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Research Article

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Evaluation of Anti-Inflammatory and Anti-Proliferative Effect of Hydroxy-, Keto-, and Epoxy-Carotenoids in RAW 264.7 and HL-60 Cells

Kariyappa Vijay, Poorigali Raghavendra-Rao Sowmya, Bangalore Prabhashankar Arathi and Rangaswamy Lakshminarayana*

Department of Biotechnology, Jnana Bharathi Campus, Bangalore University, Bengaluru-560 056, Karnataka, India

***Correspondence to:**
Rangaswamy Lakshminarayana
Department of Biotechnology
Jnana Bharathi Campus, Bangalore University
Bengaluru-560 056, Karnataka, India
Tel: +91-080-22961461
Fax: +91-080-23211020
E-mail: rlnarn21@gmail.com

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Abstract

Aim of the study was to investigate efficiency of hydroxy (lutein, LUT), Keto- (astaxanthin, AST) and epoxy- (fucoxanthin, FUCO) carotenoids on molecular/biochemical events in proliferation of RAW 264.7 and HL-60 cells. Lipopolysaccharide (LPS) induced RAW 264.7 cells treated either with 20 μM of LUT or AST or FUCO or without carotenoid for 12 h, and studied their differential influence on toxicity, inflammatory markers and nuclear factor-κB p65 (NF-κB p65) expression levels. Similarly, HL-60 cells treated for 48 h and evaluated cytotoxicity and apoptosis inducing activity. It was observed, carotenoids treatment decreased cytotoxicity of LPS induced inflammation in macrophages. Among carotenoids, FUCO protects cytotoxicity by 10.7 and 15.8% than AST and LUT, respectively. Likewise, carotenoids treatments reduced Nitric oxide (NO), Prostaglandin E_2 (PGE_2) and NF-κB p65 levels than LPS treated group, while FUCO shown to be superior to control markers of inflammation than AST and LUT. Similarly, in case of HL-60 cells, FUCO significantly reduced cell viability by 16.4 and 33.4% than AST and LUT, respectively. Further, increased apoptosis of HL-60 positively correlates with decreased glutathione, and increased malondialdehyde and oxidative stress in cells treated with FUCO than AST and LUT. Also, typical apoptotic morphological changes were observed in FUCO treated cells than other oxygenated carotenoids. These differences among oxygenated carotenoids may be due to functional group and reactivity with the cells. Further, we presumed that an increase in the number of oxygen or hydroxyl groups in the carotenoids may decide the bioactivity. This study provides a greater insight of oxygenated carotenoids to combat chemoprevention of cancers originating from inflammation.

Keywords

Astaxanthin, Lutein, Fucoxanthin, Cytotoxicity, Anti-inflammation, Anti-proliferation

Abbreviations

AST: Astaxanthin; AO: Acridine orange; DMEM: Dulbecco's modified eagle's medium; DMSO: Dimethyl sulfoxide; DCF: 2'-7'-dichlorofluorescein-diacetate; DTNB: 5, 5-dithiobis (2-nitrobenzoic acid); EtBr: Ethidium bromide; FBS: Fetal bovine serum; FUCO: Fucoxanthin; GR: Glutathione reductase; HRP: Horseradish peroxidase; LUT: Lutein; LPS: Lipopolysaccharide; MDA: Malondialdehyde; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; NAD: Nicotinamide adenine dinucleotide; NADPH: Nicotinamide adenine dinucleotide phosphate oxidase; NO: Nitric oxide; NF-κB p65: Nuclear factor-κB p65; PBS: Phosphate buffered saline; PGE_2: Prostaglandin E_2; PI:

Propidium iodide; ROS: Reactive oxygen species; RPMI 1640: Roswell park memorial institute; TMP: Tetramethoxypropane; LDH: Lactate dehydrogenase

Introduction

Carotenoids are synthesized *de novo* majorly by plants and certain microorganism including algae. In nature, more than 700 carotenoids are found and classified as carotenes (hydrocarbon carotenoids) and xanthophylls (oxygenated carotenoids). Dietary carotenoids, like xanthophylls have gained nutritional significance due of their positive association in reduction of cardiovascular disease, cancer, obesity and age related degenerative diseases. Epidemiological and clinical trials have correlated the consumption of dietary carotenoids and decreased risk of major chronic and non-communicable diseases [1-3]. The xanthophylls consists diverse group of oxygenated carotenoids with varied structural properties. Among xanthophyll carotenoids, lutein, β-cryptoxanthin, capsanthin, astaxanthin, and fucoxanthin are evident as promising molecules. Lutein and itsisomer zeaxanthin are known as macular pigments and involved in protection of ocular health [4]. Astaxanthin is found in crustaceans, certain microalgae and mould, and demonstrated strongest antioxidant molecule than other carotenoids, vitamins and other phytochemicals. Likewise, fucoxanthin another major carotenoid pigment occurs in macro algae and seaweeds have attracted due to its anti-inflammatory and anti-cancer activity [5, 6]. In addition, studies have also demonstrated that, effect of carotenoids may be due to its metabolites [7, 8]. Although, influence of oxygenated carotenoids in different animals and *in vitro* models [9] are available, however efficiency of each carotenoid with different functional group on a single cell model is not been well illustrated. Hence, this study aimed to screen the influence of oxygenated carotenoids such as, lutein (LUT, hydroxy-carotenoid) astaxanthin (AST, keto-carotenoid) and fucoxanthin (FUCO, epoxy-carotenoid) (Figure 1) on biochemical/molecular events of anti-inflammatory response in macrophages (RAW 264.7) and anti-proliferation effects in HL-60 cells.

