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Astaxanthin from shrimp efficiently modulates oxidative stress and allied cell death progression in MCF-7 cells treated synergistically with β-carotene and lutein from greens

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ABSTRACT

This study investigated the synergistic efficacy of keto-carotenoid astaxanthin (AST, from shrimp) plus hydrocarbon (β-carotene, BC) and hydroxyl (lutein, L) carotenoids (from greens) on molecular events in MCF-7 cells. MCF-7 cells were treated with either of carotenoid (20 μM, AST or BC or L) separately or the mixture of them (an equimolar concentration of carotenoids mixture, CM) or saponified carotenoid extract from shrimp (SSCE) for 48 h and analyzed cellular uptake, cytotoxicity, and apoptosis. The IC_{50} and combination-index values of AST co-treatment with a lower concentration of BC and L (5 μM) exhibited enhanced cytotoxicity and oxidative stress as compared with individual carotenoids or SSCE. Further, higher cellular uptake/accumulation of AST along with BC and L found to synergistically induce apoptosis through modulation of cyclin D1, p53, Bax and Bcl-2 expressions by arresting cell cycle at G0/G1 phase. Further, CM or SSCE treatments are unlikely to affect proliferation of normal breast epithelial cells (MCF-10A). The results of selective killing of MCF-7 cells demonstrated a greater insight on the synergistic effect of shrimp AST plus BC and L. It is concluded that consumption of shrimp along with green leafy vegetables helps in combating cancer chemoprevention.

1. Introduction

The biological significance of hydrocarbon (β-carotene, α-carotene, γ-carotene, and lycopene) and oxygenated (lutein, zeaxanthin, β-cryptoxanthin, canthaxanthin, astaxanthin, and fucoxanthin) carotenoids are recognized as potential bioactive molecules against age-related degenerative disease and cancers (Arathi et al., 2015). Among carotenoids, astaxanthin (3, 3'-dihydroxy-β, β-carotene-4, 4'-dione, AST) a keto-carotenoid found in shrimp considered as promising nutraceutical molecule due to its potential biofunctionality (Hussein et al., 2006). Previously, studies have focused on the isolation of AST from red yeast (*Xanthophyllomyces dendrorhous*), freshwater green alga (*Haematococcus pluvialis*) and other marine sources (shrimp, crayfish, and krill) (Hussein et al., 2006). Currently, exploration of marine sources for AST is one of the major concerns of nutraceuticals and food processing industry. Shrimp is an excellent source of AST apart from protein, chitin, polyunsaturated fatty acid and created a demand for its production around the world (Santos et al., 2012).

AST has gained distinct attention due to its unique chemical properties and exerted higher antioxidant activity than other carotenoids with emerging evidence of health benefits (Naguib, 2000). Furthermore, AST correlated with enhancement of immune response, and reduction of certain acute and chronic diseases (Hussein et al., 2006). Also, studies have demonstrated the possibility of AST conversion into retinol in retinol-deficient fishes and rats (Matsuno, 1991; Sangeetha and Baskaran, 2010). Studies reveal that AST is linked to anti-inflammatory, anti-tumor, anti-cancer and attenuate UV-radiation-induced photo-toxicity (Lee et al., 2003;

Abbreviations: AST, Astaxanthin; BC, β-Carotene; BHT, Butylatedhydroxytoluene; CM, Carotenoids mixture; DMEM, Dulbecco's modified eagle's medium; DMSO, Dimethyl sulfoxide; DTNB, 5, 5-dithiobis (2-nitrobenzoic acid); FBS, Fetal bovine serum; GR, Glutathione reductase; L, Lutein; MDA, Malondialdehyde; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide; NADPH, Nicotinamide adenine dinucleotide phosphate oxidase; SSCE, Saponified shrimp carotenoid extract; THF, Tetrahydrofuran; TMP, Tetramethoxypropane.

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Chew et al., 1999; Palozza et al., 2009; Camera et al., 2009). In continuation, Jia et al. (2012) demonstrated the role of AST in inhibition of cholesterol biosynthesis by modulating lipid metabolic pathways in HepG2 cells. Epidemiological studies have shown that an increased intake of carotenoids rich fruits and green vegetables associated with a decreased risk of certain cancer and chronic diseases (Riboli and Norat, 2003). Consumption of these sources may provide multiple carotenoids along with other nutrients instead of single carotenoid. Since, AST not identified as a major carotenoid in commonly consumed dietary sources, information related to its epidemiology is not studied (Østerlie et al., 2000).

