Chapter-5
Objective No.3
Chapter 5

Influence of lycopene and its major oxidative products on gap junction communication and proliferation of cancer cells

Introduction

The health benefits of lycopene (LYC) have been extensively studied, however the fate of its metabolic/oxidation products have not been much detailed. Several in vivo and in vitro studies suggested that LYC has anti-cancer properties. Reports have shown the inverse relation between carotenoid consumption with decreased risk of cancer and other chronic diseases. The possible anti-cancer properties of LYC may be due to its antioxidant activity, enhancing cell-cell communication, and modulation of signalling pathway. However, the mechanisms of LYC against chronic diseases is still not detailed. In this regard, bio-functionality of LYC may be attributed due to its oxidative/cleavage products. However, little is known about the metabolism or oxidative decomposition of LYC in mammals (Wang, 2012). In general, due to series of conjugated double bonds and high electrophilicity of carotenoids including LYC are more prone to oxidative modification, hence several oxygenated LYCs or cleavage products may be formed. Previously, Khachick et al. (1997) identified several carotenoid metabolites in human milk and serum. Subsequently, Kim et al. (2001) have shown the formation of LYC oxidation products in vitro and proposed the presence of similar compounds in vivo samples due to autoxidation. Caris-Veyrat et al. (2003), elucidated the possible formation of LYC oxidation products under several oxidative conditions in vitro and presumed the formation of the same in vivo. Zhang et al. (2003) isolated autoxidized LYC fraction and identified as (E,E,E)-4-methyl-8-oxo-2,4,6-nonatrienal (MON) and this result suggest the possible site of oxidative cleavages. Ferreira et al. (2004) demonstrated enzymatic (3-keto-apo-13-lycopene, 3,4-dehydro-5,6-dihydro-15,15'-apo-lycopenal) and non-enzymatic oxidation products (2-apo-5,8-lycopenal-furanoxide, lycopene-5, 6, 5', 6'-diepoxide, lycopene-5,8-furanoxide isomer) of LYC. Gajic et al. (2006) investigated (apo-8'-lycopenal and apo-12'-lycopenal) LYC metabolites in the rats liver. Further, they observed other very polar, short-chain compounds. Rodriguez et al. (2009) demonstrated the formation of carotenoid oxidation products in processed foods. Later, they compared the LYC oxidation products formed under in vitro (MCPBA and KMnO4) conditions in in vivo. LYC might be cleaved to a series of apolycopenooids in biological tissues under oxidative
stress or due to enzymatic reactions. Although conversion of β-carotene into retinal and retinoic acid are well documented in humans (Nagao, 2004), little information is available on the metabolism of LYC. In general, carotenoid metabolites/oxidative products are reported to be involved in the chemoprevention of cancer (King et al. 1997; Lakshminarayana et al. 2013). Likewise, oxidation product of canthaxanthin, 4-oxo-retinoic acid, could activate the retinoic acid receptor (RAR) gene promoter and enhance gap junctional communication (Hanusch et al. 1995). Hu et al. (1998) demonstrated a polar oxidation product of β-carotene, 5,8-endoperox-2,3-dihydroapocarotene-13-one, inhibited cell growth and cholesterol synthesis in MCF-7 cells. Duitsman et al. (1999) reported that 5,6-Epoxy-β-carotene had significantly greater differentiation-inducing activity than β-carotene in human leukemia cells. Later, Nara et al. (2001) demonstrated that autooxidation mixtures of LYC inhibited the HL-60 cell growth effectively than LYC. Similarly, Zhang et al. (2003) evaluated apoptosis-inducing activity of LYC cleavage product (E, E, E)-4-methyl-8-oxo-2, 4, 6-nonatrienal, in HL-60 cells. These results strongly suggest that oxidation products of carotenoids have potential biological effects on human health. The proliferation of cells is controlled by series of cell cycle regulators, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (Nahum et al. 2001; Palozza et al. 2002 and 2004). Studies have demonstrated that LYC or its derived products blocks cell cycle at G0/G1 phase with reduced expression of cyclin D and increased expression of cyclin dependent inhibitors p21 and p27 (Lian et al. 2007; Ford et al. 2011; Takeshima et al. 2014). 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 (Trosko et al. 1998; King et al. 2005). GJIC (gap junction intercellular communication) may progressively decrease during multistage carcinogenesis (Yamasaki et al. 1996). Prevention of down-regulation of GJIC by tumor promoters or up-regulation of GJIC in non-communicating cancer cells are considered as chemopreventive approaches using natural compounds (Trosko et al. 1998). Cooper et al. (1999) explored notable role of carotenoids by stimulation of GJIC. Preliminary, Zhang et al. (1991) and (1995) found that carotenoids induce GJIC through increased expression of connexin 43 (Cx43) in a mouse embryo fibroblast cell line and human dermal fibroblasts. This stimulatory activity of carotenoids is associated with their structural properties (Stahl et al. 1997; Sies et al. 1997). Further, Zhang et al. (2001) addressed the association of overexpression of Cx43 with suppressed proliferation of human osteosarcoma cells by cell cycle transition at the G1 to the S phase. The active