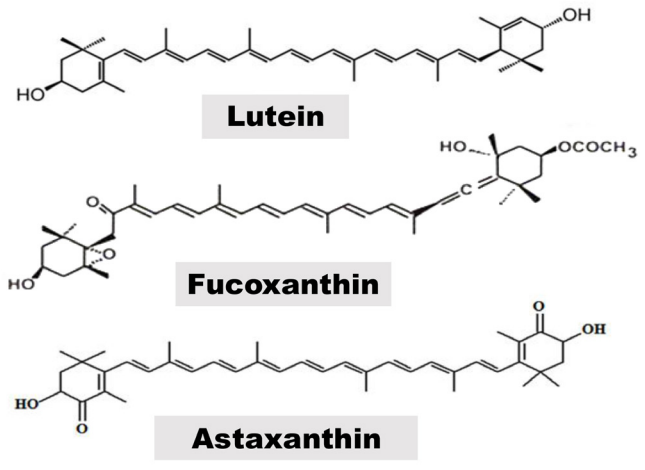

Figure 1: Structure of carotenoids used in the present study.

Materials and Methods

Standard LUT (99%), AST (99%), FUCO (98%), Bovine serum albumin, Glutathione reductase (GR), Propidium iodide, 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). Dulbecco's modified eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 Medium, fetal bovine serum (FBS), 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), antibiotic-antimycotic solution, calcium and magnesium free phosphate buffer saline, trypan blue, cell culture consumables and neutral aluminium oxide (particle size: 70-230 mesh) were obtained from Hi-Media Chemical Laboratories (Mumbai, India). All other chemicals and solvents of analytical and HPLC grades were purchased from Sisco Research Laboratories (Mumbai, India). FITC Annexin-V apoptosis detection kit was purchased from BD pharmingen (BD Bioscience, San Diego, CA). NF-κB p-65, β-actin, goat anti-rabbit/mouse IgG-HRP secondary antibodies and western blotting luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CLX-posureTM film (8 x 10 inches) and BCA assay reagent were procured from Thermo scientific.

Extraction and purification of carotenoids

LUT, a major di-hydroxy carotenoid from green leafy vegetables (spinach), keto-carotenoid, AST from shrimp (*Penaeusmondon*) samples (meat) and FUCO an epoxy-carotenoid from brown seaweeds (*Padina tetrasomatica*) were isolated. LUT was isolated from spinach as per procedure established by our laboratory [10]. Likewise, AST and FUCO were isolated from the respective samples as per the established methods [11, 12] with slight modifications. In brief, shrimp meat portion (100 g) grounded using mixer grinder to obtain fine paste. A portion of homogenized sample (10 g) mixed and soaked in ice-cold acetone (30 mL) for 5 minutes and extracted carotenoid until residue became colourless. The pooled acetone extract filtered through Whatman No.1 paper, and the filtrate was subjected for mild saponification (0.2% NaOH) for 16 h in a ratio of 5:1 (v/v). The saponified sample partitioned by adding an equal volume of 10% sodium sulfate and collected upper phase by repeated extraction. The pooled extract dried under N_2 gas and dissolved in a known volume of acetone and subjected to purification on silica gel column. Similarly, FUCO extracted with acetone/methanol (7:3, v/v) repeatedly till the sample become colourless. The crude extracts evaporated and re-dissolved in methanol/hexane/water (1:1:0.2, v/v/v). The methanol/water phase separated and re-suspended in diethyl ether/water (3:4, v/v), swirled and collected upper diethyl ether phase and subjected further purification. All the process and preparations were done at 4 °C under dim yellow light to minimize photo-oxidation of carotenoids [10].

Open column chromatography

LUT rich fraction eluted by using MeOH/DCM (1:1,v/v) through column chromatography (OCC, 20 cm x 1.5

cm) consists of neutral aluminium oxide (Al_2O_3) (particle size: 70-230 mesh, SRL, Mumbai) [4]. AST separated on silica gel column (20 cm x 1.5 cm, particle size: 60-120 mesh, SRL, Mumbai) using hexane-acetone (88:12, v/v) [11]. Likewise, FUCO rich fraction obtained using hexane/acetone (6:4, v/v) on Al_2O_3 column [13].

LC-MS $(APCI)^+$ analysis

The purity of each isolated carotenoid was quantified from the HPLC peak area of respective standards. The analysis of carotenoids done by using RP-HPLC system consisting of the photodiode array (PDA) detector (SPD-M20A, Prominence, DAD) 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 was used as mobile phase. An isocratic condition was performed at a flow rate of 1 mL/min and monitored at 450 (LUT), 480 (AST) and 445.5 nm (FUCO). The peak identities and λ_{max} of purified carotenoids were confirmed by their retention time, characteristic UV–Vis, and LC–MS $(APCI)^+$ mass spectra of reference standards recorded under similar condition. Further, the purified carotenoids used for the cell culture studies to substitute commercial or synthetic with high cost value [10].

Culture and treatments of RAW 264.7 and HL-60 cells

Cell lines were procured from NCCS (Pune, India). RAW 264.7 cells cultured as monolayer in DMEM and HL-60 cells cultured as a suspension in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U penicillin and 100 µg/mL streptomycin. Cells maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells regularly passaged (twice in a week) and between 4-20 passages considered for experiments. Exponentially growing HL-60 cells (70-80%) seeded (5 x 10^3 cells/ well) in a 96-well plate supplemented with 200 µL of culture medium and incubated for 24 h. Followed, replenish with 200 µL of serum-free media consists of 20 µM carotenoid (LUT or AST or FUCO) or without carotenoid (control) incubated for 48 h. Then, MTT assay performed as per the existing procedure [14]. Similarly, cell viability of LPS (1 µg) stimulated RAW 264.7 cells co-treated with or without 20 µM carotenoid for 12 h [15, 16].

Further, RAW 264.7 and HL-60 cells (2 x 10^4/well) seeded separately in 24-well plates (n = 4) and incubated with or without 20 µM carotenoid (LUT or AST or FUCO) delivered through DMSO or DMSO vehicle alone for 12 and 48 h, respectively. Cells harvested and quadruplicate hemocytometer counts were performed. Trypan blue dye exclusion method used to evaluate the percentage of viable and dead cells. The viability of cells treated DMSO alone (control) considered as 100%. Carotenoids samples prepared freshly in a nitrogen environment in a known volume of DMSO. The vehicle solvent system withdrawn from sealed ampoule by a syringe and purged nitrogen to prevent oxidation. Carotenoids stored at -80 °C between each experiment to minimize oxidation decay.

Lactate dehydrogenase (LDH) assay

LDH release in LPS-stimulated RAW 264.7 cells co-treated with or without carotenoid for 12 h quantitated by using commercial LDH assay kit according to manufacturer's instructions (Lab-care diagnostics Pvt. Ltd. India). Determination of cytotoxicity in experimental samples measured as % LDH release compared with control cells (without LPS treatment). Cells treated with 1% Triton X-100 for same time point served as a positive control of the test system. A value of >5% LDH released into the culture media was considered as cytotoxic.

Nitric oxide (NO) scavenging assay

NO scavenging activity of the different carotenoids done as per the established procedure with slight modification [17]. In brief, RAW 264.7 cells (10^6 cells/mL) seeded on a 24-well tissue culture plates and pre-incubated at 37 °C for 12 h to achieve stable attachment. Further, LPS stimulated cells co-treated with or without carotenoid for 12 h and monitored NO production by measuring nitrite levels in the culture media using Griess Reagent at 540 nm.

Determination of PGE_2 levels

RAW 264.7 cells (10^6 cells/ mL) incubated with LPS and carotenoids for 12 h. The level of PGE_2 in the supernatants of cultured macrophage cell lysate was determined using a competitive enzyme immunoassay kit (Thermo Fisher Scientific, India) according to the manufacturer's protocol. Celecoxib (3 µM) was used as a positive control. The levels of PGE_2 in cells were established according to the regression equation of the standard curve [18].

Western blot analysis of NF-κB p65

RAW 264.7 cells seeded in 100 mm dish (10 x 10^6) and cultured overnight. Then, cells stimulated with LPS (1 µg/mL), and co-treated either with LUT or AST or FUCO or no carotenoid for 12 h. After incubation, cells rinsed in ice-cold PBS twice and harvested by scraping. Cytoplasmic and nuclear proteins separated by using NE-PER Nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, India). The BCA assay was done to estimate total protein content. In each sample, 30 µg of protein loaded per lane for separation in 10% polyacrylamide gel and then transferred electrophoretically on to nitrocellulose membrane. After blocking for 1 h with blocking buffer, the membranes were incubated overnight at 4 °C with antibodies against β-actin (loading control) or NF-κB p65.The primary antibodies detected by using HRP labelled goat anti-rabbit/mouse immunoglobulin G secondary antibodies. A chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA) used to visualize protein bands. In each experiment, three X-ray film exposures (10 to 15 min) of the blot scanned and calculated the levels of proteins expression by densitometry analysis [19].

Measurement of glutathione and malondialdehyde (MDA) levels in HL-60 cells

Cell lysate of HL-60 cells treated with or without carotenoids for 48 h was prepared and analyzed glutathione [20] and MDA levels [21]. The processing of cell pellet, cell lysate, and other reagent preparation were done as per Sowmya et al. [14]. Protein estimation was done according to Lowry et al. [22].

Apoptosis detection by Annexin FITC and PI staining

Apoptosis detection performed with FITC Annexin V apoptosis detection kit (BD pharmingen, BD Bioscience, San Diego, CA) according to manufacturer's instructions. Briefly, after incubation LPS-stimulated RAW 264.7 (12 h) and HL-60 cells (48 h) with and without carotenoids 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 was re-suspended in an ice-cold binding buffer, followed by FITC Annexin V (1 µL/mL) and PI (10 µL/mL) solutions were added. Sample tubes incubated for 15 min in a dark at room temperature before flow cytometry analyses [14].

Measurement of reactive oxygen species (ROS)

LPS-stimulated RAW 264.7 and HL-60 cells (1 x 10^6) treated either with or without LUT, AST and FUCO were harvested and were co-activated with 2'-7'-dichlorofluorescein-diacetate (DCF) for 30 min. DCF intensity was measured using the fluorescence spectrophotometer (excitation 484 nm/emission 531 nm) [23].

Morphological evaluation by AO/EtBr staining

The effects of LUT, AST and FUCO on LPS-stimulated RAW 264.7 and HL-60 cells were determined by dual staining with Acridine orange and ethidium bromide (AO/EtBr). Cells were seeded on poly- L-lysine (0.01%) coated cover slip and kept in the CO_2 incubator for 24 h, then LUT, AST and FUCO were treated separately and incubated for 48 h and 12 h for HL-60 and RAW 264.7 cells, respectively. After incubation, the cells were washed twice with PBS, followed by 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 their combination 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) with the exposure time 5500 milliseconds under 200x magnification [24].

Statistical analysis

Values are mean ± SEM 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 (Assistant software, v.7.7). The differences between carotenoids, LPS and vehicle controls group were analyzed by Tukey's test. The differences between the experimental samples were considered significant levels at $p < 0.05$.

Results

Carotenoids content and purity from natural source

Carotenoids isolated from spinach, shrimp and seaweeds were identified and confirmed based on their λ_{max}, retention time, characteristic UV–Visible, and mass spectra. Mass spectra of LUT, AST and FUCO were compared with their respective reference standard and were used for cell culture treatments. The purity of column purified LUT, AST and FUCO were 97 ± 1, 96 ± 3, and 94 ± 2%, respectively. The results show that the levels of LUT in spinach were 78 ± 2 µg/g dry weights, while AST in shrimp was 50 ± 2 µg/g dry weights, respectively. *Padina tetrasomatica* consists of 42.8 ± 5 µg/g dry weights FUCO as the major pigment, respectively. Cell viability/toxicity levels between purified carotenoids and respective standards were comparable, and there is no significant difference in treated cultures.

Effect of LUT, AST and FUCO on RAW 264.7 cell viability and LDH assay

The cytotoxicity influence of the carotenoids treatments in LPS-stimulated RAW 264.7 cells is shown in Figure 2A. The LPS-stimulated cells decreased cell viability by 85%. Whereas, LUT, AST and FUCO delivered separately in DMSO increased cell viability by, 58.3, 63.4 and 74.1% as compared to LPS-stimulated, respectively. Also observed in the LDH assay,

