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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 IC₅₀ 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.

Keywords: Shrimp astaxanthin. β-Carotene. Lutein. Synergism. Cytotoxicity. MCF-7 cells proliferation.
Abbreviations used

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.
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 & 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; 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 & 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 (8X10 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 monodon) 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 minutes, 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 N₂ 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 x 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 N₂ 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 C₃₀ column (5 µm; 250 X 4.6 mm; Princeton, Cranbury, USA). Acetonitrile: methanol: dichloromethane (60:20:20, v/v/v) containing 0.1% of ammonium acetate used as mobile phase. An isocratic condition performed at a flow rate of 1 mL/min and monitored at 450 (BC and L) and 480 (AST) nm. The peak identities and λₘₐₓ of purified carotenoids were confirmed by their retention time, characteristic UV–Visible, and LC–MS (APCI)⁺ mass spectra of reference standards analyzed under similar condition (Lakshminarayana et al., 2008). All the procedures including extraction, purification, standards preparations and their analyses were performed under dim yellow light at 4 °C to prevent isomerization and photo-degradation of carotenoids.

2.5. **Culture conditions and viability assay for MCF-7 cells**

MCF-7 cells procured from NCCS (Pune, India) and cultured as a monolayer in DMEM (4.5 g/L glucose, 4 mM glutamine, 3.7 g/L sodium bicarbonate, and 25 mM sodium pyruvate) supplemented with 10% heat-inactivated FBS and antibiotics (100 U penicillin and 100 µg/mL streptomycin). Likewise, normal breast epithelial cells (MCF-10A, ATCC, USA) were cultured and maintained under the established culture condition (Lee et al. 2015). Cells maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Cells were regularly passaged (twice in a week) after trypsinization (0.25% trypsin containing 0.02% EDTA), and passages between 4-20
considered for conducting experiments. Exponentially, growing cells (70-80%) were seeded (5 x 10^3 cells/well) in 96-well plate supplemented with 200 µL of culture medium and incubated for 24 h. Then, cells treated for dose response study by replenished with 200 µL of serum-free medium consists of different range (10-50 µM) of AST or BC or L separately and incubated for 48 h. In case of time-dependent study, cells treated either with 20 µM (effective dose of cytotoxicity) AST/BC/L or combination of three carotenoids (AST + BC + L) represented as carotenoids mixture (CM) (2.5 µM each as per the combination index value calculated using isobologram equation followed by the viability results of different concentration ranged 1-10 µM of three carotenoids described in section 2.6) or SSCE (12 µg of sample equivalent to 20 µM AST) separately, and incubated up to 72 h. After incubation (dose and time dependence study), cell viability evaluated by MTT assay (Sowmya et al., 2015). The cell viability and toxicity of carotenoids isolated from shrimp and GLVs were compared with the reference standards separately before further cell culture experiments. Carotenoids were delivered either with 0.5% of DMSO (AST) or THF (BC and L). In the case of CM treatment, BC and L dissolved in 0.5% of THF and AST in 0.5% of DMSO. Carotenoid delivered through respective vehicle considered as controls. Based on the cytotoxic results of dose dependent study, an effective dose of 20 µM of each carotenoid selected for other cell-based assays.

The viability of vehicle treated cells (control) without carotenoid represented as 100%. Trypan blue dye exclusion method was performed to count the percent viable and dead cells. Briefly, cells were trypsinized, washed with PBS and quadruplicate hemocytometer counts done for each experiment before and after the treatments. Further, the effect of carotenoids treatments on cytotoxicity in normal breast epithelial cells (MCF-10A) was evaluated. Carotenoids were prepared freshly in a nitrogen environment in a known volume of THF/DMSO. The vehicle solvent is withdrawn from sealed ampoule by a syringe and purged with nitrogen to prevent
oxidation. Carotenoids stored at -80°C between each experiment to minimize oxidation decay. All the preparations were done under dim yellow light and at 4°C to avoid carotenoids degradation.

2.6. Combination index

In the current study, inhibitory concentration of each carotenoid was selected based on the 50% growth inhibition of MCF-7 cells. The IC\textsubscript{50} values of each carotenoid subjected further to screen the synergistic concentration of two carotenoids required to reduce 50% cancer cells proliferation. Based on these, we calculated combination index value, and studied the effect of carotenoid mixtures on cancer cell proliferation as per the following isobologram equation (Cutts et al., 2001). Combination index (CI) = (D1)/(DX1) + (D2)/(DX2), where DX1 and DX2 are the doses of individual carotenoid required to reduce 50% inhibition of cell proliferation, and D1 and D2 are the doses of combined carotenoids treatment that produce the same effect (IC\textsubscript{50}) as in the case of individual carotenoids. From this analysis, combined effects of two carotenoids represented as either additive (or zero), interaction (CI=1), synergism (CI < 1) or antagonism (CI > 1) (Table 1).

Considering the concentration of two carotenoids, we tested the combination of three different carotenoids (AST, BC, and L) concentration (ranges 1 to 10 µM) on cytotoxicity. This treatment resulted in 50% of the reduction in cell numbers at an equimolar ratio of 2.5 µM of each carotenoid. Hence, we have used similar concentration for further evaluation of cell based parameters.