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LYC oxidation product (2,7,11-trimethyl-tetradecahexaene-1,14-dial) obtained by in vitro oxidation induced by hydrogen peroxide/osmium tetroxide possess a potential role in cell signalling by enhancing cell-to-cell communication via gap junctions in rat fibroblast cells (Aust et al. 2003). In continuation, Fornelli et al. (2007) confirmed the inhibitory effects of LYC on MCF-7 cell growth and showed that LYC is involved in the modulation of the GJIC. Furthermore, formation of LYC oxidative products and precise role of them on biochemical and molecular events in cancer cells is not yet detailed. Related literatures pertaining to this hypothesis is also scanty. Therefore, in the present study, we isolated and identified a cleavage products of LYC and evaluated its role in apoptosis and molecular signalling in MCF-7 cells. Elucidation of such active component/oxidation products is important to augment prevention and disease progression.
Results

Effect of LYC oxidative products on cell viability

Based on the preliminary studies, cytotoxicity, oxidative stress and apoptotic induction, among three cell lines (PC-3, HeLa and MCF-7 cell lines) MCF-7 cells with maximum growth inhibition was chosen for further studies (refer chapter 4). This cell line was used to detail the role of LYC metabolites. Further, the cell viability assay with different concentration of LYC is also shown in chapter 4.

The influence of different fractions (1-4) of LYC oxidation products on cytotoxicity of MCF-7 cells shown in Figure 5.1 (a). The fraction 3 strongly inhibited the cell viability compared to other three fractions. The % cell viability of MCF-7 cells decreased by 57.6% (fraction 1), 44.9% (fraction 2), 77.5% (fraction 3) and 54.5% (fraction 4) compared to control. In addition, cell death measured by trypan blue dye exclusion strongly confirmed the above results. The IC$_{50}$ concentration of fraction 3 was found to be 64.5 μM (equivalent to LYC concentration). Further, LDH release in MCF-7 cells treated with different concentrations of COL (fraction 3) [Figure 5.1 (b)]. LDH release to the medium is the hallmark of cytotoxicity of the compound due to membrane lysis and oxidation reaction of lactate to pyruvate. A significant increase (51.06% and 76.59%) in LDH was observed in COL (fraction 3) treated cells with 50 and 100 μM respectively, where as no difference was found between LYC treated and control cells. Based on these the effective concentration 50 and 100 μM of COL (fraction 3) was used to evaluate the cell based assays.

![MCF-7 Cell line](image)

Figure 5.1 (a). Effect of COL different fractions (1-4) on viability of MCF-7 cancer cell lines.
The asterisk indicates a value significantly different from the control ($p<0.05$). Statistical comparisons were made by one way ANOVA followed by Tukey’s test. Values are mean ± SD ($n=5$).
Figure 5.1 (b). Effect of active COL products (fraction 3) on lactate dehydrogenase (LDH) release assay. COL (fraction 3) at 50μM and 100 μM for 24 h significantly increased LDH release from MCF-7 cells as a measure of cytotoxicity in MCF-7 cancer cell lines.

The asterisk indicates a value significantly different from the control ($p<0.05$). Statistical comparisons were made by one way ANOVA followed by Tukey’s test. Values are mean ± SD ($n=3$). (ns indicates non significance)

Effect of LYC and its oxidative products on cell migration

Cell anti-metastatic activity of LYC and its oxidative products were shown in Figure 5.2. It is observed that cells treated with LYC did not inhibit the migration of cells and found to be similar to control cells, whereas inhibition of migration is strongly evident in cells treated with COL (fraction 3) and demonstrates the anti-metastatic activity in MCF-7 cells.
Figure 5.2. Effect of LYC and COL (fraction-3) on cell migration and invasion in MCF-7 cancer cell lines. Note: LYC treated with 50 and 100 μM are not significantly different.

Apoptosis induction of lycopene oxidation products

Cell cycle analysis carried out in cells treated with LYC (50 μM) or its relative concentration of LYC oxidation products (Fraction 3) for 24 h. An increase in apoptosis was observed in MCF-7 cells treated with COL (Fraction 3). Further, cell cycle analysis demonstrates that COL treated cells showed G2/M followed by S phase arrest and increase in Sub-G1 peak representing the apoptotic cell population (Figure 5.4). The influence of COL on apoptosis is further confirmed by annexin FITC-PI staining and shown in Figure 5.5 (a) and 5.5 (b). Results of bright field and fluorescence microscopic observation showed morphological changes, an early and late apoptosis, including nuclear condensations in COL treated cells [Figure 5.3 (a) and 5.3 (b)]. The live cells stained uniformly green, early apoptotic cells were green with bright green nuclei due to chromatin condensation and nuclear fragmentation. Late apoptotic cells stained orange with marked nuclear condensation.
**Figure 5.3 (a).** Effect of COL (fraction-3) on MCF-7 cell morphology after crystal violet and giemsa staining.

**Figure 5.3 (b).** Morphological observation of COL (fraction-3) treated MCF-7 cells with dual staining (acridine orange & ethidium bromide staining). DAPI was used as a marker for nuclear condensation.
Figure 5.4. Effect of LYC and COL (fraction -3) on cell cycle progression and cell death at different concentration in MCF-7 cells.
Chapter 5. Results and discussion

**Figure 5.5 (a).** Detection of apoptosis induced by COL (fraction -3) in MCF-7 cell line. Values are mean ± SD (n=3).
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**Figure 5.5 (b).** Visualization of apoptotic cells stained with annexin V FITC using confocal microscopy (100 X) after treatment with COL-fraction 3 (50 and 100 μM) in MCF-7 cells. DAPI staining was used as nuclear marker.
Intracellular ROS generation

Influence of LYC and its oxidation products (Fraction 3) on intracellular ROS levels in MCF-7 cell lines are shown in Figure 5.6. COL treatment generated a lower level of ROS at initial concentration and decreased at higher concentration. The levels of ROS in control and LYC lower by 13.1 and 10.1% than COL treated cells in the initial concentration and was negligible amount at higher concentration. The % of ROS in positive control was found to be 81.3% in MCF-7 cells.

COL induces loss of mitochondrial transmembrane potential (Dym)

Loss of mitochondrial transmembrane potential (Dym) is well known early event during apoptosis. We measured the loss of mitochondrial membrane potential of LYC and its oxidation products (Fraction 3) treated MCF-7 cells (24 h) using flow cytometry staining with JC-1 dye. Dym was measured from the shift in the ratio of red to green fluorescence emitting cells after treatment. Results showed an increase in green fluorescence in presence of LYC oxidation products (Fraction 3) in a dose dependent manner, indicating a loss of mitochondrial transmembrane potential in treated cells (Figure 5.7). Thus, data suggests that oxidation products (Fraction 3) induces depolarization and mitochondrial transmembrane potential collapse in cells leading to activation of apoptosis.
Figure 5.6. Effect of LYC and COL (fraction -3) on production of reactive oxygen species in MCF-7 cell line.

The asterisk indicates a value significantly different from the control ($p<0.05$). Statistical comparisons were made by one way ANOVA followed by Tukey’s test. Values are mean $\pm$ SD ($n=3$).
Figure 5.7. Effect of LYC and COL (fraction -3) on mitochondrial integrity in MCF-7 cell line.
The asterisk indicates a value significantly different from the control ($p<0.05$). Statistical comparisons were made by one way ANOVA followed by Tukey’s test. Values are mean ± SD ($n=3$).
Effects of LYC and its oxidation products on GJIC

The GJIC assay resulted Lucifer yellow dye spread to about 1-2 rows away from the disrupted edge in the control cells (Figure 5.8). The transfer of dye increased markedly to a maximum of about 3-4 rows in cells incubated with effective concentration 50 and 100 μM COL (fraction 3). The dye transfer is enhanced by COL (fraction 3), than LYC and control and the effect of COL is dose dependent. The greater coupling index (quantitative measure of GJIC) represents the greater transfer of lucifer yellow dye (Figure 5.8).

Figure 5.8. Effect of LYC and COL (fraction-3) on GJC assay-scrape loading dye method.
Effect of LYC and its oxidation products on Caspase-3, 8 and 9

To elucidate the apoptotic mechanism induced by COL (fraction 3), we evaluated its effect on caspase-3, caspase-8 and caspase-9 activities. Results showed that COL (fraction 3) activated caspase-3, caspase-8 and caspase-9 after 24 h incubation in MCF-7 cells (Figure 5.9).

Figure 5.9. Caspase activity of LYC (100 μM) and COL (fraction 3, 100 μM).
The asterisk indicates a value significantly different from the control ($p<0.05$). Statistical comparisons were made by one way ANOVA followed by Tukey’s test. Values are mean ± SD ($n=3$).

Caspase 3 fluorometric assay is based on detection of cleavage of substrate DEVD-AFC. Caspase 8 fluorometric assay is based on detection of cleavage of substrate IETD-AFC. The Caspase-9 fluorometric assay is based on detection of cleavage of substrate LEHD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin).

Substrate of caspase 3, 8 and 9 emits blue light ($\lambda_{\text{max}} = 400$ nm); upon cleavage of the substrate by caspases, free AFC emits a yellow-green fluorescence ($\lambda_{\text{max}} = 505$ nm), which can be quantified using a fluorescence microtiter plate reader. Comparison of the fluorescence of AFC from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase activity.
Effects of LYC oxidation products on expression of cell cycle regulators, anti- and pro-apoptotic markers, DNA repair and GJC gene by semi-quantitative and quantitative RT PCR

To quantify cyclin D, E, A, B, p21, p27, Bax, Bcl-2, p53, Ku 70 and Cx43 mRNA expression upon exposure to COL (fraction 3), an semi-quantitative and RT-QPCR done using SYBR green method in MCF-7 cells. COL (fraction 3) significantly decreased the expression of cyclin D, A, B, Bcl-2, p53, Ku 70 and increased the expression of p21, p27 and Cx43. However, no variation in expression of cyclin E and Bax (Figure 5.10).

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Figure 5.10. Effect of COL (fraction 3) on the expression of Cell cycle regulators (Cyclin D, E, A, B, p21 and p27), Gap junction gene (Cx43), anti and pro-apoptotic genes (Bax, Bcl-2 and p53) and DNA repair gene (Ku 70) in MCF-7 cells. [Legend 1-COL 50μM (fraction 3), Legend 2- COL 100 μM (fraction 3)]. The asterisk indicates a value significantly different from the control (p<0.05). Statistical comparisons were made by one way ANOVA followed by Tukey’s test. Values are mean ± SD (n=3).
Expression of Cx43 in MCF-7 cells by LYC oxidation products

The effect of LYC oxidation products on the expression of Cx43 proteins was determined by immunoblot. β-tubulin was used for normalization and verification of protein loading. After being normalized to β-tubulin, the expression of Cx43 protein level was increased as shown in Figure 5.11.

Figure 5.11. Expression of Connexin 43 protein in MCF-7 cells treated COL (fraction-3) The cells (6 X 10^5 cells/well) were exposed to 50 and 100 μM for 24 h. The control and treated cells were lysed, and 50 μg of extracted proteins were subjected to SDS-PAGE on 12% polyacrylamide gel and blotted with anti-Connexin 43 or anti-tubulin.
Lane-1- control, Lane-2-COL (fraction 3, 50 μM), Lane-3- COL (fraction 3, 100 μM).

Figure 5.12. Proposed mechanism of action of LYC oxidation products.
Discussion

Several studies suggest that consumption of carotenoids rich diet is associated with reduced risk of several types of cancer. Among carotenoids, LYC has gained special attention due to consumption of tomato and tomato based products and its reflection in higher levels in plasma and tissues is correlated with reduction incidence of cancer. The detailed molecular mechanism which carotenoids exert its action is not well known. Many studies attempted to elucidate the role of carotenoids, majorly β-carotene, then LYC on possible role in inhibition/modulation of cancer cell proliferation. Furthermore, reports have shown the contradictory results in in vivo and in vitro studies with ambiguous and non-significant results (Forbes et al. 2003; Wu et al. 2004; Sesso et al. 2005; Burgess et al. 2008). Although, LYC has same molecular mass and chemical properties as β-carotene, however LYC is considered as a second leading molecule in carotenoid biochemistry. β-carotene mechanism on anti-cancer properties are well supported by literature on metabolism including elucidation of β-carotene metabolites. Based on these reports researchers have attempted to investigate the presence of other non provitamin A metabolites or oxidative products in in vitro, in vivo and humans (Khachik et al. 1997; Lakshminarayana et al. 2008 and 2013). Subsequently, inevitable studies on β-carotene and LYC metabolism were done to test the hypothesis whether the parent molecule or its derived products target the anti proliferative effect of cancer. However, there is no detailed studies even after two decades of carotenoid research on bioactivity of metabolites due to challenges in isolation, characterization, lower concentration with lesser stability. We attempted for the first time to compare simultaneously LYC and its oxidative rich fraction on anticancer effects. In continuation of earlier studies including our own reports shown that chemical induced LYC products inhibit growth and induce apoptosis in different cancer cell lines. Apart from these, a similar kind of molecules are identified in vitro and in vivo, and schemed the possible cleavage pattern (Khachik et al. 1997; Caris-Veyrat et al. 2003; Pennathur et al. 2010; Lakshminarayana et al. 2013; Arathi et al. 2016). In the present study, we demonstrated that LYC oxidation products (fraction-3) rich in apo-8,6′-carotendiol with apoptosis-inducing activity obtained by using open column chromatography (refer chapter 3) by combination of suitable solvent ratio.

Generally, the activity of cell cycle kinases is often upregulated in cancer due to the overexpression of cyclins and CDKs or to the inactivation of CDK inhibitors
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. Studies have reported that the growth of cancer cells was inhibited by LYC treatment induced cell cycle arrest at G0/G1 phase (Amir et al. 1999; Ivanov et al. 2007). Breast cancer cells treated with LYC 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 LYC 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; Uppala et al. 2013; Takeshima et al. 2014).

Studies have shown that inhibition of cancer by LYC degraded products and compared its influence to demonstrate the possible role of metabolites. Likewise, LYC-derived metabolite apo-10'-lycopenic acid is involved in induction of cell cycle arrest at G1/S phase in non small lung carcinoma cells. In addition, they also interpreted a decrease in cyclin E, an increase in the cyclin-dependent kinase inhibitors and the transcriptional induction of the RARβ tumour suppressor gene. Subsequently, the LYC 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 (Hwang et al. 2004; Ford et al. 2011; Soares et al. 2013). Contradictory to LYC reports, our study resulted in non-significant difference in cell cycle progression upon intact LYC treatment whereas LYC oxidation products COL (fraction 3) induced cell cycle arrest at G2/M phase followed by S phase arrest was confirmed by cell cycle analysis (Figure 5.4). Further, authentication was done by checking the expression of cell cycle regulatory genes. Results demonstrated down regulation of cyclin D, B and A, and no alteration in cyclin E levels and also an upregulation of cell cycle inhibitors p21 and p27 were observed.

LYC or its metabolites/oxidative products mediate apoptosis via death receptors or intrinsic pathway. In this regard, the metabolite (E, E, E)-4-methyl-8-oxo-2, 4, 6-nonatrienal (MON), formed by autoxidation of LYC, induces apoptosis in human promyelocytic leukaemia (HL-60) cells as evidenced by morphological
changes with chromatin condensation and DNA fragmentation, these were associated with decreased Bcl-2 and Bcl-XL expression and the activation of caspases 8 and 9. MON induced apoptosis via the mitochondrial and death receptor pathways, and 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 may 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, LYC 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). Another study suggested that apoptosis induction by LYC in prostate cancer cells is associated with increased expression of Bax and decreased expression of Bcl-2 gene (Soares et al. 2013). In the present study, COL (fraction 3) induced apoptosis via the mitochondrial and death receptor pathways, it induced the activation of both caspase 8 and 9. Further it might induce cytosolic release of cyt c by decreasing the expression of Bcl-2. Hence COL might mediate apoptosis by both intrinsic and extrinsic pathway (Figure 5.9). Further, apoptosis was confirmed by annexin FITC-PI staining, chromatin condensation and loss of mitochondrial membrane potential (Figure 5.5 and 5.7). ROS generation is considered as an intermediate step during activation of apoptosis, here in our study we observed a very low amount of ROS levels at lower concentration and no detectable levels of ROS production was observed upon COL treatment at higher concentration (Figure 5.6). The proposed mechanism of action of COL (fraction 3) is shown in Figure 5.12. Carotenoids as well as their metabolites and oxidation products stimulate gap junctional communication (GJC) between cells, which is thought to be one of the protective mechanisms related to cancer preventive activities of these compounds. Sies et al. (1997) suggested that oxidation products of carotenoids, especially retinoic acid analogs, significantly contribute to this biological property. Teicher et al. (1999) demonstrated that biological activities of canthaxanthin may be due to metabolites 4-oxo-retinoic acid known to stimulate GJC and to activate the RAR-β2 receptor. Stahl et al. (2000) evaluated the effect of LYC and its possible oxidation product, acyclo-retinoic acid on GJC. In comparison to retinoic acid, considerably higher concentrations of the acyclo analog were required for similar effects. Their data demonstrated 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 LYC oxidation product (2,7,11-trimethyl-tetradeca-hexaene-1,14-dial) on GJC in rat liver epithelial WB-F344 cells. Fornelli et al. (2007) confirmed the inhibitor effects of LYC on MCF-7 cell growth and suggest that LYC is involved in the modulation of the gap junction intercellular communication. In the present study, an enhanced gap junction communication was observed in cells treated with COL (fraction 3) by GJC assay-scrape loading dye method (Figure 5.8), and confirmed by RT-PCR and western blotting analysis of CX43. Further, we observed the LYC and COL (fraction-3) did not significantly affect the normal cells (MCF-10A) under the experimental conditions. These results strongly suggest that LYC metabolites play an important role against cancer cell proliferation. Based on the current study we hypothesized that LYC metabolites are involved in biological functions. The possible mechanism of action of LYC metabolites is shown in Figure 5.12.

The overall observation/results indicated and questioned the active role of LYC. A new observation made from this study demonstrated the possibility of a key metabolites of carotenoid like LYC in chemoprevention of cancer. Although there is a need for greater insight on the role of carotenoid metabolites, but it is more challenging to further authenticate its benefits due to various limitations such as lesser concentration and circulation in biological system. Apart from these, the life span/half life of these oxidized products/metabolites needs to be compared with isotopic labeled studies to show further potentiality of metabolites.