Fractionation and Characterization of Lycopene-Oxidation Products by LC-MS/MS (ESI)⁺: Elucidation of the Chemopreventative Potency of Oxidized Lycopene in Breast-Cancer Cell Lines

Bangalore Prabhashankar Arathi,[†] Poorigali Raghavendra-Rao Sowmya,[†] Gini Chempakathinal Kuriakose,[‡] Shivaprasad Shilpa,[†] Hulikere Jagdish Shwetha,[†] Sharath Kumar,[§] Marisiddaiah Raju,^{||} Vallikannan Baskaran,[⊥] and Rangaswamy Lakshminarayana^{*,†}

[†]Department of Biotechnology, Bangalore University, Jnana Bharathi Campus, Bengaluru 560 056, India

[‡]Department of Biochemistry, Indian Institute of Science, Bengaluru 560 012, India

[§]Himalaya Drug Company, Makali, Bengaluru 562 162, India

^{||}Department of Botany, St. Joseph's College Autonomous, PB 27094, 36 Lalbagh Main Road, Bengaluru 560 027, Karnataka, India

[⊥]Department of Biochemistry, CSIR-Central Food Technological Research Institute, Mysuru 570 020, India

S Supporting Information

ABSTRACT: Lycopene (LYC) has been correlated with the reduction of certain cancers and chronic diseases. However, the existence and biofunctionality of degraded, oxidized, and biotransformed LYC products in vivo have not been revealed. Therefore, this study aimed to screen and elucidate the potential bioactive lycopene-derived products in breast-cancer and non-cancerous cells. LYC-oxidation or -cleavage products were generated using KMnO₄. These oxidation products were separated as fractions I–III by silica column chromatography using gradient solvent systems. Further, LC-MS/MS (ESI)⁺ was used to elucidate their possible fragmentation patterns and structures. Fraction II showed higher cytotoxicity (IC₅₀ value of 64.5 μM), cellular uptake, and apoptosis-inducing activity in MCF-7 cells. This fraction consists of major peak *m/z* 323, identified as apo-8,6'-carotendial. The cytotoxicity-inducing activity may be due to partial ROS generation with mitochondrial dysfunction. Further, the role of apo-8,6'-carotendial in the induction of apoptosis is demonstrated for the first time. These results illustrated that LYC-oxidation derivatives or metabolites are involved in growth inhibition of cancer cells. Exploration of specific oxidized-carotenoid products will give further insight into the field of nutritional biochemistry.

KEYWORDS: lycopene-oxidation products, breast cancer, apoptosis, reactive oxygen species, mitochondrial membrane potential

INTRODUCTION

Several epidemiological studies have shown a relationship between lycopene (LYC) consumption and decreased risks of cancer and other chronic diseases.^{1–4} Studies have suggested that LYC decreases the risks for certain types of cancers, including breast cancer.^{5,6} The effects of LYC are attributed to its antioxidant and cell-signaling properties.⁷ The unique chemical properties of LYC are very sensitive to oxidative modification and degradation.⁸ Stability studies have shown isomerization and degradation of (all-*E*)-LYC when exposed to light, oxygen, and high temperatures.⁹ In general, carotenoids are more prone to various modifications such as hydrogenation, dehydrogenation, double-bond migration, chain shortening or extension, rearrangement, isomerization, oxidation, and combinations of these processes, resulting in numerous products.¹⁰ Apart from these, carotenoid-oxidation metabolites may also be formed as a result of the presence of monooxygenase, cyclooxygenase, and dioxygenase enzymes.¹¹ Typically, the breakdown of lycopene occurs through isomerization followed by oxidation to yield epoxides, or it undergoes eccentric cleavage by carotene-9',10'-monooxygenase (CMO-II) to form apo-lycopenoids.^{12,13} The conversion and biological functions of β-carotene metabolites are well documented in humans;¹⁴ however, the bioactivities of specific LYC-oxidation derivatives are poorly explained.

Carotenoid metabolites and oxidized-carotenoid products are reported to be involved in the chemoprevention of cancer.^{15–17} Previously, lycopene-oxidation metabolites have been identified in vitro and in vivo.^{18–24} Few notable studies support the concept that the biological activities of lycopene may be mediated by its oxidative-cleavage products.^{25–28} Even though studies claim the possible role of carotenoid metabolites, the specific functions of oxidative derivatives and metabolites or fractions rich in them are not well explained, as compared with those of the parent compound. Previously, we speculated that oxidized-LYC mixtures might have possible cytotoxic effects, resulting in the inhibition of different cancer cells.²⁹ The enzymatic- and oxidative-degradation products of LYC have ascertained health benefits; however, the fates of these derivatives and their anti-cancer activities are still not well detailed. Furthermore, elucidation of such potential components and oxidation products is considered to be important in understanding their role against chronic-disease progression. Hence, the present study was aimed at isolating and identifying the major oxidative-cleavage products of LYC and elucidating their anticancer properties.

Received: September 5, 2018

Accepted: September 27, 2018

Published: September 27, 2018

Table 1. Structures of Apo-lycopenals and Apo-carotendials Obtained from the Oxidation of LYC by Potassium Permanganate