2.7. Cellular uptake of carotenoids

MCF-7 cells (10\textsuperscript{6}) in 75-cm\textsuperscript{2} flasks (n=5) were treated with 20 µM purified carotenoids (AST or BC or L) or SSCE or CM separately. Upon incubation of 48 h, cells were harvested, washed with DMEM, and extracted carotenoid (s) with suitable solvent systems (Sowmya et al., 2015).
Carotenoids content of all the treated cells was quantified by HPLC and expressed after subtracting the content of carotenoids adherent to the cell surface determined at 4 °C (Lin et al., 2007).

2.8. Measurement of glutathione, malondialdehyde, and ROS levels in cells

Cell lysate from cells treated with or without carotenoids (48 h) was prepared and analyzed for glutathione (Tietze, 1969) and malondialdehyde (MDA) levels (Ohkawa et al., 1979). The processing of cell pellet, cell lysate, and other reagent preparations done as described previously (Sowmya et al. 2015). Protein estimation performed according to Lowry’s method (1951). Likewise, cells (1 x 10^5) treated (48 h) either with or without carotenoids were harvested, and co-activated with 2′-7′-dichlorofluorescein-diacetate (DCF-DA) and measured levels of ROS generation using flow cytometer. Cells analyzed by flow cytometry after calibration with negative (unstained) and positive control (H_2O_2) cells. The results expressed as fluorescence intensity of DCF-DA compared with control, treatment, and cells with H_2O_2 (positive control).

2.9. Cell cycle analysis

Cell cycle analyses were done using flow cytometry equipped with CELLQuest Pro software (FACS Calibur, Becton Dickinson, Mountain View, CA). In brief, cells (10^6) treated with or without carotenoids for 48 h washed and rinsed with PBS, detached with trypsin-EDTA at room temperature and centrifuged at 3000 rpm for 5 min at 4 °C. Then, cells were washed again with PBS twice, re-suspended in 1.0 mL of ice-cold hypotonic solution (0.1% Triton X-100, 0.1% citrate buffer, 0.1 mg/mL RNase, 50 µg/mL propidium iodide), incubated for 15 min and DNA content of the cell suspension was analyzed. Cell debris, doublets, sub-G1 population, and aggregates were gated out using a two-parameter plot of FL-2-Area to FL-2-Width of PI fluorescence. Forward and side light scatter (FSC and SSC, respectively) detectors and the three fluorescence channels (FL-1, FL-2, and FL-3) set on a linear scale. The acquired (20,000 events)
relative proportions of cells with different phases (Go–G1, S, G2/M) of the cell cycle analyzed by using flow jo software. The cell population (%) at a particular phase estimated by using Dean Jet Fox analysis software. Effect of carotenoids and their respective vehicles on percent cell death measured by propidium iodide staining before the cell cycle analysis.

2.10. **Apoptosis detection**

Apoptosis detection performed with FITC Annexin V apoptosis detection kit (BD pharmingen, BD Bioscience, San Diego, CA) according to manufacturer’s instructions. Briefly, after 48 h of incubation with or without carotenoids, cells were harvested and washed with the ice-cold PBS and centrifuged at 3000 rpm for 5 min at 4 °C. The cells grown as a monolayer included both harvested by trypsinization and floating in the medium. The cell pellet suspended in an ice-cold binding buffer, followed by addition of 1 µL/mL FITC Annexin V and 10 µL/mL PI solutions. Sample tubes incubated for 15 min in the dark at room temperature before flow cytometry analyses.

2.11. **Determination of cell and nuclear morphological changes**

Cells (2 x10^4) were seeded in 6 well plates either treated with or without carotenoids for 48 h. After incubation, cells were washed with PBS and observed under the inverted microscope (CKX-41, Inverted Microscope, Olympus Corporation, Japan). PBS-washed cells fixed with methanol, stained with crystal violet, cell morphology, and their changes observed and documented. Similarly, for nuclear analysis, PBS-washed cells fixed with 4% paraformaldehyde for 10 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min, and incubated with DAPI (1 µg/mL) for 5 min at room temperature. The apoptotic nuclei stained densely with DAPI (excitation with 359 nm and emission at 461 nm wavelength) viewed under a fluorescent microscope (CKX-41, Inverted Trinocular Fluorescence Microscope, Olympus Corporation,
Images were analyzed using Q-Imaging MP3.3 cooled color camera with Q-Capture Pro7 imaging software (Canada).

2.12. *Apoptosis evaluation by acridine orange and ethidium bromide staining*

The effect of individual and combination of carotenoids on MCF-7 cell death determined using acridine orange and ethidium bromide (AO/EtBr) staining. Cells seeded on poly-L-lysine (0.01%) coated cover slip and kept in the CO\(_2\) incubator for 24 h, then AST or BC or L or CM of them or SSCE were treated separately and continued the incubation for 48 h. After incubation, the cell monolayer was washed twice with PBS, followed by cells stained with 10 µL of dye mixture (100 µg/mL of AO and 100 µg/mL of EtBr) in PBS for 2-3 min. The effect of individual carotenoid or carotenoid mixtures or saponified shrimp carotenoids extract on cellular and morphological changes were documented by fluorescence microscope with excitation/emission filters (360/590 nm for EtBr and 480/510 nm for AO) exposed 5500 milliseconds under 200X magnification.

