14′-carotenal, but not other structurally related apocarotenals, represses peroxisome proliferator activated receptors (PPAR) and RXR activation. Eroglu et al. (2012) demonstrated that none of the β-apocarotenoids significantly activated RARs. However, β-apo-14′-carotenal, β-apo-14′-carotenoid acid, and β-apo-13-carotenone antagonized ATRA-induced transactivation of RARs.

Lycopene is another non-provitamin A hydrocarbon carotenoid present in human serum and tissues. Recently, lycopene has attracted much attention due to its association with a decreased risk of certain cancers, cardiovascular diseases and other chronic problems (Story et al. 2010). Several studies from in vivo and in vitro suggest
that lycopene induces apoptosis in cancer cells (Hwang et al. 2005; Tang et al. 2005; Ivanov et al. 2007). Structurally lycopene consists of open polyene chain lacking the β-ionone ring compared to β-carotene and share a same molecular mass and chemical formula. Even though the metabolism of β-carotene has been extensively studied, the metabolism and biological activities of lycopene is not much detailed. There are several reports supported the concept, biological activities of lycopene mediated by its oxidative products/cleavage products (Lindshield et al. 2007; Mein et al. 2010; Wang, 2012). The characterization of BCO-II enzyme has demonstrated that this enzyme can catalyze the excentric cleavage of lycopene to form apo-10'-lycopenoids (Hu et al. 2006). The discovery of various oxidation products or metabolites of carotenoids has questioned the active role of them compared to intact carotenoid molecules in combating various diseases. However, little is known about metabolites or oxidative products of LYC on health benefits. Nara et al. (2001) have demonstrated that autoxidation mixtures of LYC inhibited the HL-60 cell growth effectively than LYC. Similarly, Zhang et al. (2003) identified a cleavage product of LYC (E, E, E)-4-methyl-8-oxo-2, 4, 6-nonatrienal, and evaluated its apoptosis-inducing activity in HL-60 cells. Consequently, Aust et al. (2003) reported the role of LYC degraded products in enhancing cell communication and cell signaling. Lian et al. (2007) demonstrated that apo-10'-lycopenoic acid is a biologically active metabolite of lycopene and suggest that apo-10'-lycopenoic acid is a potential chemopreventive agent against lung tumorigenesis. Ford et al. (2011) demonstrated that lycopene and apo-12'-lycopenal reduce the proliferation of prostate cancer cells. In the recent study, it is reported that apo-10'-lycopenoic acid led to the increase in SIRT1 (Sirtuin 1) enzyme activity by treatment with this metabolite in mice, resulting in prevention of fatty liver (Chung et al. 2012). Due to its high antioxidant activity lycopene is more unstable and needs more advanced analytical techniques to identify and characterize lycopene metabolites and their biological activities.