compd	<i>m/z</i>	proposed structure	formula	app. molecular weight	name
A	390.26		C ₂₇ H ₃₄ O ₂	391.14	(2E,4E,6E,8E,10E,12E,14E,16E,18E)-2,6,11,15,19-pentamethyldocosa-2,4,6,8,10,12,14,16,18-nonaenal
B	467.17		C ₃₂ H ₄₈	468.38	(4E,6E,8E,10E,12E,14E,16E,18E,20E,22E)-2,6,10,15,19,23,27-heptamethyloctacos-2,4,6,8,10,12,14,16,18,20,22,26-dodecaene
C	338.22		C ₂₃ H ₃₀ O ₂	338.22	(4E,6E,8E,10E,12E,14E,16E)-4,8,12,17-tetramethyl-18-oxononadec-4,6,8,10,12,14,16-heptaenal
D	428.31		C ₃₁ H ₄₀ O	429.27	(2E,4E,6E,8E,10E,12E,14E,16E)-4,9,13,17,21-pentamethyldocosa-2,4,6,8,10,12,14,16,20-nonaenal
E	449.2		C ₂₉ H ₃₆ O ₄	448.59	(2E,4E,6E,8E,10E,12E,14E,16E,18E,20E)-4,8,13,17,21-pentamethyltetracos-2,4,6,8,10,12,14,16,18,20-decaenedioic acid
F	323.1		C ₂₂ H ₂₆ O ₂	322.1	(2E,4E,6E,8E,10E,12E,14E,16E)-2,6,11,15-tetramethyloctadec-2,4,6,8,10,12,14,16-octenal
G	350.26		C ₂₅ H ₃₄ O	351.13	(2E,4E,6E,8E,10E,12E,14E)-2,7,11,15,19-pentamethylcosa-2,4,6,8,10,12,14,18-octenal (apo-12'-lycopenal)
H	349.2		C ₂₄ H ₂₈ O ₂	348.2	apo-5',6'-carotendial
I	416.31		C ₃₀ H ₄₀ O	417.09	(2E,4E,6E,8E,10E,12E,14E,16E,18E)-2,6,11,15,19,23-hexamethyltetracos-2,4,6,8,10,12,14,16,18,22-decaenal (apo-8'-lycopenal)
J	376.28		C ₂₇ H ₃₆ O	377.10	(2E,4E,6E,8E,10E,12E,14E,16E)-4,9,13,17,21-pentamethyldocosa-2,4,6,8,10,12,14,16,20-nonaenal (apo-10'-lycopenal)

MATERIALS AND METHODS

Chemicals. (all-*E*)-Lycopene (>90%), potassium permanganate, dichloro-dihydro-fluorescein diacetate (DCHF-DA) dye, 4,6-diamidino-2-phenylindole dilactate (DAPI), butylated hydroxytoluene (BHT), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1 dye), and tetrahydrofuran (stabilized with BHT) were purchased from Sigma-Aldrich. HPLC-grade methanol, acetonitrile, dichloromethane (DCM), isopropyl alcohol, and tetrahydrofuran (THF) and PVDF syringe filters (0.45 μ m) were procured from Merck. Minimum essential medium (MEM), fetal-bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), antibiotic-antimycotic solution, calcium magnesium free phosphate-buffered saline (PBS), and cell-culture consumables were purchased from Hi-Media Chemical Laboratories. The FITC-annexin V apoptosis-detection kit was purchased from BD Pharmingen (BD Bioscience). All other chemicals and solvents were of analytical grade and were purchased from Sisco Research Laboratories.

Isolation of LYC from Tomato. Ripened tomatoes were utilized for LYC extraction (Indian hybrid) as per our earlier method.³⁰ The peak identities, absorption maxima (λ_{max}), and characteristic UV-visible spectrum of isolated LYC were confirmed by LC-MS/MS analysis using a reference standard.³⁰ Extraction and preparation of LYC from sample and standard were carried out under dim, yellow light at 4 °C to prevent isomerization and degradation.

Oxidation of LYC. LYC isolated from tomato was subjected to oxidation by using KMnO₄ as per the procedure of Caris-Veyrat et al.²⁰ In brief, LYC (30 mg) and cetyltrimethylammonium bromide (6 mg) was dissolved in 30 mL of DCM/toluene (1:1 ratio). The oxidation of LYC was initiated by adding 1.5 mL of aqueous solution of KMnO₄ stock (135 mg in 9 mL of water) and stirring with a magnetic stirrer for 30 min at 26 $\pm$ 2 °C. The organic phase containing LYC-oxidation products was separated by repeated washings with double distilled water (10 mL), filtration through a PVDF filter (0.45 μ m), and drying on sodium sulfate under a nitrogen environment. The residue of the LYC-oxidation mixture redissolved in petroleum ether and was filtered with a PVDF filter to remove the remaining KMnO₄. LC-MS/MS ESI⁺ mode was applied to analyze crude LYC-oxidation products and different fractions obtained by gradient open-column chromatography. Aliquots of the oxidized

fractions were used for cell-culture treatments or sealed under a nitrogen environment and stored at -80 °C in amber vials.

Fractionation of LYC-Oxidation Products. Open-column chromatography (OCC, 20 $\times$ 1.5 cm) on silica G (particle size 70–230 mesh) was employed to separate LYC-oxidation products using specific gradient solvent systems. In brief, an aliquot of the LYC-oxidation products was dried under a nitrogen environment and redissolved in a known volume of hexane/acetone (50:50, v/v), and applied to OCC. The oxidation products were eluted using hexane/acetone in the ratios 90:10, 80:20, and 70:30 (v/v), and each fraction was collected separately in amber vials and subjected to HPLC analysis. LC-MS/MS analysis confirmed the peak identities and the respective spectra of each fraction. Further, the bioactive-rich fraction was subjected to MS/MS analysis to understand its fragmentation pattern.

HPLC Analysis of LYC-Oxidation Products. Chromatographic separation of the LYC-oxidation products was standardized using an AB SCIEX API 2000 system (Applied Biosystems) with a C30 column (5 μ m, 250 $\times$ 4.6 mm; Princeton Chromatography Inc.). The mobile phase, consisting of acetonitrile/methanol/isopropyl alcohol (40:40:20, v/v/v) under isocratic conditions with a flow rate of 0.8 mL/min, was monitored at 420 and 450 nm using a UV detector. An aliquot (20 μ L) of LYC and its oxidation products was injected on the column, which was maintained at 40 °C. Data acquisition and processing were carried out using Analyst version 1.5 software (Applied Biosystems).