2.13. *Western blot analysis of cyclin D1, phospho-p53, Bax, and Bcl-2 expression*

Cells (10 x 10\(^6\)) treated either with or without AST, BC, L, their combination CM or SSCE were harvested and washed twice with ice-cold PBS and gently lysed in ice-cold lysis buffer for 30 min, then centrifuged (10,000 rpm) for 10 min at 4°C to obtain the supernatant. The total protein content estimated using BCA assay. In each sample, 30 µg of total protein was loaded per lane for separation in 10% polyacrylamide gel and then transferred electrophoretically to a nitrocellulose membrane. After blocking for one hour with blocking buffer, the membranes were incubated overnight at 4°C with antibodies against β-actin (loading control) Cyclin D1 or Bcl-2 or Bax or Phospho-p53. The primary antibodies detected by using HRP labeled goat anti-rabbit or anti-mouse immunoglobulin G secondary antibodies. A chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA) was used to visualize protein bands. In each experiment, three X-
ray film exposures (10 to 15 min) of the same blot were scanned and calculated the expression levels of proteins by using densitometry analysis.

2.14. **Statistical analysis**

Values are mean ± SD of five samples. Data tested for homogeneity of variances by the Bartlett test. When homogenous variances confirmed, the data were further analyzed by using ANOVA (Assistat software, v.7.7). The difference between carotenoids and vehicle control treated group was analyzed by Tukey’s test. The differences between the experimental samples were considered significant levels at $p < 0.05$.

3. **Results**

3.1. **Influence of AST from shrimp with BC and L from spinach on cytotoxicity and cell death in MCF-7 cells**

Carotenoids isolated from spinach and shrimp were identified and confirmed based on their $\lambda_{\text{max}}$, retention time, characteristic UV–Visible, and mass spectra. Mass spectra of AST, BC, and L were compared with their respective reference standard and upon confirmation, used for cell culture treatments. The purity of column purified BC, L and AST were 97 ± 1, 96 ± 3, and 94 ± 2%, respectively. The results show that the levels of BC and L in spinach were 37 ± 5 and 78 ± 2 (µg/g dry weight) while AST in shrimp was 50 ± 2 µg/g dry weight, respectively. Whereas, SSCE comprises, AST (77.2%), BC (3.3%) and lutein + zeaxanthin (19%) (Since, lutein and zeaxanthin have an identical chemical formula and are isomers, the content represented as L+Z). Cell viability/toxicity levels between purified and standard carotenoids were comparable, and there is no significant difference in treated cultures. MCF-7 cells were treated with increasing concentration of carotenoids (10–50 µM) and examined cell viability. Carotenoids drastically decreased the cell viability in a dose-dependent manner after 48 h of incubation (Fig. 2a). The
carotenoids (10–50 µM) delivered through suitable vehicle reduced cell viability from 78 to 42 (BC), 89 to 48.2 (L), and 62 to 32% (AST), respectively (Fig. 2a). The influence of carotenoids treatments on cell viability at different time intervals (12-72 h) shown in Fig. 2b. The viability of MCF-7 cells treated for 48 h with BC, L, AST, SSCE, or CM reduced significantly by 52, 44.8, 59.9, 62 and 75% than control, respectively. Interestingly, CM (57.1% dead cells) and SSCE (55%) effectively ($p<0.05$) reduced the cell viability than individual carotenoid over 48 h exposure as observed by trypan blue dye exclusion method (Fig. 2c). These results indicate that AST co-treated with BC and L decreased cell viability ($p>0.05$) compared to other groups. No significant difference in cell number was found between untreated and vehicle control, suggesting that DMSO or THF (0.5%) did not affect cell growth. Carotenoid’s concentration required for inhibition (IC$_{50}$) of MCF-7 cells in 48 h were 20 ± 0.2 (AST) 35 ± 0.5 (BC) and 40 ± 0.4 µM (L) respectively. The CI results demonstrated that the combination of AST with BC or L, and BC with L in MCF-7 cells is much lower than one (Table 1). It is evident from the result that at physiological concentration (2.5 µM) of carotenoids studied in combination is more highly effective than individual carotenoid at higher than physiological concentration. The higher inhibition of cancer cell growth by a mixture of carotenoids may be due to their synergistic action.

The cellular uptake of carotenoids treated either with individual carotenoid or CM or SSCE was in the order of AST > BC > L (Fig 3a). The levels of glutathione reduced in cells treated (48h) with individual carotenoid, CM, and SSCE than control. The glutathione level in CM treated group was lower by 16.3, 26.4, 43.6 and 55.4% than the SSCE, AST, L and BC groups, respectively (Fig. 3b). Whereas, in the case of lipid peroxides, MDA levels increased in carotenoids treated cells compared to control cells. CM treated cells showed higher MDA levels (21.9, 35.1, 46.9 and 59.4%) than SSCE, AST, BC and L treated cells (Fig. 3b). The values of glutathione and MDA levels represented after subtracting the control values unless otherwise mentioned. Also, CM
exhibited higher levels of ROS generation than SSCE, individual carotenoid’s, and control treated cells indicating the higher toxic effect of CM (Fig. 4).