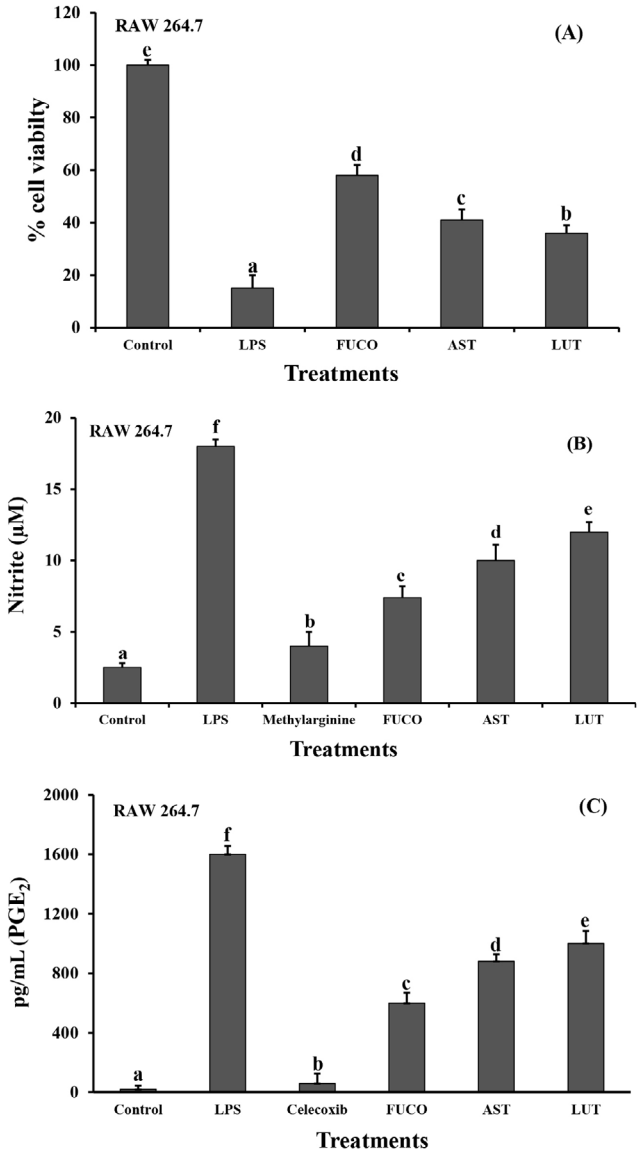

Figure 2: Effect of purified carotenoids (FUCO/AST/LUT) (20 µM) from seaweeds on LPS-induced cell viability (A), NO production (B) and PGE_2 production (C) in RAW 264.7 cells for 12 h. [a,b,c,d,e,f]Values were the means ± SEM of three different experiments. Values with different letters were significantly different ($p < 0.05$).

a clear increase of % LDH in the culture medium was found in LPS treated group. The lowest % LDH release was observed in FUCO (8%) treated followed by AST (12%) and LUT (18%). These results indicate that FUCO treated group increased cell viability significantly compared to other groups by protecting cells from LPS-stimulated inflammation.

Effect of LUT, AST and FUCO in nitric oxide scavenging and PGE_2 production in RAW 264.7 cells

Carotenoids used in the present study effectively suppress the LPS-stimulated NO production (Figure 2B). LUT, AST, and FUCO decreased NO by 33.3, 44.4 and 58.8% as compared to LPS-stimulated cells, respectively. Similarly, PGE_2 production increased in the culture medium treated with LPS. Whereas, LUT, AST and FUCO significantly inhibited PGE_2 levels by 37.5, 45 and 62.5% as compared to LPS-stimulated cells in RAW 264.7 cells, respectively (Figure 2B and 2C).

Effect of LUT, AST and FUCO on NF-κB p65 level

In order to understand the mechanism underlying the inhibitory effect of the LUT, AST and FUCO on LPS-stimulated inflammatory mediators, we studied the NF-κB p65 level in the nuclear fraction of the treated cells by western blotting. The amount of NF-κB p65 in the nucleus of RAW 264.7 dramatically increased upon stimulation with LPS. Carotenoids treatment reduced the expression of nuclear NF-κB p65, whereas FUCO shown a more evident inhibition. All the three carotenoids exerted their anti-inflammatory actions by down-regulating of NF-κB p65 level in macrophage cells. The fold difference was found to be 2.1, 3.3 and 6.8 in LUT, AST and FUCO as compared to LPS-stimulated group, respectively (Figure 3).

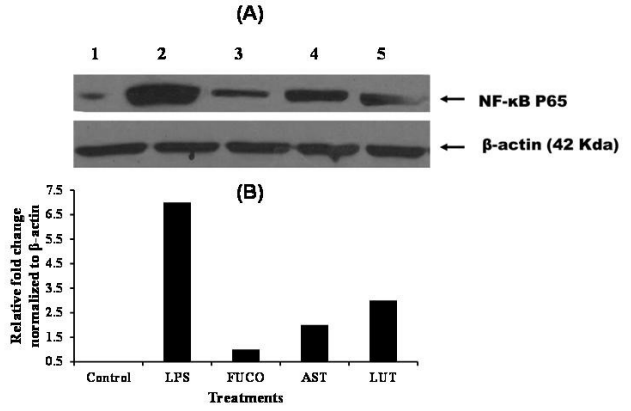

Figure 3: Effect of purified carotenoids (FUCO/AST/LUT) (20 μM) on LPS induced NF-κB expression in RAW 264.7 cells for 12 h. Lane 1-Control, Lane 2-LPS-stimulated, Lane 3-FUCO, Lane 4-AST, Lane 5-LUT (A). The results are expressed as relative fold change to basal group and demonstrated as histogram (B). The experiments were performed in triplicate and the results are expressed as the mean ± S.D.