LC-MS/MS Conditions for Analysis of LYC-Oxidation Products. Analysis of LYC-oxidation products was performed using an AB SCIEX API 2000 mass spectrometer (Applied Biosystems) via an ESI source operated in positive-ion mode. An aliquot (20 μ L) of LYC and its oxidation products (~20 μ g/mL) was injected onto the column. The conditions used are the same as those mentioned for HPLC analysis. The instrument conditions were the following: an ion-source voltage of 3500 V, a declustering potential of 20 V, a focusing potential of 400 V, an entrance potential of 10 V, a source temperature of 420 °C, GS-I at 50 psi, GS-II at 60 psi, and a curtain gas of nitrogen at 30 psi. Mass spectra of the LYC-oxidation products were recorded with an *m/z* 50–1000 scan range at 450 nm. Data were processed by using Analyst version 1.5 software (Applied Biosystems). Further, the collision-induced-ion-dissociation spectra were recorded and compared with those of the molecular ions of the LYC-oxidation

Figure 1. LC-MS/MS analysis and elucidation of possible LYC-oxidation products (fraction III, compound A and B; fraction II, compounds C–F; fraction I, compounds G–J). MS/MS scan 391 > 331, 463 > 377, 338 > 321, 429 > 359, 449 > 312, 323 > 251, 351 > 281, 349 > 331, 417 > 347, 377 > 307.

products. A collision-induced-dissociation energy of 6 V was used to check the fragmentation pattern. Because of the constraint in the sample size and the unavailability of standards for LYC-oxidation products, the possible structures were elucidated using ChemsSketch 8.0 software (ACD Laboratories).

Furthermore, the samples were subjected to a Bruker impact HD (Q-TOF) high-resolution mass spectrometer to confirm the LYC-oxidation products with higher accuracy (Supplementary Figure 1).

Culture Conditions and Viability Assay. MCF-7 cells were obtained from the National Centre for Cell Science in Pune, India,

Figure 2. LC-MS/MS analysis of LYC-oxidation products (fraction II major peak, m/z 323).

and cultured in standardized conditions.²⁹ Cells were grown in MEM medium supplemented with 10% heat-inactivated fetal-bovine serum, 4 mM glutamine, 40 g/mL penicillin, and 400 U/mL streptomycin. Similarly, normal breast epithelial cells (MCF-10A, ATCC) were cultured and maintained as per the previous report.³¹ Preliminarily, we screened different concentrations of lycopene (1–200 μ M) and its oxidation products (fractions I to III) for the cytotoxic assay. This study demonstrated that rich fractions of LYC-oxidation products showed cytotoxicity at higher concentrations (50, 100, and 200 μ M equivalent LYC concentrations). Hence, in the current study, we used above optimal level (100 μ M) of LYC concentration to compare its activity with different fractions of LYC-oxidation products for cell-based assays. LYC and the different fractions of LYC-oxidation products obtained by OCC were dissolved in cell-culture-grade THF and added to the cells in the medium at different doses (quantified on the basis of the equivalent concentrations of standard LYC, as per the Zhang et al.²¹ method). The final concentration of THF in the culture medium was 0.5% (v/v). THF at this concentration did not induce cytotoxicity. The cells were seeded into 96-well microtiter plates (5000 cells/well). After 24 h of cultivation, cell viability was measured using the MTT method as described previously.¹⁶

Apoptosis Detection. Apoptosis detection was performed with an FITC–annexin V apoptosis-detection kit according to the manufacturer's instructions. Briefly, control and treated cells (1×10^5 cells/well in 12-well plates) incubated for 24 h were collected, then washed with ice-cold PBS, and centrifuged at 2500 rpm for 5 min. The cell pellet was resuspended in the ice-cold 1 $\times$ binding buffer, incubated with FITC-conjugated annexin V and propidium iodide (PI) for 15 min at room temperature in the dark. Before the analysis, the instrument was calibrated with internal controls, such as unstained cells (negative control) and stained cells (annexin V-FITC, PI, and annexin V-FITC with PI control cells). The samples were immediately analyzed on a FACS Verse flow cytometer using the Diva analysis software.

Confocal Microscopy. LYC-oxidation-products (50 and 100 μ M) untreated and treated cells were harvested after 24 h of incubation and used for annexin V–FITC–PI and DAPI staining. Then, the cells were observed under an inverted confocal laser-scanning microscope (Zeiss LSM 510 MK4), and the images were captured.

ROS Detection by Flow Cytometry. Control and treated cells (2×10^5) were harvested after 24 h of incubation, washed with PBS, and suspended in 1 mL of PBS. Afterward, DCFH-DA (10 μ M) was added, and the solution was incubated for 15 min in 5% CO₂ at 37 °C. After incubation, the cells were washed and resuspended in PBS and analyzed within 1 h on a FACS Verse flow cytometer after calibration with negative-control (unstained) and positive-control (H₂O₂ treated) cells. The results were expressed as the fluorescence intensities of dichlorofluorescein, and the negative-control, treatment, and H₂O₂-treated (positive control) cells were compared.

Detection of Loss of Mitochondrial Transmembrane Potential (Dym). Modifications of the mitochondrial membrane potential were analyzed by flow cytometry using JC-1 dye, as described earlier.³² Briefly, MCF-7 cells were treated with either LYC or its oxidation products (50–200 μ M concentration) for 24 h. Then, cells were incubated with JC-1 (0.5 mM) for 15 min at 37 °C and subjected to flow-cytometric analysis. The ratio of red to green fluorescence was measured for each treatment and plotted. Cells treated with 2,4-dinitrophenol (2,4-DNP) were considered the positive control.