Lutein and its isomer zeaxanthin are the two major carotenoids found in the human eye and are associated with vision protective properties. Intakes of fruits and vegetables rich in lutein and zeaxanthin have specific biological functions in decreasing AMD and cataract (Bone et al. 2003). Although, conversions of β-carotene to retinoids documented in animals and humans, only little is known about the metabolism of xanthophyll carotenoids. In general, carotenoid metabolites are involved in chemoprevention of cancer. Lutein metabolites such as 3'-epilutein, 3'-
oxolutein, 3’-dehydrolutein, meso-zeaxanthin, methoxy-zeaxanthin, oxime derivatives of 3-hydroxy-β-ionone and 3-hydroxy-14-apocarotenal are reported in human tissues and serum (Khachik et al. 1997; Prasain et al. 2005; Bhosale et al. 2007). Characterization of these lutein and zeaxanthin metabolites in vitro and in vivo is warranted to address their biological functions. The health benefit of these lutein metabolites is not studied in detail. The oxidized lutein may be highly reactive when combined with similar reactive oxygen species and presumed to enhance antioxidant property (Lakshminarayana et al. 2013), but the mechanism of action of these xanthophylls (lutein) metabolites remains unanswered. Several noteworthy studies have explored lutein metabolites in vivo (Khachik et al. 1997; Prasain et al. 2005; Bhosale et al. 2007; Lakshminarayana et al. 2008; Yonekura et al. 2010). However, mechanism of action and other functional aspects of these metabolites/oxidative cleavage products needs further research. We reported the possible protective effect of lutein oxidation products in cervical cancer cell lines (Lakshminarayana et al. 2010). Further, we also elucidated the formation of lutein oxidation products mediated through peroxyl radicals and screened the antioxidant and cytotoxic effects of oxidized lutein on HeLa cancer cells (Lakshminarayana et al. 2013). Previously, we identified the apocarotenals, diepoxides, and other oxidative degradation products of lutein in liver. Further, we presumed that these products are formed ay due to the peroxyl radical mediated oxidation in the body (Lakshminarayana et al. 2013; Lakshminarayana et al. 2008). These results are significant in chain breaking peroxyl radical or quenching of singlet oxygen. Furthermore, existence of these oxidized molecules in vivo is significant in view of free radical chemistry and oxidative stress (Woodall et al. 1997). Possibly, oxidized lutein may reduce the cancer cells viability through induction of apoptosis. Further, result from our study demonstrated the inhibitory effect of these compounds on cancer cell growth, which may be due to the effect on signaling pathway-involved in apoptosis. The biological activity of intact lutein may be different than its metabolites. Hence it is important to address, that the beneficial role of lutein and zeaxanthin in delaying and possibly protecting against ascribed chronic diseases may be due to their metabolites. In the case of other oxygenated carotenoids, such as astaxanthin, Wolz et al. (1999) identified the metabolized products such as 3-hydroxy-4-oxo-β-ionone and 3-hydroxy-4-oxo-7, 8-dihydro-β-ionone in primary rat hepatocytes. Later, Kistler et al. (2002) reported four radiolabeled metabolites of astaxanthin including 3-hydroxy-4-oxo-β-ionol, 3-
hydroxy-4-oxo-β-ionone, 3-hydroxy-4-oxo-7, 8-dihydro-β-ionol and 3-hydroxy-4-

hydroxy-7, 8-dihydro-β-ionone in human and primary human hepatocytes. Further, they
demonstrated that incubation of astaxanthin with microsomes from liver containing
detoxifying enzymes did not generate astaxanthin metabolites. Sangeetha et al. (2010)
hypothesized that astaxanthin may be converted into retinol via β-carotene in retinol
deficient rats. However, the formation of astaxanthin metabolites and their biological
functions is not much explored. Further, studies related to astaxanthin metabolites and
their biological functions are not detailed and explored.