Cell viability and oxidative status of HL-60 cells treated with LUT, AST and FUCO

Carotenoids drastically decreased cell viability after 48 h of incubation (Figure 4A). LUT, AST and FUCO reduced cell viability by 22, 39 and 55.4% in HL-60 cells, respectively. Also,

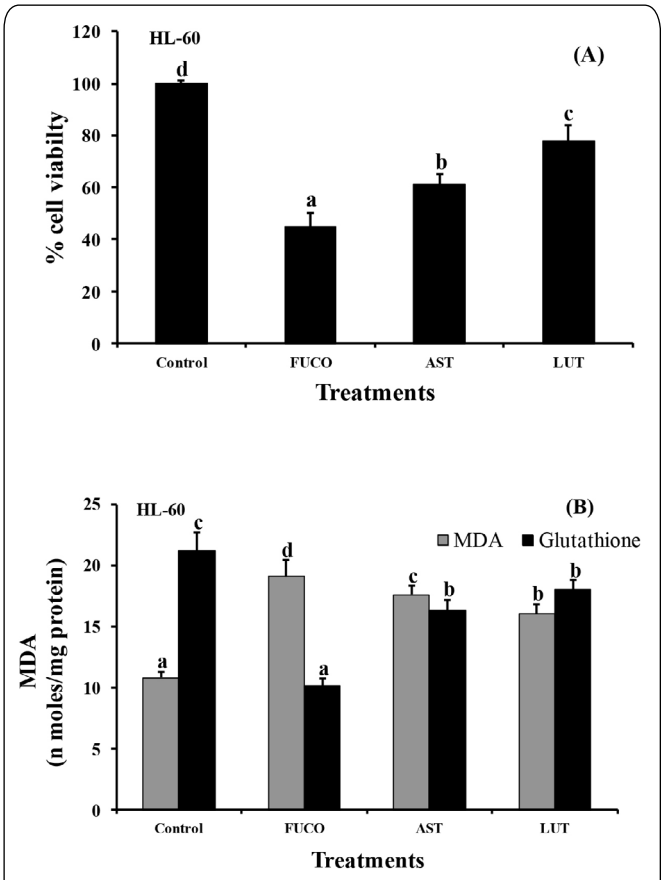

Figure 4: Effect of purified carotenoids (20 μM) treatments (48 h) on cell viability (A), glutathione and MDA levels in HL-60 cells (B).[a,b,c,d]Values were the means ± SEM of three different experiments. Values sharing different letters are significantly different ($p < 0.05$).

FUCO decreased glutathione (37.3 and 18%) and increased MDA levels (55.8 and 71.1 %) significantly in HL-60 cells as compared to LUT and AST treated groups (Figure 4B).

Effect of LUT, AST and FUCO on induction of apoptosis and ROS generation in RAW 264.7 and HL-60 cells

Induction of apoptosis was confirmed by using FITC Annexin V and PI staining. The relative % of apoptotic cells and ROS generation in carotenoids treatment groups is shown in Table 1. The % apoptosis in RAW 264.7 cells decreased in LUT (26.6%), AST (34.7%), and FUCO (41.8%) than LPS treated group. Likewise, decreased levels of ROS generation observed in LUT (22.1%), AST (25.4%), and FUCO (36.5%) than LPS treated group.

In case of HL-60 cells, the % apoptotic cells higher in LUT (5%), AST (11.5%) and FUCO (18.1%) than control treatment. In addition, FUCO shown slightly higher levels of ROS generation than LUT (4.7%) and AST (8.6%) treated group.

Effect of LUT, AST and FUCO on HL-60 and RAW 264.7 cells morphology

Morphological changes in cells were confirmed by acridine orange and ethidium bromide staining (AO/EtBr staining) in cells treated with LUT, AST and FUCO. Cells stained with fluorescence green, yellow and reddish /orange represented viability, early apoptosis, and late apoptosis, respectively

(Figure 5). HL-60 cells treated with carotenoids showed changes in cellular morphology, FUCO being more effective compared to AST and LUT and similar trend was observed as in case of cell viability assay. Whereas, LPS-stimulated RAW 264.7 cells induces apoptotic/necrosis, upon treatment with carotenoids cells retained towards normal morphology in the following order FUCO>AST>LUT.

Table 1: Effect of carotenoids on induction of apoptosis and ROS levels in HL-60 and RAW 264.7 cells.

Experimental groups	Apoptosis (%)	ROS (%)
	HL-60	
CN	2.22 ± 1.4[a]	1.8 ± 0.6[a]
FUCO*	20.30 ± 0.6[d]	17.3 ± 0.9[d]
LUT*	7.2 ± 1.7[b]	6.5 ± 0.8[b]
AST*	13.7 ± 0.8[c]	10.4 ± 0.4[c]
	RAW 264.7	
CN	3.22 ± 1.6[a]	4.62 ± 0.6[a]
LPS	70.3 ± 2.7[e]	63.8 ± 1.3[e]
FUCO*	28.5 ± 0.7[b]	27.3 ± 0.9[b]
LUT*	43.7 ± 1.2[d]	41.7 ± 1.5[d]
AST*	35.6 ± 0.8[c]	38.4 ± 1.6[c]

*20 µM of individual carotenoid of OCC purified LUT from GLVs, AST from shrimp and FUCO from seaweed. The values are means ± of SEM of three experiments.Values not sharing a common superscript letter within a column under each treatment are significantly different from its respective control at $p < 0.05$.