Cellular Uptake of LYC versus Cellular Uptake of Its Oxidized Products. MCF-7 cells (10^6) in 75 cm² flasks ($n = 3$) were treated with 100 μ M purified LYC or its bioactive oxidation products (fraction II). After incubation for 24 h, cells were harvested, washed using MEM, and extracted with suitable solvent systems. The LYC and oxidized-LYC contents of all the treated cells were quantified by HPLC and expressed after subtracting the contents of carotenoids adherent to the cell surfaces as determined at 4 °C.³³

Statistical Analysis. The data were tested for homogeneity of variances by the Bartlett test. When homogeneous variances were confirmed, the data were further analyzed using ANOVA (GraphPad Prism 5.1. software). Tukey's test evaluated the differences between the treatment and control groups. Significant differences between the experimental samples were considered at $P < 0.05$.

RESULTS

Lycopeneals Formed with Potassium Permanganate. LC-MS/MS analysis of KMnO₄ induced LYC-oxidation products showing the presence of single-oxidative-cleavage and

double-oxidative-cleavage products of LYC. The single-oxidative-cleavage compounds contained an aldehyde or a ketone group at one end and were identified as apo-lycopenal and apo-lycopenone, respectively. The second category of oxidation products contained two aldehyde end groups and was identified as apo-carotendials. The chemical structures of the apo-lycopenals, apo-lycopenones, and apo-carotendials are shown in Table 1. Treatment with KMnO_4 resulted in cleavage of the carbon–carbon double bonds of LYC, yielding a series of apo-lycopenals and apo-carotendials. The separation of these cleaved-LYC products was achieved using a C30 column (Figure 1). The lycopeneals and apo-carotendials were eluted on a C30 column in the following order: (2E,4E,6E,8E,10E,12E,14E,16E,18E)-2,6,11,15,19-pentamethyldocosa-2,4,6,8,10,12,14,16,18-nonaenedial (A, 19.73%), (4E,6E,8E,10E,12E,14E,16E,18E,20E,22E)-2,6,10,15,19,23,27-heptamethyloctacos-2,4,6,8,10,12,14,16,18,20,22,26-dodecaene (B, 7.5%), (4E,6E,8E,10E,12E,14E,16E)-4,8,12,17-tetramethyl-18-oxononadeca-4,6,8,10,12,14,16-heptaenal (C, 13.5%), (2E,4E,6E,8E,10E,12E,14E,16E)-4,9,13,17,21-pentamethyldocosa-2,4,6,8,10,12,14,16,20-nonaenal (D, 3.9%), (2E,4E,6E,8E,10E,12E,14E,16E,18E,20E)-4,8,13,17,21-pentamethyltetracos-2,4,6,8,10,12,14,16,18,20-decaenedioic acid (E, 4.2%), (2E,4E,6E,8E,10E,12E,14E,16E)-2,6,11,15-tetramethyloctadeca-2,4,6,8,10,12,14,16-octaenedial (F, 23.2%), (2E,4E,6E,8E,10E,12E,14E)-2,7,11,15,19-pentamethylcosa-2,4,6,8,10,12,14,18-octaenal (apo-12'-lycopenal, G, 10.8%), apo-5,6'-carotendial (H, 4.9%), (2E,4E,6E,8E,10E,12E,14E,16E,18E)-2,6,11,15,19,23-hexamethyl tetracos-2,4,6,8,10,12,14,16,18,22-decaenal (apo-8'-lycopenal, I, 0.29%), (2E,4E,6E,8E,10E,12E,14E,16E)-4,9,13,17,21-pentamethyldocosa-2,4,6,8,10,12,14,16,20-nonaenal (apo-10'-lycopenal, J, 0.15%; Figure 1). The elution of the rich fraction of LYC-oxidation products was obtained by OCC using a hexane/acetone gradient solvent system with the following ratios: 90:10, 80:20, and 70:30 (v/v). A major peak in fraction 1 was identified and designated as apo-12'-lycopenal (G). Fraction 2 consisted of apo-8,6'-carotendial (F, (2E,4E,6E,8E,10E,12E,14E,16E)-2,6,11,15-tetramethyloctadeca-2,4,6,8,10,12,14,16-octaenedial), and fraction 3 consisted of (2E,4E,6E,8E,10E,12E,14E,16E,18E)-2,6,11,15,19-pentamethyldocosa-2,4,6,8,10,12,14,16,18-nonaenedial (A), as a major peak. Fractions of LYC-cleavage products (fractions I–III) were further evaluated in terms of their bioactive roles in cancer and normal cell lines. Further, MS/MS analysis of the bioactive LYC-cleavage products was done to understand their fragmentation patterns (Figure 2).

Effect of LYC-Oxidation Products on Cell Viability. The cytotoxicity of fractions I to III of LYC-oxidation products on MCF-7 cells is shown in Figure 3. Among the fractions evaluated, fraction II, rich in apo-8,6'-carotendial, strongly inhibited cell viability. The percent cell viability of MCF-7 cells was decreased by 57.6 (fraction I), 77.5 (fraction II), and 54.5% (fraction III) as compared with the control. The IC_{50} concentration of fraction II was found to be 64.5 μM equivalent LYC concentration in MCF-7 cells.

Apoptosis Induction of LYC-Oxidation Products. An increase in apoptosis was observed in MCF-7 cells treated with fraction II (Figure 4). The influence of fraction II on apoptosis was further confirmed by annexin V-FITC–PI staining as shown in Figure 4. Results of confocal-microscopic observation showed more early and late apoptosis and nuclear condensations in the treated cells than in the control (Figure 5).

Intracellular ROS Generation. ROS generation is considered one of the intermediate steps during activation of

Figure 3. (a) Effects of LYC and oxidized fractions I–III (1–200 μM) on viability of MCF-7 cancer cell lines. (b) Effects of LYC and oxidized fractions I–III (1–200 μM) on viability of MCF-10A cell lines. The asterisks indicate values that are significantly different from the control (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$). Statistical comparisons were made by one-way ANOVA followed by Tukey's test. Values are means $\pm$ SD ($n = 3$).

apoptosis. The levels of ROS were examined on the basis of the fluorescence of DCFDA. The influence of LYC and its oxidized fraction II on intracellular ROS levels in MCF-7 cells are shown in Figure 6. Fraction II increased ROS levels at the lower concentration (50 μM) and decreased them at a higher concentration (100 μM). Flow-cytometric evaluation showed no detectable levels of ROS production upon treatment with fraction II. The levels of ROS in the control cells and LYC-treated cells were lower by 13.1 and 10.1% than those in the fraction II treated cells at the lower concentration, and differences not detected at the higher concentration. The percentage of ROS in the positive control was found to be 81.3%.

Induction of the Loss of Mitochondrial Transmembrane Potential (Dym) by LYC-Oxidation Fraction II. Loss of mitochondrial transmembrane potential (Dym) is well-known to be an early event during apoptosis. We measured the loss of mitochondrial membrane potential of LYC-treated and fraction II treated MCF-7 cells (24 h) by flow cytometry using JC-1 dye. Dym was measured from the shift in the ratio of red- to green-fluorescence-emitting cells after treatment. Results showed an increase in green fluorescence in the fraction II treated cells in a concentration-dependent manner, indicating a loss of mitochondrial transmembrane potential in treated cells (Figure 7). Thus, the data suggest that the oxidation products (fraction II) induce depolarization and mitochondrial-transmembrane-potential collapse in the cells, leading to activation of apoptosis.

Figure 4. Detection of apoptosis induced by LYC (100 μM) and oxidized LYC (fraction II, 50–200 μM) in the MCF-7 cell line. Values are means $\pm$ SD ($n = 3$). Ctrl, control; EA, early apoptosis; LA, late apoptosis.

Cellular Uptake of LYC versus Cellular Uptake of Its Oxidized Products. The cellular uptake of LYC and its oxidation products (fractions I–III) was in the order of fraction II > fraction I > fraction III > LYC (Figure 8). The oxidation products showed higher cellular uptake compared with intact LYC.

DISCUSSION

Studies have reported that carotenoid-rich diets are associated with reduced risks of certain cancers. Among carotenoids, LYC has gained special attention because of the worldwide consumption of tomato and its products. Further, higher levels of LYC in plasma and tissues are positively correlated with the reduction of chronic diseases, including cancer.^{34,35} In this regard, many studies have attempted to elucidate the possible

roles of carotenoids, mainly β -carotene, LYC, and lutein, on the modulation of cancer progression. In contrast, few reports have shown insignificant effects of carotenoids on cancers both in vitro and in vivo.^{36–39} Subsequently, studies were undertaken on β -carotene and LYC metabolites to test the hypothesis that the metabolites render higher antiproliferative effects on cancer cells than parent molecules. However, there is no detailed study, even after two decades of research, on the bioactivities of carotenoid metabolites or oxidation products because of the unavailability of standards, low concentrations and stabilities, and challenging issues in isolation and characterization from biological samples. In the present study, we demonstrated LYC-oxidation products, purified by column chromatography using suitable solvent ratios and optimized conditions, and the anticancer properties of fraction II, which

Figure 5. Apoptotic MCF-7 cells stained with annexin V–FITC–PI and examined under a microscope (100 $\times$) after treatment with fraction II (50 and 100 μ M). DAPI was used as nuclear marker.

Figure 6. Effects of LYC (100 μ M) and oxidized LYC (fraction II, 50–200 μ M) on the production of reactive oxygen species in the MCF-7 cell line. The asterisks indicate values that are significantly different from the control (* P < 0.05, *** P < 0.0001). Statistical comparisons were made by one-way ANOVA followed by Tukey's test. Values are means $\pm$ SD (n = 3).

Figure 7. Effects of LYC (100 μM) and oxidized LYC (fraction II, 50–200 μM) on mitochondrial integrity in the MCF-7 cell line. The asterisks indicate values that are significantly different from the control (** $P < 0.001$, *** $P < 0.0001$). Statistical comparisons were made by one-way ANOVA followed by Tukey's test. Values are means $\pm$ SD ($n = 3$).

Figure 8. Cellular uptake of LYC and its oxidation products at a 100 μM equivalent LYC concentration. The asterisks indicate values that are significantly different from those of LYC (** $P < 0.001$, *** $P < 0.0001$). Statistical comparisons were made by one-way ANOVA followed by Tukey's test. Values are means $\pm$ SD ($n = 3$).

is rich in apo-8,6'-carotendial. The apoptotic properties of fraction II indicate an opportunity for its potential application. We also attempted for the first time to compare the anticancer effects of LYC with those of its oxidized fraction II. Several reports have proposed possible cleavage patterns of LYC to obtain its metabolites in vitro and in vivo.^{17,18,20,40}

Studies have suggested that lycopene decreases the risk of breast cancer.^{6,41–43} Further, Sharoni et al.²⁷ suggested that some of the cellular effects of carotenoids are mediated through their derivatives, formed either by chemical oxidation or by enzymatic cleavage inside the cells. Also, Ford et al.⁴⁴ demonstrated that LYC and its metabolite apo-12'-lycopenal significantly suppressed the proliferation of cells through alteration of cell-cycle regulation. However, our results are contradictory,

showing a non-significant difference between intact LYC treatment and control cells on apoptosis induction in MCF-7 cells. The apoptosis-inducing activity of LYC-oxidation products (fraction II with major peak apo-8,6'-carotendial) was confirmed by annex V-FITC-PI and JC-1 staining (Figures 4–7) and may be due to changes in polarity and cellular uptake (Figure 8) when compared with LYC and other fractions. It is evident from Figure 8 that fraction II cellular uptake is comparatively higher than that of LYC. Studies demonstrated that uptake rates and intracellular concentrations in a different stages of cells might play important roles in the chemopreventive effects of lycopene.⁴⁵ Cellular uptake of fraction II is higher than that of other fractions and LYC, which may be due to differences in their structures, polarities, and solubilities.⁴⁶ Further, in the present study, a strong relationship among fraction II accumulation, cytotoxicity, and cell antiproliferation is evident. It is possible that cell growth modality, metabolic status, and membrane composition may influence carotenoid accumulation depending on cell type.⁴⁷ Further, ROS generation is considered one of the intermediate steps during the activation of apoptosis. In our study, we observed slightly higher ROS levels at 50 μM concentrations and minimal levels of ROS production upon fraction II treatment at higher concentrations (Figure 7). We determined that ROS was generated with fraction II at 50 μM , as ROS levels were suppressed when treated in combination with *N*-acetylcysteine (NAC), an ROS scavenger (Supplementary Figure 2). The higher oxidative stress by fraction II with the 50 μM treatment may be due to an imbalance in oxidants and antioxidants in the cells. In case of the 100 and 200 μM concentration, decreased ROS levels were evident as compared with in the 50 μM fraction II treated cells (Figure 7). These contrasting results were clarified with repeated experiments. The higher ROS

levels at 50 μM may trigger apoptosis either through the extrinsic or intrinsic pathway within a short period of exposure. This could be the reason for minimal levels of ROS at 100 and 200 μM fraction II (similar to in the control), showing an ROS-independent pathway. Interestingly, the percent apoptosis ratio and loss of mitochondrial potential increase significantly in a dose-dependent manner. The cell-death-induction mechanism in the fraction II treated cells may be through either an ROS-dependent or -independent pathway and still needs to be detailed. The cell-viability results confirmed that LYC and its oxidized fractions did not affect the normal cells (MCF-10A) under the experimental conditions, suggesting that LYC-oxidation products or metabolites have an important function against cancer-cell proliferation. On the basis of the current study, we propose that LYC-derived products, especially apo-8,6'-carotenoid, may have biological functions. The overall results indicated the potentiality of LYC-derived products as chemopreventive molecules. This observation gives a platform to address the active roles of carotenoid-derived products. Although the roles of specific carotenoid metabolites need to be explored, this is challenging because of the following limitations: the unavailability of standards, coelution, lower concentrations, and circulation in a biological system. Furthermore, studies related to the anticancer properties and molecular mechanisms of specific oxidation products, such as cyclins, inhibitors of apoptosis proteins and caspase activities, need to be addressed (under progress). The in vivo studies related to the roles of these specific metabolites also necessitate addressing. Apart from these, the lifespans (half-lives) of these oxidation products and metabolites need to be compared in isotopic-labeling studies to show further potentiality.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04850.

LC-MS/MS analysis and elucidation of possible LYC-oxidation products and effects of LYC and oxidized LYC in combination with NAC on the production of reactive oxygen species in the MCF-7 cell line (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rlnarn21@gmail.com. Tel.: +91-080-22961461. Fax: +91-080-23211020.

Funding

A.B.P. acknowledges DST (Government of India) for a grant from the Women Scientists Fellowship (F.NO.SR/WOS-A/LS-35/2012). The authors acknowledge DST-SERB (NO.SB/EMEQ-233/2013) for a project grant, Department of Microbiology and Biotechnology (Bangalore University), UGC-SAP, DST-FIST.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge Dr. Ravi Sundaresan, Department of Microbiology and Cell Biology, Indian Institute of Science, for permitting us to use the FACS and central analytical-spectroscopy facilities.

■ ABBREVIATIONS USED

LYC, lycopene; KMnO_4 , potassium permanganate; ROS, reactive oxygen species; RAR, retinoic acid receptor; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DCM, dichloromethane; FBS, fetal-bovine serum; MEM, minimum essential medium; PVDF, polyvinylidene difluoride; FITC, fluorescein isothiocyanate; DCFH-DA, dichloro-dihydro-fluorescein diacetate; DAPI, 4',6-diamidino-2-phenylindole; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide; LC-MS/MS, liquid chromatography–mass spectrometry; MCPBA, meta-chloroperoxybenzoic acid; NAC, N-acetylcysteine