3.2. Influence of mixture of AST from shrimp with BC and L from spinach on cell cycle progression and induction of apoptosis in MCF-7 cells

To elucidate the possible mechanism(s) responsible for the suppressed cell viability, we examined the reduction of cell number and its association with cytostatic effect and changes in cell cycle progression. In control group, most of the cells accumulated in S-phase due to the higher proliferation than the treated groups (Table 2). In the case of carotenoids treated groups, a net increase in the percentage of cells in G0/G1 phase accompanied with the corresponding decline in the S and G2/M phase. In the case of cells treated with CM, percent level of S phase was decreased significantly than control, followed by a net increase at G0/G1 phase. A similar trend observed in other carotenoid treatments which found to be in the order of SSCE > AST > BC > L. Also, induction of apoptosis confirmed by using FITC Annexin V and PI staining. The relative % of apoptotic cells in carotenoid treated groups is given in Table 2. The % apoptotic cells were higher in CM treated cells (21.3%) than SSCE (17.5%), AST (13.6%), BC (10.3%) and L (7.2%), respectively. Further, the apoptosis induction confirmed by acridine orange and ethidium bromide staining (AO/EtBr staining). Cells were stained with fluorescence green, yellow and reddish/orange represented the cell viability, early apoptosis, and late apoptosis, respectively (Fig. 5). Cells treated with carotenoids displayed changes in morphology, including chromatin condensation, membrane blebbing, and fragmented nuclei. In specific, cells treated at a lower concentration of AST with BC and L exhibited late stage apoptosis compared to SSCE.

Effect of a mixture of carotenoids on cell cycle progression was studied by measuring the expression of G0/G1 phase-related cyclin D1 and apoptosis-regulating proteins. The expression of phospho-p53 and Bax as apoptosis promoter and Bcl-2 as a suppressor of programmed cell death
were measured by western blotting (Fig. 6). Cells treated with individual carotenoid, or CM or SSCE affected the anti- and pro-apoptotic protein expression. Increased phospho-p53 and Bax expression by AST (53.6, 54.5%), BC (45.8, 54.5%), L (35, 50%), SSCE (75, 66.6%) and CM (74 and 69.7%) were evident compared to control. Likewise, cyclin D1 and Bcl-2 expression were decreased by 41.2 and 56.2 (AST), 5.8 and 18.7 (BC), 23.6 and 15.6 (L), 52.9 and 34.4 (SSCE) and 58.8 and 56.2% (CM) respectively compared to control (Fig. 6).

Further, treatment of individual carotenoid or CM or SSCE did not affect the morphology, cell proliferation, oxidative status, cell cycle regulation, and induction of apoptosis in normal breast epithelial cells (MCF-10A) (Fig. 7 & 8, Table 3).

4. Discussion

This study evaluated the antiproliferative activity of shrimp carotenoid (AST) with co-treatment of BC and L from spinach in MCF-7 cells. At a physiological concentration, a mixture of AST, BC, and L found to exhibit a higher anti-proliferative effect in MCF-7 cells as compared to individual carotenoids at higher doses signifying synergetic effect. Earlier, antitumor effect of AST (Chew et al. 1999) and L (Chew et al. 2003) have been reported in BALB/c mice. Available studies attributed cytotoxic effect and induction of apoptosis by individual carotenoids including AST in various cancer cell lines (Chew et al., 1999; Niranjana et al., 2015). Also, BC and canthaxanthin have shown to inhibit UV and X-ray induced transformation of C3H10T1/2 cells proliferation (Kennedy & Krinsky, 1994). Apart from the distinct inhibitory effect of carotenoids on cancer cells, Yang et al. (2014) demonstrated the synergistic effect of sorafenib and all-trans retinoic acid on connexin-dependent gap junction in human hepatocellular carcinoma cell lines. Likewise, Linnewiel-Hermoni et al. (2015) showed a synergetic influence of lycopene, phytoene, and phytofluene with polyphenols and vitamin D at physiological concentration against prostate and breast cancer cells proliferation. In this study, we have shown a dose and time dependent
cytotoxicity of carotenoids combination in MCF-7 cells. Further, results show that the level of apoptosis is significantly higher in cells treated with CM, followed by SSCE, AST, BC, and L. As noted previously (Lakshminarayana et al., 2013), under the experimental conditions adopted in this study, individual carotenoids or their combination is unlikely to affect the normal cells. Further, cellular uptake of carotenoid is positively associated with their cytotoxicity. Cellular uptake of AST is higher than other carotenoids, which may be due to difference in their structure, polarity, and solubility (van den Berg, 1999). Further in the present study, a strong relationship between carotenoids accumulation, cytotoxicity, and cell proliferation is evident. It is possible that cell growth modality, metabolic status and membrane composition, may influence carotenoid accumulation depending on cell type (Grolier et al., 1992; Kennedy & Krinsky, 1994; Palozza et al., 2004). Earlier, Palozza et al. (1998) also showed a correlation between the cellular level of canthaxanthin and proliferation in WiDr and MEL-2 cells. Kennedy and Krinsky (1994) have revealed that transformed cells reached maximum canthaxanthin uptake within 24 h of carotenoid exposure.