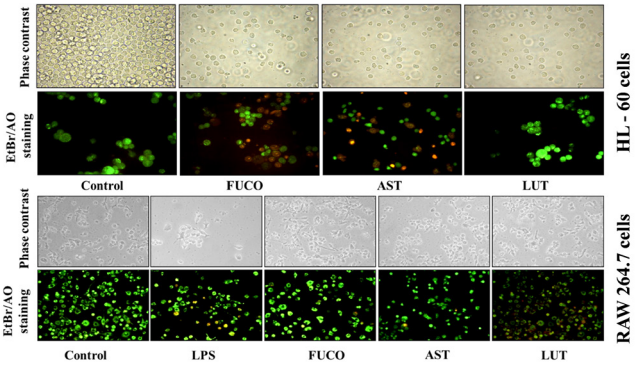

Figure 5: Effect of carotenoids (20 µM of LUT/AST/ FUCO) on HL-60 and RAW 264.7 cell morphology incubated for 48 and 12 h, respectively. Cells were stained with and without AO/EtBr and observed under phase contrast and fluorescence microscope (200×). Green live cells show normal morphology; green early apoptotic cells show nuclear margination and chromatin condensation. Late orange apoptotic cells showed fragmented chromatin and apoptotic bodies. These are representative results of at least three independent experiments (n = 3).

Discussion

Carotenoids role in several degenerative diseases have been focused from past two decades, however effect of specific xanthophyll are less focused. Although, carotenoids are linked to anti-inflammation, oxidative stress and anti-proliferation of cancer cells, perhaps many aspects are poorly understood. Therefore, in the present study, the potentiality of three selected oxygenated carotenoids on inflammation and cancer cell proliferation were evaluated. Several scientific reports demonstrated the carotenoids influence on markers of oxidative stress and inflammation indicating positive health benefits, by persuading transcription factors, and their downstream targets [25-29]. NO have been shown to cause DNA damage that can ultimately leads to development of chronic diseases and cancer [30]. Macrophages play an important role in both, host-defense mechanisms and inflammation. Activated macrophages secrete a number of different inflammatory mediators, including NO, TNF-α, IL-1β and IL-6. The overproduction of these mediators has been implicated in several inflammatory diseases and cancer [31]. Thus, inhibition of activation of these cells appears to be an important target for the treatment of inflammatory diseases. NO is one ubiquitous cellular mediator of physiological and pathological processes, being largely released at inflammatory sites [32]. In this study, it is evident that epoxy-carotenoid like FUCO exerted higher anti-inflammatory property in RAW 264.7 and demonstrated its superior role in controlling HL-60 cell proliferation. Leukemia cells considered as a bipotent cell line with the capacity to differentiate into granulocytes and macrophages/monocytes [33], hence, we tested our hypothesis using RAW 264.7 and HL-60 cell lines.

In the current study, we observed that oxygenated carotenoids shown to control inflammation effectively in RAW 264.7 cells, further each kind of carotenoid at similar concentration inhibits production of NO, PGE_2 and ROS with different potencies. Anti-proliferation activity of each oxygenated carotenoid on higher percentages of apoptotic/necrotic cells may be due to inflammation triggered by LPS. Whereas, cells co-treated with carotenoids for 12 h decreased the induction of necrosis/ apoptosis and retains its proliferation. These results positively correlated with decreased production of ROS generation in carotenoid treated groups. Among these parameters, FUCO exhibited to shown better inhibition than hydroxy and keto-carotenoids. Further the key biomarker of inflammation, NF-κB p65 inhibits the expression by 3.5 and 4.7 folds in FUCO than AST and LUT. These results are well supported by earlier report with inhibition of NO production, prostaglandins, and related expression of inflammatory genes by suppressing the activation of transcription factor NF-κB p65 by hydrocarbon carotenoid β-carotene [34].

Violaxanthin is recently known to be potent anti-inflammatory agents *in vitro* and *in vivo* in responses to bacterial LPS [18]. Shiratori et al. [35] reported that effect of FUCO is comparable with commercially anti-inflammatory steroidal drug prednisolone. Heo et al. [36] screened inhibitory effect of NO production from nine species of brown algae and confirmed that inhibition of NO production correlates with FUCO contents. FUCO treatment attenuates the productions of NO and PGE_2 by inhibiting inducible NO synthase (iNOS) and cyclooxigenase-2 (COX-2) expressions.