■ REFERENCES

- (1) Rao, A. V.; Agarwal, S. Role of antioxidant lycopene in cancer and heart disease. *J. Am. Coll. Nutr.* **2000**, *19*, 563–569.
- (2) Hwang, E. S.; Bowen, P. E. Cell cycle arrest and induction of apoptosis by lycopene in LNCaP human prostate cancer cells. *J. Med. Food* **2004**, *7*, 284–289.
- (3) Beilby, J.; Ambrosini, G. L.; Rossi, E.; de Klerk, N. H.; Musk, A. W. Serum levels of folate, lycopene, β -carotene, retinol and vitamin E and prostate cancer risk. *Eur. J. Clin. Nutr.* **2010**, *64*, 1235–1238.
- (4) Riccioni, G.; Scotti, L.; Di Ilio, E.; Bucciarelli, V.; Ballone, E.; De Girolamo, M. D.; Orazio, N.; Martini, F.; Aceto, A.; Bucciarelli, T. Lycopene and preclinical carotid atherosclerosis. *J. Biol. Regul. Homeost. Agents* **2011**, *25*, 435–441.
- (5) Levy, J.; Bosin, E.; Feldman, B.; Giat, Y.; Miinster, A.; Danilenko, M.; Sharoni, Y. Lycopene is a more potent inhibitor of human cancer cell proliferation than either α -carotene or β -carotene. *Nutr. Cancer* **1995**, *24*, 257–266.
- (6) Nahum, A.; Hirsch, K.; Danilenko, M.; Watts, C. K.; Prall, O. W.; Levy, J.; Sharoni, Y. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27 Kip1 in the cyclin E-cdk2 complexes. *Oncogene* **2001**, *20*, 3428–3436.
- (7) Trejo-Solís, C.; Pedraza-Chaverrí, J.; Torres-Ramos, M.; Jiménez-Farfán, D.; Cruz Salgado, A.; Serrano-García, N.; Osorio-Rico, L.; Sotelo, J. Multiple molecular and cellular mechanisms of action of lycopene in cancer inhibition. *J. Evid. Based Complementary Altern. Med.* **2013**, *2013*, No. 705121.
- (8) Britton, G. Structure and properties of carotenoids in relation to function. *FASEB J.* **1995**, *9*, 1551–1558.
- (9) Angelova, T.; Warthesen, J. Lycopene stability in tomato powders. *J. Food Sci.* **2000**, *65*, 67–70.
- (10) Rodriguez-Amaya, D. B.; Kimura, M. *HarvestPlus Handbook of Carotenoid Analysis*; HarvestPlus, 2004.
- (11) Yeum, K. J.; Leekim, Y. C.; Yoon, S.; Lee, K. Y.; Park, I. S.; Lee, K. S.; Kim, B. S.; Tang, G. W.; Russell, R. M.; Krinsky, N. I. Similar metabolites formed from β -carotene by human gastric mucosal homogenates, lipoxygenase, or linoleic-acid hydroperoxide. *Arch. Biochem. Biophys.* **1995**, *321*, 167–174.
- (12) Hu, K. Q.; Liu, C.; Ernst, H.; Krinsky, N. I.; Russell, R. M.; Wang, X.-D. The biochemical characterization of ferret carotene-9', 10'-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. *J. Biol. Chem.* **2006**, *281*, 19327–19338.
- (13) Wang, X. D. Lycopene metabolism and its biological significance. *Am. J. Clin. Nutr.* **2012**, *96*, 1214–1222.
- (14) Nagao, A. Oxidative conversion of carotenoids to retinoids and other products. *J. Nutr.* **2004**, *134*, 237–240.
- (15) King, T. J.; Khachik, F.; Borkiewicz, H.; Fukushima, L. H.; Morioka, S.; Bertram, J. S. Metabolites of dietary carotenoids as potential cancer preventive agents. *Pure Appl. Chem.* **1997**, *69*, 2135–2140.
- (16) Lakshminarayana, R.; Aruna, G.; Sathisha, U. V.; Dharmesh, S. M.; Baskaran, V. Structural elucidation of possible lutein oxidation products mediated through peroxyl radical inducer 2, 2'-Azobis (2-

methylpropionamidine) dihydrochloride: Antioxidant and cytotoxic influence of oxidized lutein in HeLa cells. *Chem.-Biol. Interact.* **2013**, *203*, 448–455.

(17) Arathi, B. P.; Sowmya, P. R.; Vijay, K.; Baskaran, V.; Lakshminarayana, R. Metabolomics of carotenoids: The challenges and prospects - A review. *Trends Food Sci. Technol.* **2015**, *45*, 105–117.

(18) Khachik, F.; Spangler, C. J.; Smith, J. C.; Canfield, L. M.; Steck, A.; Pfander, H. Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. *Anal. Chem.* **1997**, *69*, 1873–1881.

(19) Kim, S. J.; Nara, E.; Kobayashi, H.; Terao, J.; Nagao, A. Formation of cleavage products by autoxidation of lycopene. *Lipids* **2001**, *36*, 191–199.

(20) Caris-Veyrat, C.; Schmid, A.; Carail, M.; Böhm, V. Cleavage products of lycopene produced by in vitro oxidations: characterization and mechanisms of formation. *J. Agric. Food Chem.* **2003**, *51*, 7318–7325.

(21) Zhang, H.; Kotake-Nara, E.; Ono, H.; Nagao, A. A novel cleavage product formed by autoxidation of lycopene induces apoptosis in HL-60 cells. *Free Radical Biol. Med.* **2003**, *35*, 1653–1663.

(22) Gajic, M.; Zaripheh, S.; Sun, F. R.; Erdman, J. W., Jr Apo-8'-lycopenal and apo-12'-lycopenal are metabolic products of lycopene in rat liver. *J. Nutr.* **2006**, *136*, 1552–1557.

(23) Rodriguez, E. B.; Rodriguez-Amaya, D. B. Lycopene epoxides and apo-lycopenals formed by chemical reactions and autoxidation in model systems and processed foods. *J. Food Sci.* **2009**, *74*, 674–682.

(24) Kopeck, R. E.; Riedl, K. M.; Harrison, E. H.; Curley, R. W., Jr; Hruszkewycz, D. P.; Clinton, S. K.; Schwartz, S. J. Identification and quantification of apo-lycopenals in fruits, vegetables and human plasma. *J. Agric. Food Chem.* **2010**, *58*, 3290–3296.

(25) Reynaud, E.; Aydemir, G.; Rühl, R.; Dangles, O.; Caris-Veyrat, C. Organic synthesis of new putative lycopene metabolites and preliminary investigation of their cell-signaling effects. *J. Agric. Food Chem.* **2011**, *59*, 1457–63.

(26) Goupy, P.; Reynaud, E.; Dangles, O.; Caris-Veyrat, C. Antioxidant activity of (all-E)-lycopenol and synthetic apo-lycopenols in a chemical model of oxidative stress in the gastro-intestinal tract. *New J. Chem.* **2012**, *36*, 575–587.

(27) Sharoni, Y.; Linnewiel-Hermoni, K.; Zango, G.; Khanin, M.; Salman, H.; Veprik, A.; Danilenko, M.; Levy, J. The role of lycopene and its derivatives in the regulation of transcription systems: implications for cancer prevention. *Am. J. Clin. Nutr.* **2012**, *96*, 1173–1178.

(28) Narayanasamy, S.; Sun, J.; Pavlovic, R. E.; Eroglu, A.; Rush, C. E.; Sunkel, B. D.; Li, C.; Harrison, E. H.; Curley, R. W., Jr. Synthesis of apo-13-, and apo-15-lycopenols, cleavage products of lycopene that are retinoic acid antagonists. *J. Lipid Res.* **2017**, *58*, 1021–1029.