Fucoxanthin is a major epoxy carotenoid found in marine source (seaweeds)
and explored for its anticancer, anti-allergic and anti-obese activities (Woo et al.
2009; Kim et al. 2010). Dietary fucoxanthin is metabolised to fucoxanthinol in
gastrointestinal tract by digestive enzymes and further it is converted to
amaroucixanthin in liver (Asai et al. 2004). The bioactivity of fucoxanthin is
attributed to its metabolites fucoxanthinol and amaroucixanthin A. There are reports
demonstrated that fucoxanthinol is more effective and plays an important role in
health benefits than intact fucoxanthin (Rwigemera et al. 2015; Martin, 2015). Anti-
proliferative and cancer preventing influences of fucoxanthin and fucoxanthinol are
mediated through different signaling pathways (Martin, 2015). Also, others have
reported fucoxanthin and its metabolites regulate adipogenic gene expression and
inhibit the adipocyte differentiation of 3T3-L1 cells through down-regulation of
PPARγ. The suppressive effect of fucoxanthinol is superior on adipocyte
differentiation than its parent molecule (Maeda et al. 2006). In MDA-MB-231 cells,
fucoxanthinol reduced nuclear levels of NF-κB members and indicated an effective
treatment and/or prevention of breast cancer (Rwigemera et al. 2015). However, there
are no much reports on epoxy-carotenoids identified in blood and tissues, may be due
to the less dietary importance. This is an active area of research and deserves further
study. The overview of bio-functionality of carotenoid cleavage products/metabolites
is shown in Table 1.3.
<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Metabolites identified</th>
<th>Bio-functionality of carotenoid metabolites</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycopene</td>
<td>Oxidation products of lycopene, Acetylo retinol acid, 2,7,11-trimethyl-tetradecahexene-1,14-dial (E,E,E)-4-methyl-8-hydroxy-2,4,6-nonatrienal (MON) apo-10'-lycopenoic acid, apo-12'-lycopenal apo-10'-lycopenoic acid, 5,8-endoperoxyl-2,3-dihydro-beta-apocarotene-13-one, 5,6-monoepoxide</td>
<td>Inhibits the growth of HL-60 human promyelocytic leukemia cells, Activates RAR to inhibit mammary cancer cells growth, Enhance gap-junction communication, Induce apoptosis by downregulation of Bel-2 and Bel-XL, and activated caspase cascades in HL-60 cells, Chemopreventive effect on lung carcinogenesis, Reduced androgen-independent prostate cancer cells, Influence on SIRT1 enzyme activity and prevention of fatty liver, Inhibits cell growth and cholesterol synthesis in MCF-7 cells, Greater differentiation-inducing activity than beta-carotene toward NB4 human leukemia cells, Arrest HL-60 cell proliferation and induce their differentiation into mature granulocytes, Stimulates the differentiation of U937 leukemia cells, Inhibits the proliferation of HL-60 cells, Inhibits adipocyte differentiation through RAR up-regulation and suppression of PPARY, Inhibits MCF-7 growth, and Hs578T growth, Represses PPAR and RXR activation, Antagonized ATRA-induced transactivation of RARs</td>
<td>[Nara et al. 2001], [Ben-Dor et al. 2001], [Austi et al. 2003], [Zhang et al. 2003], [Lian et al. 2007], [Ford et al. 2011], [Chung et al. 2012], [Hu et al. 1998], [Duitsman et al. 1999], [Zile et al. 1987], [Winum et al. 1997], [Suzuki et al. 1995], [Kawada et al. 2000], [Tibaduiza et al. 2002], [Ziouzenkov et al. 2007], [Eroglu et al. 2012]</td>
</tr>
<tr>
<td>Beta-carotene</td>
<td>5,8-endoperoxyl-2,3-dihydro-beta-apocarotenone-13-one, 5,6-monoepoxide</td>
<td>Greater differentiation-inducing activity than beta-carotene toward NB4 human leukemia cells, Arrest HL-60 cell proliferation and induce their differentiation into mature granulocytes, Stimulates the differentiation of U937 leukemia cells,</td>
<td>[Duitsman et al. 1999], [Zile et al. 1987], [Winum et al. 1997], [Suzuki et al. 1995], [Kawada et al. 2000]</td>
</tr>
<tr>
<td>Lutein</td>
<td>Photodegraded and peroxy methyl mediated lutein oxidized products</td>
<td>Higher antioxidant and cytotoxic effects in HeLa cells.</td>
<td>[Lakshminarayana et al. 2013], [Lakshminarayana 2010]</td>
</tr>
<tr>
<td>Fucoxanthin</td>
<td>Fucoxanthin and amarouciaxanthin A, Halocynthia xanthin and fucoxanthin</td>
<td>Anti-proliferative effect on human prostate cancer cells MCF-7 and MDA-MB-231 cells, Induces apoptosis in human leukemia, breast and colon cancer cells, Fucoxanthin metabolites reduced the growth of primary effusion lymphoma tumor cells, Induction of gap junctional communication in murine fibroblasts</td>
<td>[Asai et al. 2004], [Rwigemera et al. 2015], [Konishi et al. 2006], [Yamamoto et al. 2011], [Hausch et al. 1995]</td>
</tr>
</tbody>
</table>
Proposed future line of research

Till now, carotenoid research has typically focused on characterization of carotenoids in food and various natural sources. Currently, hyphenated analytical tools are also applied for the identification of carotenoids and their metabolites in body fluids and biological tissues. The comprehensive investigation of carotenoid metabolites drives further progress to develop a novel or advance analytical techniques for versatile analysis. The development of metabolomics database and its integration into genomics, transcriptomics, and proteomics have made links between different levels of biological systems leading to understanding systems biology. Furthermore, concept of metabolomics helps to explore thousands of new components with beneficial or harmful effects to humans. Notably, carotenoids are rarely absorbed and excreted in their ingested forms, but extensively metabolized in the body. The fate of metabolites and its role in comparison with intact carotenoids related to their health benefits needs to be addressed. Although, sensitive analytical techniques are used for carotenoids analysis, further these techniques could also be explored for metabolites too. Most of the bioactive degraded/oxidized/metabolites of carotenoid products may likely to have lesser life span, determination of such components is really challenging. The acquiring of entire spectrum of metabolites and their characterization helps to understand its structure-function relation. Hence, exploration of a suitable analytical method with optimization, validation and reproducibility is required to reveal unknown carotenoids and their metabolites. Further, studies of our laboratory have an opinion that cellular effects of carotenoids are mediated through their derivatives formed either by chemical oxidation or by enzymatic cleavage in vivo system (Lakshminarayana et al. 2008 and 2013). Hence, elucidation of oxidation products/metabolites and evaluation of their biological significance is warranted.
Chapter 1

Aim and scope of the study

Lycopene is a hydrocarbon non pro-vitamin A carotenoid and recognized as efficient singlet oxygen quenchers among the natural carotenoids. In general, carotenoids consist of tetraterpenoid (40 carbon atoms) structure with series of alternative conjugated double bonds, which makes them highly electrophilic in nature and acts as superior antioxidants. Further, these unique chemical properties are very sensitive to oxidative modification/degradation. There are five major carotenoids (β-carotene, α-carotene, lycopene, β-cryptoxanthin, lutein) found in human plasma and tissues. Among carotenoids, β-carotene has been well studied. In case of xanthophylls (oxygenated carotenoid) lutein/zeaxanthin are recognized as front line defense for age related macular degeneration and cataract. More recently, lycopene has attracted attention due to its association with decreased risk of number of chronic diseases. Naturally lycopene is present in various fruits like tomato, watermelon, guava and papaya. Epidemiological studies have shown that consumption of carotenoids rich diet is associated with a lower risk for cancer and cardiovascular disease. Lycopene is effective in quenching of free radicals, especially singlet oxygen species formed during normal metabolic processes. Since lycopene plays an important role in preventing oxidative damage to the membrane lipids, thereby influencing the strength and fluidity of the membranes and increases the gap junction communication. Although conversion of β-carotene into retinal and retinoic acid are well documented in humans, little information is available on the metabolism of lycopene. In general, carotenoid metabolites/oxidative products are reported to be involved in the chemoprevention of cancer. Studies have suggested that higher intakes of tomato rich products are associated with a reduced risk of prostate cancer, breast cancer, lung cancer, colon cancer. The proliferation of cells is controlled by series of cell cycle regulators, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. Further, gap junction communication (GJC) has been implicated in the control of cell growth via adaptive responses i.e. differentiation, proliferation and apoptosis. Reports have evident that loss of GJC is hallmark of carcinogenesis. However, mechanism of carotenoids underlying these biological effects remain unclear. Furthermore, formation of lycopene oxidative products and their differential effects on cancer and other biological functions are yet to be illustrated. Related literatures pertaining to this
study is also scanty. Although, studies have correlated the influence of lycopene on prostate cancer and breast cancer development, nonetheless the mechanism is poorly understood. Against this background, the present work was undertaken to elucidate the possible role of lycopene and its oxidative products on regulation and anti-proliferation of prostate (PC-3) and breast (MCF-7) cancer cells.

With this the following objectives were proposed

- Isolation and characterization of lycopene oxidative products in vitro by using HPLC and LC-MS techniques.
- Evaluation of antioxidant and cytotoxic influence of lycopene and its major oxidative products in vitro.
- Influence of lycopene and its major oxidative products on gap junction communication and proliferation of cancer cells.