The anti-inflammatory activities of selected oxygenated carotenoids may be due to the suppression of NF-κB as similar to previous observation [5, 34]. NF-κB plays a decisive role in the transcriptional regulation of a wide range of genes that are involved in regulation of inflammatory and immunity. NF-κB proteins exist in the cytoplasm of inactive cells, but upon stimulation they become active and translocate into the nucleus, inducing transcription of diverse inflammatory genes. In this study we found that FUCO from seaweed lowers the transactivation of NF-κB p65 in the nucleus more prominently as compared to hydroxy and keto-carotenoids treatments in LPS-stimulated macrophage cells. Production of pro-inflammatory mediators has been continuously reported in many inflammatory tissues, along with increased expression of their mRNAs and proteins. Therefore, inhibition of pro-inflammatory mediators by these carotenoids suggesting that its potentiality to treat the inflammatory and other related diseases.

This study also evaluated the anti-proliferative activity of FUCO, AST and LUT in HL-60 cells. Previously, carotenoids demonstrated the range of biological properties, including, antioxidant, cytotoxicity, modulation of gene expression and signalling [9]. In this study, FUCO affects significantly on anti-proliferation of HL-60 cells than hydroxy and keto-carotenoids at similar concentration. Earlier, Chew et al. [37] have reported antitumor effect of AST in mouse models. Others have also attributed the role of AST and other carotenoids against cancer in various cell lines [38, 39]. Similarly, chemo-preventive action of di-hydroxy carotenoid LUT has been recognized in tumour-induced female BALB/c mice [2]. In the present study, we shown the effect of structurally different oxygenated carotenoids on cytotoxicity in HL-60 cells. Further, we also observed that, FUCO treated cells significantly affects induction of apoptosis followed by AST, and LUT. This difference may be due to structurally different carotenoids having variable polarity and solubility in the medium [40]. Apart from these, uptake of carotenoid and its level in cells may also be accountable in the biochemical events of cell toxicity [7, 41, 42]. The different sensitivities of the cells to carotenoids are presumably due to the difference in absorption of carotenoids [43]. It is possible that cell growth modality, as well as cell metabolic status and membrane composition, may influence carotenoid accumulation. Oxidative stress plays important role in the induction of apoptosis in diverse models [44]. We believe that reduced glutathione and increased MDA levels may induce apoptosis. Since glutathione serves as an endogenous antioxidant, reduced glutathione levels reported to facilitating apoptosis in leukemic cancer cells [45] FUCO was found to perform superior bioactive molecule and inhibits greatly on proliferation of cancer cells than AST, and LUT. Hence, FUCO induces higher cytotoxicity and percent apoptosis of HL-60 cells due to increase oxidative stress in cancer cells as indicated by the higher accumulation of MDA and depleted levels of glutathione [8]. Further, support to this observation, FUCO treated group showed higher ROS level followed by AST, LUT and control (Figure 4 and Table 1). Earlier, studies have demonstrated that epoxy xanthophylls such as fucoxanthin and neoxanthin reduce the viability of prostate cancer cells by inducing apoptosis to a greater extent than the lutein, β-carotene and lycopene [46]. In addition, Palozza et al. [42] demonstrated that under the experimental condition, carotenoids acts as pro-oxidants predominate over the antioxidant and induce the formation of free radical species, including peroxyl radicals, and responsible for an induction of apoptosis. In this study, oxygenated carotenoids inhibited HL-60 cells proliferation by accumulation of ROS. This difference among oxygenated carotenoid may be due to functional group and reactivity of carotenoids or its breakdown products with cancer cells. Previously, it has been suggested that an increase in the number of oxygen or hydroxyl groups in the carotenoids decides the efficiency of antioxidant activity [47]. Apoptosis detection by Annexin/FITC staining confirmed the anti-proliferative effect of oxygenated carotenoids. The higher apoptosis of cells may be due to the increased oxidative stress in cells treated with FUCO followed by AST and LUT. Carotenoids may act as antioxidants or as pro-oxidants in biological systems, depending on their concentration and oxygen tension [7, 48]. Also, the observation of apoptotic morphological features in carotenoids treated group strongly suggested the influence of oxygenated carotenoids in cancer cell proliferation. Further, apoptosis process may be due to, at least in part, to the formation of its oxidative breakdown products, while reported to render higher toxicity to cancer cells [7, 8].

Conclusion

This study demonstrated the variable potentiality of individual oxygenated carotenoid related to anti-inflammation and anti-proliferation activities, the difference presumed may due to its chemical and structural properties. Interaction of bioactive FUCO with hydrocarbon carotenoids and other phyto-components from intra specific food/natural source is deserved for further exploration of characteristics carotenoid. This observation provides a greater insight of oxygenated carotenoids consumption from green vegetables and marine food sources to combat chemoprevention of cancers originating from inflammation.

Conflict of Interest

The authors declare no competing financial interest.

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