We observed significantly decreased glutathione levels in MCF-7 cells treated with carotenoids in the following order; CM > SSCE > AST > L > BC. We believe that reduced glutathione levels may induce apoptosis. Since glutathione serves as an endogenous antioxidant, its reduced levels mediate apoptosis in breast cancer cells (Franco & Cidlowski, 2009). The increased MDA levels observed in the CM-treated group compared to SSCE, AST, BC, and L groups. Cytotoxicity and apoptosis could be due to increase oxidative stress in cancer cells as indicated by an elevated MDA and depleted glutathione levels (Lakshminarayana et al., 2013). An increased ROS level in CM group than SSCE, individual carotenoids, and control supports the above results (Fig. 3 & 4). Oxidative stress plays an important role in the induction of apoptosis (Sen & Packer, 1996). The elevated oxidative stress in CM treated cells may be also due to the lower carotenoids concentration under higher cellular oxygen stress/tension, and different
localization of carotenoids in the bio-membranes (Fig. 9). Carotenoids behave differently as they are incorporated into cell membranes differently which is reported to alter the ability of carotenoid interaction with ROS or other antioxidants (Agamey et al., 2004).

Based on the results, we presumed that equimolar concentration of carotenoids mixtures used in this study synergistically enhanced the pro-oxidant activity over their antioxidant mechanism in cancer cells. Furthermore, the present results strongly suggest that the efficiency of carotenoids is higher when co-ingested than individual carotenoids. Further, the differential structural characteristics (cyclic or acyclic, functional end groups, redox properties), oxidation or cleavage products, nature of radical species, reactive mechanism, and their aqueous or lipid environment may also be one of the reasons for higher activity of mixture of carotenoids (Agamey et al., 2004; Lakshminarayana et al., 2013).

The antioxidant or pro-oxidant actions of carotenoids reported in the biological system depend on their concentration and oxygen tension (Burton & Ingold, 1984; Palozza et al., 2004). Palozza et al. (1998) demonstrated that the pro-oxidant activity of canthaxanthin predominates over the antioxidant mechanism owing to generation of chain carrying peroxyl radicals, responsible for an induction of apoptosis. Increased metabolic rate associated with higher oxidative stress; such levels are less harmful to cancer cells. Cancer cells can resist an optimal degree of increased ROS by adopting a new redox balance. In contrast to oxidative stress mediating tumor growth, it can also increase the sensitivity to treatment (Gorrini et al., 2013). Also, higher ROS level in cancer cells may overthrow the endogenous antioxidant capability that leads to oxidative damage-driven death. The current study, address the effect of individual and mixtures of carotenoids on the possibility of ROS-mediated oxidative induced cell death (Fig. 9). Furthermore, the anti-proliferative activity of carotenoids is not only related to their antioxidant or pro-oxidant properties but also due to their effect on signaling pathway (Palozza et al., 2004; Lakshminarayana et al., 2013; Arathi et al., 2015). In the present study, cells treated with CM at
equimolar ratios of low concentration resulted in higher ROS generation than other treated groups. Based on the results, we postulate that carotenoids are highly reactive with ROS (imbalanced at low antioxidants Vs higher oxygen stress) that may lead to the formation of carotenoid peroxyl radicals which further initiates oxidation propensities to increased oxidative stress (Fig. 4 & 9).

Carotenoids involved in cell cycle arrest, resulting in an increase in G0/G1 phase accumulation (Upadhyaya et al., 2007). These results were in agreement with the earlier reports with other carotenoids, where, lycopene and vitamin D3 synergistically blocked the G0/G1 phase of the cell cycle (Amir et al., 1999). We found G0/G1 accumulation accompanied with a reduction in the percentage of cells in S and G2/M phase and induction of apoptosis in carotenoids treated cells. The results revealed higher apoptosis in cells delivered with AST in combination with BC and L than other treated groups. Paolini et al. (1999) have suggested that BC at higher concentration induce oxidative stress by increasing cytochrome p450 enzymes. In contrast to our observation, Li, Wang and Mo. (2002) ascribed that AST is less efficient than BC and lycopene in the proliferation of human breast cancer cells. However, we show a pronounced antiproliferative influence of shrimp AST when co-treated with GLVs BC and L than individual carotenoid treatment. SSCE also efficiently induced cell cycle arrest and apoptosis next to the CM treatment indicating a synergistic influence of carotenoids. The higher apoptosis of cells by shrimps extract is due to co-existence of other carotenoids in minor quantity along with AST than individual carotenoids. Furthermore, the increased cellular uptake and oxidative stress in cells treated either with AST or AST with BC and L could also be a reason for apoptotic effect (Table 2 & Fig. 4). Hence, in this study apoptosis induction is associated with increased levels of ROS in CM treated cells than other treatments (Fig. 4 & 9).

In the case of normal MCF-10A breast epithelial cells, CM or SSCE did not affect the proliferation (Fig. 7 & 8, Table 3), indicating that individual carotenoid or combination of those at physiological concentration selectively kills MCF-7 cells (Fig. 1-6). These results were further
validated in another breast cancer cell line (MDA-MB-231 cells) to rule out the possibility of cell line specific activity and establish generality. We observed the similar trend of results in MDA-MB-231 cells as in the case of MCF-7 cells; however levels of cytotoxicity, MDA, GSH and ROS generation varied in MDA-MB-231 cells (Fig. S1 & S2). It is evident that MCF-7 cells are sensitive to carotenoids treatments particularly CM treated group as compared to MDA-MB-231 cells. These observations indicate selective killing of carotenoids on cancer cells but not normal cells, this may be due to balanced oxidative defence mechanism in normal cells compared to distorted redox mechanism in cancer cells. Hence, we observed the balanced levels of glutathione, MDA and ROS in the normal MCF-10A breast epithelial cells as compared to cancer cells (Fig. 7c & d). Generally, normal cells have much lower oxidative stress and balanced endogenous antioxidant system when compared to cancerous cells (Van der Paal et al., 2016). Increasing oxidative stress and decreasing endogenous GSH are shown to be toxic to tumor cells (Weydert et al., 2008). These results provide basic information about the synergistic action of carotenoids for the first time to the best of our knowledge. However, insight on the molecular level why carotenoids selectively target cancer cells but not normal cells needs to be addressed.

Furthermore, the upregulation of phospho-p53, Bax and downregulation of cyclin D1 and Bcl-2 supports the synergism of AST co-treatments with BC and L. Further, the cell cycle arrest at the G0/G1 phase accompanied with a down-regulation of cyclin D1 (Fig 6). Carotenoids have been reported to inhibit tumor cell growth by suppressing the cyclin D1 expression, which results in arresting cell cycle progression at G0/G1 phase (Palozza et al., 1998; Amir et al., 1999). Also, expression of anti-apoptotic gene Bcl-2 decreased significantly in cells treated with carotenoid mixtures and AST compared to SSCE (1.6 fold) and other treatment groups (≥3 fold). The down-regulation of anti-apoptotic protein Bcl-2 may be due to dysregulation in an intricate network of apoptotic signaling in cancer cells (Reed, 1995). In contrast to Bcl-2 expression, pro-apoptotic gene phospho-p53 expression found higher in SSCE than carotenoid mixtures (1 fold) and other
treatment groups (>2.6 fold). Similarly, Bax expression is higher in cells treated CM than SSCE (1.15 fold) and other treatment groups (>1.9 fold). The reflection of expression levels of phospho-p53 and Bax postulated due to DNA damage and other biochemical events such as cellular stress, p53 protein stabilized and activated via post-transcriptional modifications including phosphorylation (Sakaguchi et al., 1998). It is noted that an inverse relationship exists between p53 and Bcl-2 levels in breast tumors and also demonstrated the ability of p53 to down-regulate Bcl-2 in a panel of breast cancer cell lines (Reed, 1995; Yang & Korsmeyer, 1996). The present finding clearly demonstrates that AST in combination with BC and L on the Bcl-2/p53 functional interface represents a vital role of CM on the regulation of apoptosis.

Overall, results demonstrated that SSCE rich in AST induce apoptosis strongly in MCF-7 cells as compared to individual BC and L treatment. Dietary consumption of shrimp provides majorly mono- and di-ester forms of AST. AST esters found to enhance the cellular uptake of AST compared to free AST. Though consumption of shrimp provides AST esters while absorption the esters are hydrolyzed by esterase and lipases. Therefore, carotenoids esters are not detected in blood and tissues (Chitchumroonchokchaï & Failla, 2006). Hence, we have chosen shrimp as a main source of AST and studied its synergistic influence with BC and L against cell proliferation at the molecular level. We conclude that co-consumption of AST with BC and L-rich in dark leafy vegetables like spinach may render higher beneficial effect as shown in this study. Shrimp is also considered as a rich source of essential ω-3 PUFA and found to be suitable dietary lipids to help in solubilization, micellization, and carotenoids bioavailability (Raju et al., 2006). Furthermore, dietary intervention studies need to be conducted to validate the synergistic health benefits of carotenoids in the real biological complex situation. However, the interaction of carotenoids with other phyto-components and among structurally different carotenoids from an intraspecific food source, and their synergistic influence needs to be detailed. We conclude that AST, a keto-carotenoid from shrimp potentiate anti-proliferation of MCF-7 cells when co-treated with other
carotenoids of green leafy origin (BC and L). In continuation, results of dietary studies with optimized food composition are further required to enhance health benefits.

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Conflict of interest
The authors declare no competing financial interest.

References


Figure captions

Fig. 1 - Structure of carotenoids used in the present study

Fig. 2 - Influence of carotenoids treatment on MCF-7 cell viability.

(a) Effect of different concentration of carotenoids (AST or BC or L) on cell viability of MCF-7 cells treated for 48 h. Values are mean ± SD ($n = 5$). Values not sharing common superscript letters are significantly different ($p < 0.05$) between treatments, as analyzed by two-way ANOVA followed by Tukey’s test.

(b) Effect of 20 µM of purified carotenoids (AST or BC or L) or CM (2.5 µM of AST, BC, and L) or SSCE on MCF-7 cell viability. The cells exposed to with or without carotenoids for different time points (12-72 h). Values are mean ± SD ($n = 5$). Values not sharing common superscript
letters are significantly different ($p<0.05$) between treatments, as analyzed by two-way ANOVA followed by Tukey’s test.

(c) Effect of 20 µM of purified carotenoids (AST or BC or L) or CM (2.5 µM of AST, BC, and L) or SSCE or without carotenoids on percent cell viability of MCF-7 cells treated for 48 h. CN, control; AST, astaxanthin; BC, β-carotene; L, lutein; CM, carotenoid mixtures; SSCE, saponified shrimp carotenoids extract.

**Fig. 3** The cellular uptake of carotenoids-(a) and their influence on glutathione and malondialdehyde levels (b) in cells treated either with individual carotenoid (20 µM of AST or BC or L) or CM (2.5 µM of AST, BC and L) or SSCE for 48 h. Values are mean ± SD ($n = 5$). Values not sharing common superscript letters are significantly different ($p<0.05$) between treatments, as analyzed by ANOVA followed by Tukey’s test. CN, control; AST, astaxanthin; BC, β-carotene; L, lutein; CM, carotenoid mixtures; SSCE, saponified shrimp carotenoids extract.

**Fig. 4** - Flow cytometry analysis showing levels of intracellular ROS generation in MCF-7 cells treated either with or without individual carotenoid (20 µM of AST or BC or L) or CM (2.5 µM of AST, BC and L) or SSCE using DCF-DA assay. The cells treated with $H_2O_2$ (ROS, 72.4%) considered as positive control (data not shown). Values are mean ± SD of three samples. CN, control; AST, astaxanthin; BC, β-carotene; L, lutein; CM, carotenoid mixtures; SSCE, saponified shrimp carotenoids extract.

**Fig. 5** - The typical morphological changes and nuclear condensations in the MCF-7 cells treated with or without carotenoids. (a & b) Cells stained with and without crystal violet observed under phase contrast microscope (200×). The number of cells decreased after carotenoids treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing. (c) Apoptosis induction confirmed by AO/EtBr staining. Cells stained with fluorescence green, yellow and reddish/orange
represented viability, early apoptosis, and late apoptosis, respectively. (d) The nuclei were stained with DAPI and observed chromatin condensation under a fluorescence microscope (200×). Typical apoptotic cells indicated by arrows. These are representative results of at least three independent experiments (n = 3). CN, control; AST, astaxanthin; BC, β-carotene; L, lutein; CM, carotenoid mixtures; SSCE, saponified shrimp carotenoids extract. Note: The microscopic images for AO/EB and DAPI are different with the same cell lineages.

Fig. 6 - Western blot analyses of cyclin D1, Bcl-2, Bax, and phospho-p53 in the MCF-7 control and experimental groups. (a) Western blot analyses performed with antibodies against Bcl-2, phospho-p53 and β-actin as mentioned in materials and methods. Lane 1-Control, Lane 2-BC, Lane 3-L, Lane 4-AST, Lane 5-CM (2.5 µM of AST, BC, and L) and Lane 6-SSCE. (b) Quantitative data expressing the corresponding protein levels was assessed using densitometry and expressed in relative density. Values are mean ± SD (n = 3). Values not sharing common superscript letters are significantly different (p < 0.05) between treatments, as analyzed by one-way ANOVA followed by Tukey’s test. CN, control; AST, astaxanthin; BC, β-carotene; L, lutein; CM, carotenoid mixtures; SSCE, saponified shrimp carotenoids extract.

Fig. 7 - Effect of 20 µM of purified carotenoids (AST or BC or L) or CM (2.5 µM of AST, BC, and L) or SSCE on cytotoxicity and cell viability (a & b), GSH and MDA levels (c) and intracellular ROS generation (d) in MCF-10A cells. Values are mean ± SD (n = 5). Values not sharing common superscript letters are significantly different (p<0.05) between treatments, as analyzed by ANOVA followed by Tukey’s test. CN, control; AST, astaxanthin; BC, β-carotene; L, lutein; CM, carotenoid mixtures; SSCE, saponified shrimp carotenoids extract.
Fig. 8 - The typical morphological changes and nuclear condensations in the MCF-10A cells treated with or without carotenoids. Refer details of treatment groups and morphological features as described in Fig. 5.

Fig. 9 - A scheme of possible synergistic influence of carotenoid mixtures on cytotoxicity and apoptogenic properties in MCF-7 cells.
Table 1 - Combination Index values for the inhibition of MCF-7 cells growth by combinations of carotenoids.

<table>
<thead>
<tr>
<th>Carotenoids (1) + (2)</th>
<th>DX1 (µM)</th>
<th>DX2 (µM)</th>
<th>D1 (µM)</th>
<th>D2 (µM)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST+BC</td>
<td>20</td>
<td>35</td>
<td>5</td>
<td>7</td>
<td>0.45</td>
</tr>
<tr>
<td>AST+L</td>
<td>20</td>
<td>40</td>
<td>5</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>BC+L</td>
<td>35</td>
<td>40</td>
<td>7</td>
<td>10</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^{\text{Y}}\text{DX1 and DX2-IC}_{50}\text{ values of carotenoids in growth inhibition of MCF-7 cells are 20 ± 0.2, 35 ± 0.5 and 40 ± 0.4 µM for AST, BC and L, respectively. Combination index (CI) = (D1)/(DX1) + (D2)/(DX2), where DX1 and DX2 are the doses of individual carotenoids required to produce an effect (IC}_{50}\text{, and D1 and D2 are the doses of combined carotenoids treatment that produce the same effect (IC}_{50}\text{ as in the case of individual carotenoids. From this analysis, combined effects of two carotenoids assessed as either additive (or zero) interaction (CI=1), synergism (CI < 1) or antagonism (CI > 1). AST, astaxanthin; BC, β-carotene; L, lutein.}


Table 2 - Effect of carotenoids on cell cycle distribution and induction of apoptosis in MCF-7 cells.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Cell cycle distribution (%)</th>
<th>% Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G_0/G_1$</td>
<td>$S$</td>
</tr>
<tr>
<td>CN</td>
<td>$49.1 \pm 0.5^a$</td>
<td>$25.24 \pm 1.8^c$</td>
</tr>
<tr>
<td>BC*</td>
<td>$53.73 \pm 1.0^b$</td>
<td>$16.53 \pm 0.2^b$</td>
</tr>
<tr>
<td>L*</td>
<td>$50.96 \pm 0.7^a$</td>
<td>$20.15 \pm 0.8^a$</td>
</tr>
<tr>
<td>AST*</td>
<td>$55.64 \pm 1.5^c$</td>
<td>$17.1 \pm 2.2^b$</td>
</tr>
<tr>
<td>CM**</td>
<td>$58.25 \pm 0.6^d$</td>
<td>$10.03 \pm 1.2^a$</td>
</tr>
<tr>
<td>SSCE***</td>
<td>$55.76 \pm 1.6^c$</td>
<td>$17.1 \pm 0.5^b$</td>
</tr>
</tbody>
</table>

*20 µM of individual carotenoid. **CM prepared by using equimolar concentration (2.5 µM of AST, BC, and L) of OCC purified BC and L from GLVs and AST from shrimp. ***The SSCE consists of AST (77.2%), BC (3.3%) L + Zeaxanthin (19%). The values are means ± of SD of three experiments. Values not sharing a common superscript letter within a column under each treatment are significantly different from control at $p < 0.05$. Note: CN, control; CM, carotenoid mixtures; SSCE, saponified shrimp carotenoids extract.
Table 3 - Effect of carotenoids on cell cycle distribution and induction of apoptosis in MCF-10A cells.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Cell cycle distribution (%)</th>
<th>% Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Go/G1</td>
<td>S</td>
</tr>
<tr>
<td>CN</td>
<td>73.7 ± 0.6a</td>
<td>18.1 ± 1.9a</td>
</tr>
<tr>
<td>BC*</td>
<td>72.9 ± 1.2a</td>
<td>17.4 ± 0.9a</td>
</tr>
<tr>
<td>L*</td>
<td>74.8 ± 0.7a</td>
<td>15.5 ± 1.8a</td>
</tr>
<tr>
<td>AST*</td>
<td>71.4 ± 1.5a</td>
<td>20.6 ± 2.1ab</td>
</tr>
<tr>
<td>CM**</td>
<td>72.0 ± 1.6a</td>
<td>19.3 ± 1.3a</td>
</tr>
<tr>
<td>SSCE***</td>
<td>73.3 ± 1.6a</td>
<td>17.1 ± 0.5a</td>
</tr>
</tbody>
</table>

a Refer details of treatment groups and number of samples as described in Table 2.
Fig. 1

- **Astaxanthin (Keto-carotenoid) M. W. 596.85**
- **β-Carotene (Hydrocarbon carotenoid) M. W. 536.89**
- **Lutein (Hydroxyl carotenoid) M. W. 568.87**
Fig. 2

(a) Cell viability (%) vs. Concentration (µM)

(b) Cell viability (%) vs. Incubation time (h)

(c) Percent cells: Viable cells vs. Dead cells

Treatments: CN, BC, L, AST, CM, SSCE
Fig. 3

(a) Cellular uptake (p moles/10⁶ cells)

(b) MDA/GSH (n moles/mg protein)
Fig. 4

- CN: 17.3%
- BC: 34.8%
- L: 26.8%
- AST: 53.3%
- CM: 68.1%
- SSCE: 64.7%
Fig. 5

(a) Phase contrast staining
(b) Crystal violet staining
(c) EtBr/AO staining
(d) DAPI staining

<table>
<thead>
<tr>
<th></th>
<th>CN</th>
<th>BC</th>
<th>L</th>
<th>AST</th>
<th>CM</th>
<th>SSCE</th>
</tr>
</thead>
</table>

- CN
- BC
- L
- AST
- CM
- SSCE
Fig. 6

(a) Western blot analysis showing protein expression levels of Cyclin D1 (34 KDa), Phospho-p53 (53 KDa), Bcl-2 (25 KDa), Bax (20 KDa), and β-actin (42 KDa).

(b) Bar graph demonstrating the relative density of Cyclin D1, Bcl-2, Bax, and Phospho-p53 under different treatments (Control, BC, L, AST, CM, SSCE). The treatments are compared, with letters indicating significant differences (a, b, c, d, e).
Fig. 7

(a) CN, BC, L, AST, CM, SSCE

(b) Viable cells: CN, BC, L, AST, CM, SSCE

(c) MDA/GSH (n moles/mg protein)

(d) SSC-A DCF-DA

43
Fig. 8
Fig. 9

*Synergistic interaction increases cancer cells sensitivity to oxidative stress

IC$_{50}$ and Combination Index of carotenoids

Influence of astaxanthin with β-carotene and lutein

Efficient order of cell proliferation inhibition
(carotenoids mixture > shrimp carotenoids > astaxanthin > β-carotene > Lutein)

* Equimolar ratio’s of 2.5 µM of carotenoids combinations
Highlights

- Cytotoxic effect of astaxanthin with β-carotene and lutein is superior as compared to individual carotenoid in MCF-7 cells.
- Carotenoids mixture/shrimp carotenoids modulate apoptogenic properties by increasing ROS with depleted GSH and higher MDA levels.
- Carotenoid mixture from shrimp and green leafy vegetables is advisable to achieve maximum health benefits.