Studies have correlated the action of individual carotenoid with reduced risk of cancer, however, clinically the health benefit of diverse nature of a mixture of carotenoids from different dietary sources not explored in detail. Stahl et al. (1998) demonstrated the synergistic influence of carotenoid mixtures against oxidative damage in the membrane model. Further, they have used concentration of carotenoids as per the theoretical calculation based on physiological levels. Studies have also focused on the synergistic influence of carotenoids with other phytonutrients on antiproliferation of cancer cells (Yang et al., 2014; Linnewiel-Hermoni et al., 2015). However, effect of carotenoids at physiological concentrations on cytotoxic mechanism through oxidative stress in cancer cells is not detailed. Burton & Ingold (1984) and Palozza et al. (2004) have demonstrated the pro-oxidant activity of carotenoids and their mediated cytotoxicity at increased concentration under higher oxygen tension. Recently, regulation of oxidative stress considered as a crucial factor in both tumor development and responses to anticancer therapies. In this regard, many signaling pathways of cancer metabolism are linked to reactive oxygen species (ROS) through direct or indirect mechanisms (Gorrini et al., 2013). Earlier, we have studied the effect of a composite lutein and lycopene oxidation products and revealed an enhanced inhibitory activity of cancer cell lines by modulation of oxidative status compared to its individual parent carotenoid (Lakshminarayana et al., 2013; Arathi et al., 2016). However, the synergistic efficacy of hydrocarbon and oxygenated carotenoids in amelioration of oxidative stress and its mediated cancer complications are not studied. In this context, we hypothesized that an exploration of promising natural compounds and development of nutraceutical products or food-based strategies might play a greater role in the prevention of cancer. Although considerable attention has been made to elucidate the functions of major carotenoids *in vitro* and *in vivo*, there is no detailed study on the anti-cancer property of the mixture of marine carotenoid (AST) with BC and L from greens at a physiological dose. Therefore, the synergistic effect of structurally different carotenoids (Fig. 1) and the concept of consumption of multi-carotenoids rich food sources/therapeutic supplementation are necessary to modulate anticancer strategy. Hence, we aimed to investigate the influence of co-treatment of purified AST with BC and L on cytotoxicity, the oxidative status including generation of reactive oxygen species (ROS) and mediated signaling pathway of apoptosis in MCF-7 cells. This study also validated the use of shrimp carotenoids extract. Breast cancer cells used as a cellular model, which has been established previously for carotenoid research and other anti-proliferative drugs. This study expected to signify the combined effects of hydrocarbon and oxygenated carotenoids as a potential anti-cancer therapeutic mixture.

2. Materials and methods

2.1. Chemicals

Standard β-carotene (98%), lutein (99%), astaxanthin (99%), bovine serum albumin, glutathione reductase (GR), propidium iodide, tetrahydrofuran (THF) (stabilized with 0.25% BHT), tetramethoxypropane (TMP), sodium dodecyl sulfate (SDS), n-butanol, Poly-D- lysine, ethidium bromide (EtBr), acridine orange (AO), 4,6-diamidino-2-phenylindole, dilactate (DAPI), and cell culture grade dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tissue culture plastic ware, cell culture media and reagents, and neutral aluminum oxide (particle size: 70–230 mesh) obtained from Hi-Media Chemical Laboratories (Mumbai, India). All other chemicals and solvents of analytical and HPLC grades purchased from Sisco Research Laboratories (Mumbai, India). FITC Annexin-V apoptosis detection kit purchased from BD pharmingen (BD Bioscience, San Diego, CA). β-actin, Cyclin D1, Bcl-2, and Phospho-p53 (Ser 15) (16G8) primary antibodies purchased from Cell Signaling Technology, Inc. Goat anti-rabbit or anti-mouse IgG-HRP secondary antibodies and western blotting luminolreagent purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CLX-posure™ film (8 × 10 inches) and BCA assay reagent procured from Thermoscientific.

2.2. Preparation of carotenoids

BC and L extracted using acetone from spinach leaves as per procedure established by our laboratory (Lakshminarayana et al., 2005). AST isolated from shrimp (*Penaeus mondon*) samples (meat) obtained from a local market (Bengaluru, India) according to Lin et al. (2005) with slight modification. In the current study, shrimp meat portion (100 g) was homogenized to obtain fine paste using mixer grinder in a cold condition under dim light. A portion of shrimp homogenized sample (10 g) was mixed and soaked in acetone (30 mL) for 5 min, then extracted until the residue became colorless. The pooled acetone extract filtered through Whatman No.1 paper and the filtrate was subjected for saponification to prepare free AST. Since shrimp comprises with mono and di-esters form of AST, we adopted mild saponification (0.2% NaOH for 16 h in a ratio of 5:1, v/v) procedure for the complete hydrolysis of AST esters. Further carotenoids composition in saponified shrimp extract and removal of esters confirmed by HPLC before cell culture treatment. A portion of the saponified sample was taken and partitioned by adding an equal volume of 10% sodium sulfate. The supernatant was separated and dried using N_2 gas and considered as saponified shrimp carotenoids extract (SSCE) for the cells treatment. Likewise, a portion of saponified sample was dried and re-dissolved in a known volume of acetone and subjected to purification of AST on silica gel column.

2.3. Isolation of L, BC, and AST by open column chromatography (OCC)

BC and L from acetone extract of spinach purified through activated neutral alumina column (70–230 mesh size) (Lakshminarayana et al., 2005). AST separated on silica gel column (20 cm × 1.5 cm, particle size: 60–120 mesh, SRL, Mumbai) using specific solvent systems as per Lin et al. (2005) method with slight modifications, especially in the separation of BC and L from shrimp sample. The rich fraction of *trans*-astaxanthin and its *cis*-isomers eluted with hexane-acetone (88:12, v/v). The eluent was dried using N_2 gas and re-dissolved in a known volume of mobile phase and analyzed by HPLC-MS $(APCI)^+$ (Lakshminarayana et al., 2008).

2.4. LC-MS $(APCI)^+$ analysis

The purity of each isolated carotenoid was quantified from the HPLC peak area of respective reference standards. The analysis of carotenoids was done by using RP-HPLC on C_{30} column (5 μm; 250 × 4.6 mm; Princeton, Cranbury, USA). Acetonitrile: methanol: