Chapter-6
General discussion and summary
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Carotenoids are biotransformed to yield several cleavage/oxidation products by enzymatic or non-enzymatic (oxidation) breakdown at various physiological conditions. In addition, isomerization or oxidation can occur during food processing and other chemical reactions. In general, central cleavage of carotenoids gives $C_{20}$ compounds retinoids, eccentric cleavages gives smaller fragments, notably $C_{10}$, $C_{13}$ and $C_{15}$ compounds with end group. Carotenoids are known to reduce incidence of major health related problems such as vitamin-A deficiency, age related macular degeneration, atherosclerosis, cancers and other chronic diseases (Cooper et al. 1999). Several epidemiological studies have addressed the precise role of carotenoids function in animal models and humans from past three decades. Primarily, the role of carotenoids came into existence from identification of active metabolites of provitamin A carotenoids as retinal, retinol and retinoic acid. Enzymatic (central or eccentric) cleavages of $\beta$-carotene, $\alpha$-carotene, and $\beta$-cryptoxanthin leads to the formation of retinoids (Bendich et al. 1989). These evidences demonstrate that carotenoids cleavage products are involved in major biological functions. Recently, omics has attracted much attention of researchers due to active role of secondary compounds or metabolites at cellular levels than parent compounds (Arathi et al. 2015). Similarly, many studies including our own, support the concept that biological functions are mediated by carotenoid metabolites (Khachik et al.1997; Stahl et al. 2000; Lian et al. 2007; Lakshminarayana et al. 2008 and 2013; Arathi.et al. 2016). Generally, oxidation products of carotenoids might be formed by reacting with reactive oxygen species (Khachik et al. 1997; Stratton et al. 1997). A polar oxidation product of $\beta$-carotene, 5, 8-endoperoxy-2, 3-dihy-dro-\(\beta\)-apocarotene-13-one was reported to inhibit cell growth and cholesterol synthesis in MCF-7 mammary cancer cells (Hu et al. 1998). However, in case of $\beta$-carotene and LYC or its cleavage products may act as pro-oxidants under circumstances like higher $O_2$ tension especially in smokers when supplemented with high doses (Ommen et al. 1996). Likewise, retinoids potentiates the hepatotoxicity with consumption of alcohol in various animal models and humans (Leo et al. 1999). Hence, formation and charaterization of oxidative/isomerised/enzymatic cleavage products of carotenoids
is evaluated to explore its role against various biological functions, particularly cancer chemoprevention. Furthermore, biochemical characterization of BCO-II demonstrates eccentric cleavage of non-provitamin A carotenoids like LYC and lutein (Mein et al. 2011). Several key metabolites of LYC, such as 2,6-cyclolycopene-1,5-diol, apo-6', 8', 10', 12' and 14'-lycopenal were identified in human tissue and milk (Khachik et al. 1997; Kopec et al. 2010). A cleavage product of LYC (E, E, E)-4-methyl-8-oxo-2, 4, 6-nonatrienal, was shown to be involved in apoptosis-inducing activity in HL-60 cells (Zhang et al. 2003). Consequently, Aust et al. (2003) shown the role of LYC degraded products in modulation of cell signalling by enhancing cell to cell communication. They hypothesized that, formation of metabolites may be involved in biological actions towards reduction of chronic diseases such as cancer. However, the mode of action/mechanism of these metabolites is not well detailed.

In xanthophyll’s, functional groups (hydroxy-, epoxy- and keto groups) may react rapidly with peroxyl radicals and forms number of oxidative products. Woodall et al. (1997) suggested that position of substitution of radicals is an important factor that influences the rate of bleaching. The oxidized forms of carotenoids may be highly reactive, since oxidation results in radical ions, which can combine with similar reactive oxidative species that could lead to higher antioxidant effect under the decreased partial oxygen tension (Lakshminarayana et al. 2013). Similarly, carotenoids with oxo- and hydroxyl groups may be highly reactive with the radicals (Seims et al. 1999; Hurst et al. 2004). In case of lutein metabolism, 3'-epilutein, 3'-dehydrolutein, (3R, 3'S)-meso-zeaxanthin, 3'-oxolutein, 3-methoxy-zeaxanthin and other isomers/metabolites have originated inherently due to oxidation (Khachik et al. 1997; Bhosale et al. 2007). Oxidative metabolites of these carotenoids could be involved in signal transduction, photo sensitization and act as antioxidant to protect the eyes from phototoxicity (Bernstein et al. 2001; Lakshminarayana et al. 2008).

In case of keto-carotenoids, such as astaxanthin and canthaxanthin, found in certain algae, microorganisms and marine animals are considered as potent antioxidants (Guerin et al. 2003). Metabolites of astaxanthin like, 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-oxo-7, 8-dihydro-β-ionone were shown to be involved in xenometabolism in humans (Kistler et al. 2002). It was also hypothesized that, xanthophyll’s may also yield retinoid like molecules in marine fish and rats (Matsuno, 1991). However,
responsible enzymes for formation of astaxanthin metabolites and their bioactivity is not been elucidated. Similarly, Bausch et al. (1999) detailed a major urinary metabolite 3-hydroxy-4-oxo-7, 8-dihydro-β-ionone in rats after administration of radiolabelled canthaxanthin. Furthermore, Hanush et al. (1995) shown the involvement of 4-oxo-retinoic acid, an oxidation product of canthaxanthin in activation of RAR and enhanced cell communications.

Fucoxanthin, found in brown seaweeds is attributed as a promising molecule against obesity and cancer. During intestinal absorption, the fucoxanthin is metabolized into fucoxanthinol and amarouciaxanthin-A in rodents (Sugawara et al. 2002). In case of sea squirt, fucoxanthinol metabolite was further metabolized into halocynthiaxanthin (Konishi et al. 2006). Recently, Rwigemera et al. (2015) reported the pronounced influence of fucoxanthinol on NF-κB pathway than fucoxanthin in MCF-7 and MDA-MB-231 cells.

The diversity of major carotenoid metabolites found in biological samples demonstrates, the necessity of their modifications through various interactions mediated either by chemically or physically or enzymatically to serve the key bio-functionality. There are > 750 carotenoids predicted and documented from various natural sources, investigation of these carotenoids and their products needs to be explored. This gives a platform and wide scope for the nutritional biochemistry to obtain better molecules than existing or known carotenoids or its active metabolites. The role of notable metabolites towards modulation of cell communication, cellular signalling and molecular targeting needs to be further detailed to understand beneficial and detrimental effects in normal and cancer/chronic conditions. Apart from elucidation of molecular function of carotenoids or metabolites, the isolation and characterization of such molecules is more challenging in biological entity. Thus advancement in analytical techniques and optimization is more essential towards the identification of such molecules that are present in nanomolar/picomolar range in human serum and tissues.

With this background, the present study undertaken to isolate and characterize the LYC oxidation products/ metabolites (obtained by in vitro method) to understand the bioactive role of these products Vs LYC using cancer cells. In addition, we also screened the effect of LYC and their oxidative products on antioxidant and cytotoxic activity in different cancer cell lines. Further, we also detailed the action mechanism
of major LYC oxidative product on gap junction communication and molecular signalling pathway towards apoptosis in cancer cells. This study outlines the chemistry, bioactivity and molecular mechanisms of major LYC oxidative products.

Summary

- LYC isolated from ripened tomato puree, confirmed by its characteristic UV-spectra, $\lambda_{\text{max}}$, retention time, mass spectra and comparable with analytical standard. The LYC content in the tomato puree was $9.01 \pm 0.65 \text{ mg/100 g}$ wet weight and the purity of LYC was found to be $93 \pm 5\%$ under the experimental set up, analyzed by UPLC.

- The LYC was eluted at 5.56 minutes with shorter run time compared to previous studies. The LOD and LOQ of standard LYC analysed were 0.436 and 0.931 picomoles on CSH Phenyl-Hexyl column under standardized conditions and % RSD (n=6) were 3.01 and 4.81 respectively. The LYC standard curve shown least-squares linear regression analysis of the data, providing an excellent coefficient of determination value ($r=0.999$ and $r^2=0.998$)

- The autoxidation of LYC resulted in isomerization and there is no degradation observed.

- The LYC oxidized with KMnO$_4$ readily oxidized to form the mixture of oxidative products due to break in its conjugated double bonds in the polyene chain. These degraded products were further used for cell culture studies.

- LYC showed effect on cancer cell lines (PC-3, MCF-7, HeLa and A549) only at below physiological concentration (5 $\mu$M) and no significant difference was observed at higher concentration.

- Upon LYC treatment, the percent cell viability decreased in different cancer cell lines in the order of PC-3 (47.3%) < HeLa (33.7%) < MCF-7 (29.8%) < A549 (27.3%) < HepG2 (22.9%) < A431 (17.8%). Among cancer cell lines, a maximum reduction in cell viability was found in PC-3, HeLa and MCF-7 cells.
The effect of LYC oxidative products on % cell viability were evaluated, % cell viability of PC-3 cells were decreased by 29.6 (LYC), 39.2 (AOL) and 71.4% (COL) compared to control. Likewise, 29.3, 44.2 & 68.4% decrease in MCF-7 and 32.6, 29.4 & 56.2% decrease in HeLa cells was evident compared to control.

Catalase levels in PC-3 cell lines was higher by 11.08 (LYC), 8.3 (AOL) and lower by 30.0% (COL) respectively than control. Similarly, the effect of LYC oxidized products on CAT levels of control cells were higher by 16.0, 10.9 and lower by 37.8% than LYC, AOL and COL, respectively in MCF-7 cells. Similarly, in the case of HeLa cells, the levels of catalase levels in control cells were higher by 3.17 and lower by 8.9, and 32.8% than LYC, AOL and COL, respectively.

The influence of LYC oxidized products on SOD levels in PC-3 treated cells were higher by 1.2% (LYC) and lower by 11.7% (AOL) and 49.5% (COL) than control. Likewise, SOD levels in MCF-7 cells treated with LYC, AOL and COL was lower by 2.07, 5.19 and 52.7% than control. In the case of HeLa cells, SOD activity was higher by 8.7, 5.79 and lower by 36.9% than control respectively.

Glutathione levels in control cells were higher by 30.4, 36.0 and 50.4% than LYC, AOL and COL, respectively. Similarly, in the case of HeLa cells, the levels of GSH in control cells were higher by 14, 22.2, and 59.2% than LYC, AOL and COL, respectively.

The MDA levels in PC-3 cells treated with COL were significantly higher by 30.3, 39.7 and 36.2 % than control, LYC and AOL respectively. Likewise, MDA levels in MCF-7 cells treated with COL was higher by 20.4, 33.0 and 30.2% than control, LYC and AOL. In the case of HeLa cells, COL treated cells shown higher levels of MDA formation by 14.2, 33.5 and 25.8 % than control, LYC and AOL, respectively.

The apoptosis inducing activity of COL is confirmed by FITC annexin-V apoptosis detection kit, AO & EB and DAPI.
Influence of LYC and COL on the levels of intracellular ROS production in three different cell lines were documented. In all the cases, COL increased the ROS levels than the LYC and control treated cells. In the case of MCF-7 cells, ROS levels in control and LYC were lower by 16.3 and 15.5% than COL treated cells. Further, we presumed that intermediate ROS generation could be the reason for higher cell death. Similarly, in PC-3 cells, the ROS levels decreased by 13.6% in control and 10.7% in LYC compared to COL. In case of HeLa cells, ROS levels is found to be lower by 12.8% in control and 8.2% in LYC as compared to COL treated cells respectively.

COL showed pronounced effect in comparison in cytotoxicity and apoptosis in different cancer cell lines compared to intact LYC.

Further, the crude COL sample was fractionated by OCC using different gradient solvent system with hexane/acetone in the following ratios (90:10, 80:20, 70:30 and 60:40 v/v), and eluted four different COL rich fractions.

Among three cell lines studied (MCF-7, PC-3 and HeLa), COL showed better activity in MCF-7 cells, and was used for screening different OCC fractions.

The influence of different fractions (1-4) of LYC oxidation products were evaluated on cytotoxicity of MCF-7 cells, fraction 3 (apo-8,6'-carotendial, apo-5,6'-carotendial & (2E,4E,6E,8E,10E)-3,7,11,15-tetramethylhexadeca-2,4,6,8,10, 14-hexaenal) strongly inhibited the cell viability compared to other fractions.

It is observed that the cells treated with LYC non significantly inhibited the migration of cells and was almost similar to control cells whereas inhibition of migration was evident in cells treated with COL (fraction 3) and demonstrated the anti-metastatic activity in MCF-7 cells.

FACS analysis clearly demonstrated that COL (fraction 3) treated cells showed G2/M followed by S phase arrest and increase in Sub-G1 peak representing the apoptotic cell population.

The levels of ROS in control and LYC were lower by 13.1 and 10.1% than COL (fraction 3) treated cells in the initial concentration and there was no increase in ROS levels found at higher concentration.
- COL (Fraction 3) induces depolarization and mitochondrial transmembrane potential dysfunction in cells leading to activation of apoptosis.
- COL (fraction 3) enhanced GJIC in comparison to LYC and control.
- COL (fraction 3) treatment activated caspase-3, caspase-8 and caspase-9 after 24 h incubation in MCF-7 cells, whereas there was no significant difference between cells treated with LYC and control.
- 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. No variation in expression in cyclin E and Bax was observed in MCF-7 cell lines.
- After being normalized to β-tubulin, the expression of Cx43 protein level was increased in COL (fraction 3) treated MCF-7 cells.
- Further, we observed the LYC and COL (fraction-3) did not significantly affect the normal cells (MCF-10A) under the experimental conditions.
- Based on the results, we hypothesized that COL (fraction 3) may mediate apoptosis via both extrinsic and intrinsic pathway or by enhancing GJC. There may be a cross talk between extrinsic and intrinsic pathway via Bid and t-Bid.
- Further, stability and in vivo studies are warranted with major COL rich fractions.