(29) Arathi, B. P.; Sowmya, P. R.; Kuriakose, G. C.; Vijay, K.; Baskaran, V.; Jayabaskaran, C.; Lakshminarayana, R. Enhanced cytotoxic and apoptosis inducing activity of lycopene oxidation products in different cancer cell lines. *Food Chem. Toxicol.* **2016**, *97*, 265–276.

(30) Arathi, B. P.; Sowmya, P. R.; Vijay, K.; Dilshad, P.; Saikat, B.; Gopal, V.; Lakshminarayana, R. An improved method of UPLC-PDA-MS/MS analysis of lycopene isomers. *Food Anal. Method.* **2015**, *8*, 1962–1969.

(31) Sowmya, P. R.; Arathi, B. P.; Vijay, K.; Baskaran, V.; Lakshminarayana, R. 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. *Food Chem. Toxicol.* **2017**, *106*, 58–69.

(32) Srivastava, M.; Hegde, M.; Chiruvella, K. K.; Koroth, J.; Bhattacharya, S.; Choudhary, B.; Raghavan, S. C. Sapodilla plum (*Achras sapota*) induces apoptosis in cancer cell lines and inhibits tumor progression in mice. *Sci. Rep.* **2015**, *4*, 6147.

(33) Lin, C.; Huang, C.; Hu, M. The use of fetal bovine serum as delivery vehicle to improve the uptake and stability of lycopene in cell culture studies. *Br. J. Nutr.* **2007**, *98*, 226–232.

(34) Re, R.; Mishra, G. D.; Thane, C. W.; Bates, C. J. Tomato consumption and plasma lycopene concentration in people aged 65 y and over in a British national survey. *Eur. J. Clin. Nutr.* **2003**, *57*, 1545–54.

(35) Fielding, J. M.; Rowley, K. G.; Cooper, P.; Dea, K. Increases in plasma lycopene concentration after consumption of tomatoes cooked with olive oil. *Asia Pac. J. Clin. Nutr.* **2005**, *14*, 131–136.

(36) Forbes, K.; Gillette, K.; Sehgal, I. Lycopene increases urokinase receptor and fails to inhibit growth or connexin expression in a metastatically passaged prostate cancer cell line: a brief communication. *Exp. Biol. Med.* **2003**, *228*, 967–971.

(37) Wu, K.; Erdman, J. W.; Schwartz, S.; Platz, E. A.; Leitzmann, M.; Clinton, S. K.; DeGroff, V.; Willett, W. C.; Giovannucci, E. Plasma and dietary carotenoids, and the risk of prostate cancer: A nested case-control study. *Cancer Epidemiol., Biomarkers Prev.* **2004**, *13*, 260–269.

(38) Sesso, H. D.; Buring, J. E.; Zhang, S. M.; Norkus, E. P.; Gaziano, J. M. Dietary and plasma lycopene and the risk of breast cancer. *Cancer Epidemiol., Biomarkers Prev.* **2005**, *14*, 1074–1081.

(39) Burgess, L. C.; Rice, E.; Fischer, T.; Seekins, J. R.; Burgess, T. P.; Sticka, S. J.; Klatt, K. Lycopene has limited effect on cell proliferation in only two of seven human cell lines (both cancerous and noncancerous) in an in vitro system with doses across the physiological range. *Toxicol. In Vitro* **2008**, *22*, 1297–1300.

(40) Pennathur, S.; Maitra, D.; Byun, J.; Sliskovic, I.; Abdul Hamid, I.; Saed, G. M.; Diamond, M. P.; Abu-Soud, H. M. Potent antioxidative activity of lycopene: A potential role in scavenging hypochlorous acid. *Free Radical Biol. Med.* **2010**, *49*, 205–213.

(41) Teodoro, A. J.; Oliveira, F. L.; Martins, N. B.; Maia, G. D. A.; Martucci, R. B.; Borojevic, R. Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. *Cancer Cell Int.* **2012**, *12*, 36.

(42) Takeshima, M.; Ono, M.; Higuchi, T.; Chen, C.; Hara, T.; Nakano, S. Anti-proliferative and apoptosis inducing activity of lycopene against three subtypes of human breast cancer cell lines. *Cancer Sci.* **2014**, *105*, 252–257.

(43) Dos Santos, R. C.; Ombredane, A. S.; Souza, J. M. T.; Vasconcelos, A. G.; Plácido, A.; Amorim, A. D. G. N.; Barbosa, E. A.; Lima, F. C. D. A.; Ropke, C. D.; Alves, M. M. M.; Arcanjo, D. D. R.; Carvalho, F. A. A.; Delerue-Matos, C.; Joanitti, G. A.; Leite, J. R. S. A. Lycopene-rich extract from red guava (*Psidium guajava* L.) displays cytotoxic effect against human breast adenocarcinoma cell line MCF-7 via an apoptotic-like pathway. *Food Res. Int.* **2018**, *105*, 184–196.

(44) Ford, N. A.; Elsen, A. C.; Zuniga, K.; Lindshield, B. L.; Erdman, J. W., Jr. Lycopene and apo-12'-lycopenol reduce cell proliferation and alter cell cycle progression in human prostate cancer cells. *Nutr. Cancer* **2011**, *63*, 256–263.

(45) Liu, A.; Pajkovic, N.; Pang, Y.; Zhu, D.; Calamini, B.; Mesecar, A. L.; van Breemen, R. B. Absorption and subcellular localization of lycopene in human prostate cancer cells. *Mol. Cancer Ther.* **2006**, *5*, 2879–85.

(46) van den Berg, H. Carotenoid interactions. *Nutr. Rev.* **1999**, *57*, 1–10.

(47) Palozza, P.; Serini, S.; Di Nicuolo, F.; Calviello, G. Modulation of apoptotic signalling by carotenoids in cancer cells. *Arch. Biochem. Biophys.* **2004**, *430*, 104–109.