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9-*cis*-canthaxanthin exhibits higher pro-apoptotic activity than all-*trans*-canthaxanthin isomer in THP-1 macrophage cells

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Abstract
All-*trans*-canthaxanthin (4, 4′-diketo β-carotene) but not 9-*cis*-canthaxanthin has been shown to induce apoptosis in some cell lines. In this study apoptotic activity of 9-*cis*-canthaxanthin on THP-1 macrophage is reported. Comparison of apoptotic activities of the two canthaxanthin isomers on this cell line by annexin V-cy3 and TUNEL assays indicated the higher pro-apoptotic activity of 9-*cis*-isomer than the all-*trans*-isomer. Canthaxanthin-induced apoptosis in this cell line was found to be accompanied by increased caspase-3 and caspase-8 activities, indicating its progression via caspase cascade. Induction of both caspase activities was higher by 9-*cis*-canthaxanthin than that by *trans*-canthaxanthin. All these results suggest that canthaxanthin stereoisomers differentially induce apoptosis of THP-1 monocyte/macrophage.

Keywords: *Apoptosis, differentiation, canthaxanthin isomers, carotenoids*

Introduction

Canthaxanthin (4, 4′-diketo β-carotene), a keto-carotenoid, is used extensively in poultry and aquaculture as feed additive [1]. This xanthophyll possesses no provitamin A activity, but is reported to be a more potent antioxidant than other carotenoids [2]. It has been shown to induce apoptosis in murine melanoma, fibrosarcoma and human squamous carcinoma cell lines [3] besides human adenocarcinoma and melanoma cell lines [4], but did not affect the growth of prostate cancer cells [5] and T-lymphoblast cell line [6]. All these studies have employed synthetic all-*trans*-canthaxanthin. However, carotenoid isomers are reported to differ in their biological properties, such as altered bioavailability and antioxidant capacity [7,8].

In the present study, we examined the apoptotic activity of canthaxanthin isomers in the human monocyte/macrophage cell line THP-1. Monocytes/macrophages are an integral part of the immune response during inflammation [9], but display delay or resistance to apoptosis in chronic inflammatory diseases such as asthma [10] and rheumatoid arthritis [11]. Our results strongly suggest the higher pro-apoptotic activity of 9-*cis*-canthaxanthin than the all-*trans*-canthaxanthin in THP-1 macrophage.

Materials and methods

Materials

Growth media (RPMI 1640 containing 2 mM L-glutamine) and antibiotic-anti-mycotic solution were purchased from HiMedia Laboratories (India). Foetal bovine serum (FBS), phorbol myristic acid (PMA), trypan blue and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO). HPLC grade tetrahydrofuran (THF) was procured

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from E-Merck (India). Synthetic all-*trans*-canthaxanthin (s-AT-cx) was procured from CaroteNature GmbH (Switzerland). Natural all-*trans*-canthaxanthin (n-AT-cx) and 9-*cis*-canthaxanthin (*cis*-cx) were extracted from *Dietzia* sp. K44 (Microbial Type Culture Collection, India; Accession No. MTCC 7402) under dark conditions using chloroform:methanol (1:1, v/v). Total carotenoids were concentrated, resuspended in cold acetone and were subjected to silica gel (100 mesh) chromatography using a linear gradient of hexane-chloroform-methanol. All-*trans*-canthaxanthin and 9-*cis*-canthaxanthin isomers were separated by HPLC (W 600 controller pump, PDA 2996 photo diode array detector, Waters, USA) with analytical Sunfire^{TM} C_{18} (5 μm; 4.6 × 150 mm) column using acetonitrile:methanol (7:3, v/v, 1 mL/min) and were stored at −20°C under nitrogen. Other chemicals and solvents were of reagent grade.

Cell culture and MTT assay

The non-adherent human myelomonocytic THP-1 cell line was obtained from National Centre for Cell Science (Pune, India) and propagated in RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat inactivated FBS. Cells were grown to confluence at 37°C in a humidified incubator supplied with 5% CO_2. Monocytes were allowed to differentiate into macrophages in the presence of 50 ng/mL PMA for 36 h prior to canthaxanthin treatment. Stock solutions of canthaxanthin isomers (8 mM) were freshly prepared in distilled THF. Aliquots were rapidly added to the culture medium to give the desired final concentration. The final concentration of THF in the culture medium was always kept below 0.5% (v/v). Control cultures received an amount of THF equal to that present in canthaxanthin-treated culture. To evaluate the effect of canthaxanthin isomers on the viability of THP-1 monocyte or macrophage, 5×10^4 cells/well were seeded in a 96-well plate and treated with 10, 20 and 30 μM canthaxanthin isomer for 24, 48 and 72 h. Cell viability was estimated by MTT assay [12] by recording the absorbance at 595 nm in a microplate reader (Molecular Devices, USA).

Annexin V–Cy3.18/6-Carboxyfluorescein diacetate (6-CFDA) staining

Apoptosis in macrophages was quantified by labelling with the APOAC Annexin V-Cy3 apoptosis detection kit (Sigma-Aldrich Co.), 72 h following treatment with 30 μM canthaxanthin isomers according to the manufacturer's protocol. The numbers of live, apoptotic and dead cells were calculated by counting cells in at least four fields, each having 100 or more cells.

TUNEL (Tdt-mediated dUTP nick end labelling) assays

DNA fragmentation in macrophage (1×10^6 cells per well) treated with 30 μM canthaxanthin isomers for 72 h was detected by means of DeadEnd^{TM} Colorimetric TUNEL system kit (Promega, Madison, WI) according to the manufacturer's instructions. Images were taken under inverted light microscope (Nikon, Japan) at 40× magnification using digital camera DXM 1200F and ACT-1 software system. The percentage of TUNEL-positive cells were determined from the mean of four random fields in each experimental set.

Caspase-3 and caspase-8 assays

Caspase assays were individually performed on macrophages (2×10^6 cells per assay) treated with 30 μM canthaxanthin isomer for 72 h, using caspase-3 and caspase-8 colorimetric assay kits (R & D Systems, Inc. MN). Caspase enzymatic activities were quantified spectrophotometrically at 405 nm in a microplate reader (Molecular devices, USA).

Statistics

Data are expressed as the means ± SD from at least three independent experiments, unless otherwise indicated. Statistical analysis was done with Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

Results and discussion

Canthaxanthin reduced viability of monocytes/ PMA-differentiated macrophages

Viability of both the cell types significantly decreased with increasing concentration of each canthaxanthin isomer compared to untreated controls (Figure 1A and B). However, monocytes showed increased cell death compared to the macrophages. The canthaxanthin isomers did not cause significant difference in the viability of cells at 10 μM up to 24 h. However, 9-*cis*-canthaxanthin showed significantly higher activity than its all-*trans* counterpart at all concentrations greater than 10 μM after 24 h of incubation. At 30 μM all-*trans* canthaxanthin reduced the viability of THP-1 macrophages to 42.2% while the *cis* isomer reduced the same to 23.1%. Similarly, 19.6% and 13.7% of monocytes were found viable when incubated with all-*trans* and *cis*-canthaxanthin isomer, respectively. Synthetic and natural all-*trans*-canthaxanthin did not show appreciable difference in their effects on the viability of either cell type.

Morphological changes in monocyte as well as macrophage were inconspicuous at 10 μM canthaxanthin. However, at 20 μM, both the isomers were found to induce differentiation in monocytes after 48 h. Macrophages also demonstrated augmented maturation at 20 μM concentration with increased

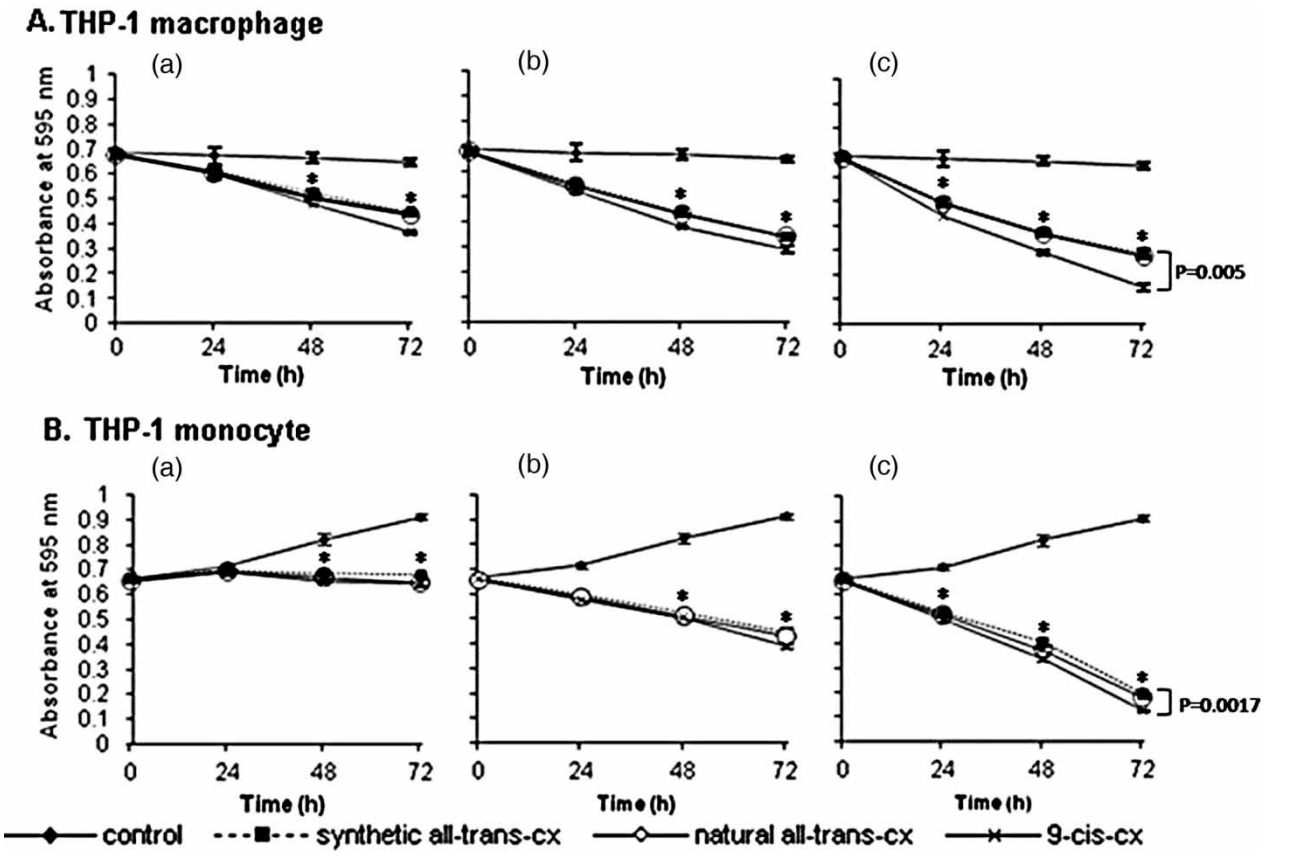

Figure 1. Viability of PMA differentiated THP-1 macrophage (A) and THP-1 monocytes (B) on incubation with different canthaxanthin (cx) isomers as determined by MTT assay. a, b, and c represent 10, 20 and 30 μM concentrations, respectively. Cells without canthaxanthin treatment are referred as control. Each value is mean ± SD ($n = 3$). *Values significantly different from the respective controls ($p < 0.05$, student's t-test).

cytoplasmic protrusions and cytoplasm/nuclei ratio, as compared to those observed in control cells. Major morphological changes indicative of apoptosis progression, including cell shrinkage, cytoplasmic condensation with surface detachment (floaters) and blebbing became very prominent at 30 μM concentration, particularly in the case of macrophages incubated with 9-*cis*-canthaxanthin for 48 h (Figure 2). In agreement, Nikawa et al. [13] reported that all-*trans*-canthaxanthin induced differentiation in F-9 mouse embryonal carcinoma cells. They suggested that this effect was triggered by chemical or enzymatic conversion of canthaxanthin to 4-oxoretinoic acid isomers and further oxidation products. The half-life of all-*trans*-canthaxanthin in cell culture medium has been accounted as 101 h [14]. Thus, it is likely that the effective concentration of its decomposition products in the cell medium is attained only after 24 h, causing differentiation and cell death in a concentration- and time-dependent manner in THP-1 cells.

Canthaxanthin-induced apoptosis in THP-1 macrophage

Staining canthaxanthin treated macrophages with annexin-cy3/6-CFDA produced labelling of cells with both dyes. The cells appeared yellow in the merged images, signifying induction of apoptotic pathway by all the isomers (Figure 3A). Control cells acquired only 6-CFDA staining indicating cell viability. Higher apoptotic activity, in terms of percentage apoptotic cells, of 9-*cis*-canthaxanthin (94 ± 3.47%) than that of all-*trans*-canthaxanthin (synthetic, 86 ± 4.66%; natural, 88 ± 4.52%) was evident from the merged images in Figure 3A. The increased percentage of TUNEL-positive cells in 9-*cis*-canthaxanthin-treated macrophage (84 ± 3.86%) than that with synthetic (56 ± 4.53%) or natural (58 ± 4.08%) all-*trans*-canthaxanthin-treated cells also reaffirmed the higher pro-apoptotic potential of 9-*cis*-canthaxanthin over the all-*trans* isomers (Figure 3B). It is well known that annexin-cy3 staining indicates plasma membrane phosphotidylserine translocation, an early event in apoptosis, while TUNEL positive nuclei are markers for DNA fragmentation, a late event in apoptosis. Thus, the higher percentage of annexin-Cy3-6-CFDA positive cells than TUNEL positive nuclei indicated the progression of apoptosis in all the cells.

Caspase-3 and caspase-8 activities in canthaxanthin-treated cells

Caspase-3 and caspase-8 activities in THP-1 cells increased significantly on treatment with either

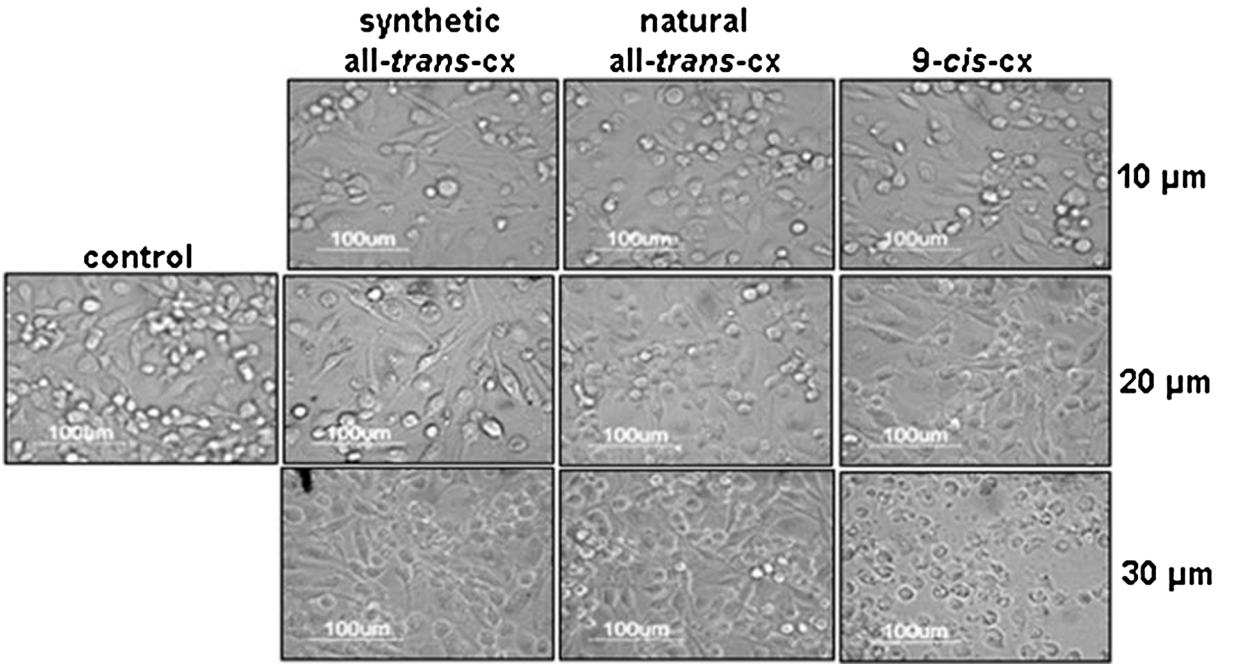

Figure 2. Appearance of PMA differentiated THP-1 macrophage, after 72 h of canthaxanthin (cx) treatment at three different concentrations. Cells without canthaxanthin treatment are referred as control. Representative images from four independent experiments are shown as seen under light microscope (40 ×).

canthaxanthin isomers, but the increase was more with 9-*cis* isomer. Induction of these two enzyme activities by natural and synthetic all-*trans*-canthaxanthin isomers were almost the same (Figure 4).

Activation of caspases in canthaxanthin-treated cells indicated the progression of apoptosis via caspase cascade. The effector caspase, caspase-3 is responsible either partially or totally for the proteolytic cleavage of many key proteins during apoptosis [15]. In tumour cells activation of caspase-8 by β-carotene treatment is reported to initiate the caspase cascade [16]. Oxidative stress has been suggested to be a mediator of apoptosis and several independent observations in diverse models support the role of oxidative mechanisms in induction of apoptosis [17,18]. Activation of caspase-8 in β-carotene-mediated apoptosis is hypothesized to be due to its pro-oxidant activity [19]. Carotenoids are reported to act as pro-oxidant or anti-oxidant, in the biological system depending on their concentration and oxygen tension [20–23]. The two canthaxanthin isomers were found to act as anti-oxidants at 10 µM up to 24 h and no conspicuous alteration in the THP-1 cell morphology or viability was observed. However, at higher concentration and increased incubation time 9-*cis*-canthaxanthin isomers were found to have greater pro-oxidant activity than the all-*trans*-canthaxanthin (our unpublished observations). The pro-oxidant activity of the canthaxanthins is reported to be due to the generation of free radical species carrying peroxyl radicals [21].

Canthaxanthins were found to be sparingly soluble in the RPMI 1640 medium used in this study. Solubility of 9-*cis*-canthaxanthin (9.81 nmol/ml) was observed to be higher than its all-*trans* isomer (6.49 nmol/ml) in the incubation medium (Supplementary Figure 1A). Intracellular content of 9-*cis* or all-*trans*-canthaxanthin in THP-1 cells was observed to be 0.0154 and 0.0087 nmol/10^5 cells, respectively, after 72 h of incubation (Supplementary Figure 1B). Synthetic and natural all-*trans*-canthaxanthin did not show any significant difference in solubility and cellular uptake. It has been reported that the amount of canthaxanthin incorporated to cells is lower when compared to the other carotenoids [24]. Use of high dosage of canthaxanthin for medical purposes is reported to cause reversible crystalline retinopathy in humans and does not cause any vision loss. However, this phenomenon is associated only with very high doses (> 0.2 mg/kg body wt/d) of canthaxanthin [25].

The biological activity of 9-*cis*-canthaxanthin was found to be distinctly more intense than its all-*trans* isomer in all the assays, irrespective of the source of canthaxanthin. Carotenoids predominantly occur in their stable all-*trans* configuration as the presence of a *cis* double bond creates greater steric hindrance between nearby hydrogen atoms and/or methyl groups [26]. The *cis* bonds possess higher potential energy and hence are more susceptible to various reactions than the *trans* bonds [8]. Also, the *cis*-isomers have lower melting points than their all-*trans* counterparts, due to a decreased tendency to

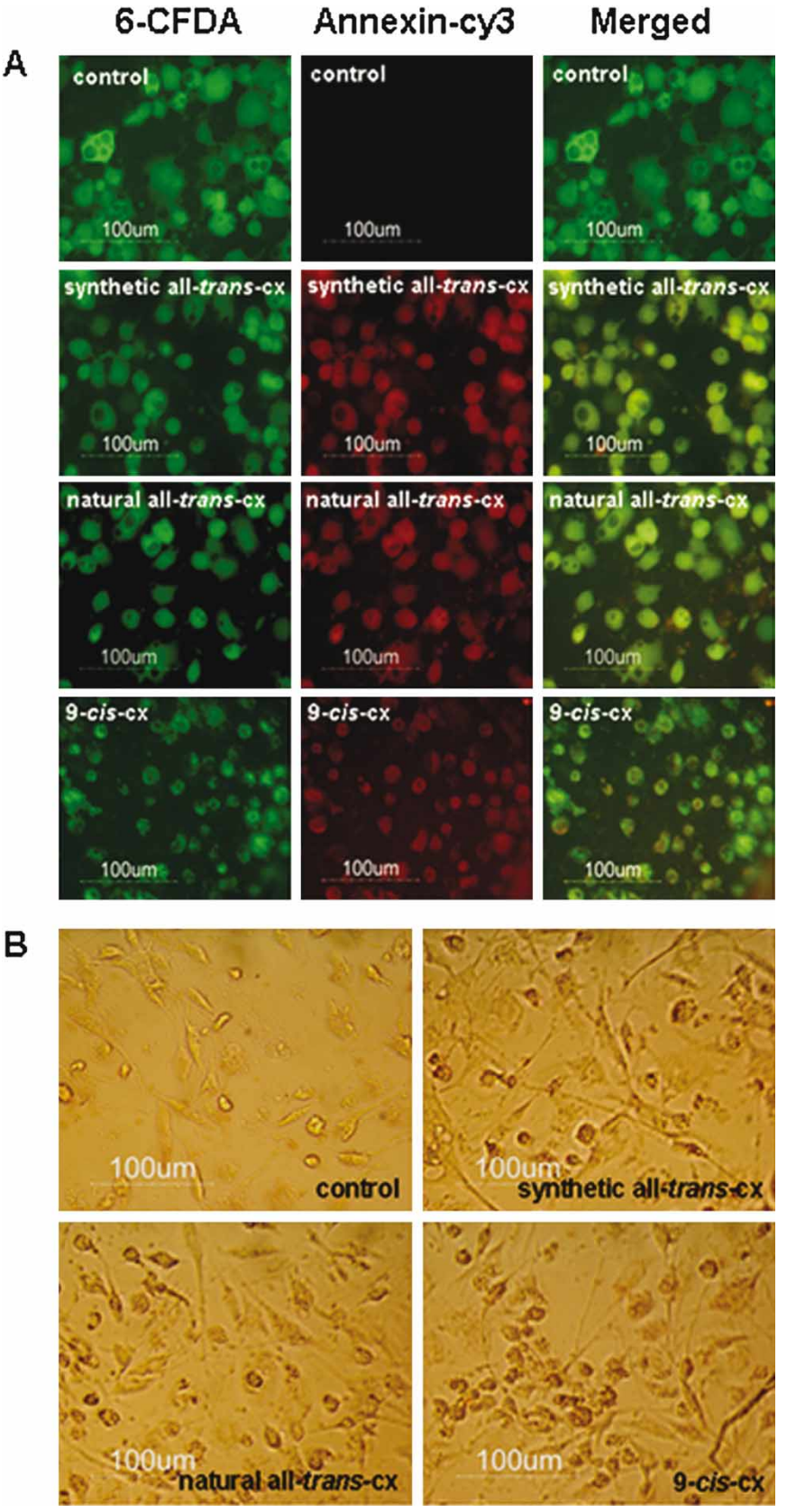

Figure 3. (A) 6-CFDA-annexin-Cy3.18 staining in THP-1 macrophage. Cells in the panel without canthaxanthin (cx) treatments are designated as control. Green, yellow and red cells are indicative of viable, apoptotic and dead cells, respectively. The composite panels in the right column were obtained by merging panels of the left columns (6-CFDA stained) with the corresponding panels of the middle column (annexin-Cy3 stained) using Adobe Photoshop 7.0 software. (B) The canthaxanthin isomer induced apoptosis of THP-1 macrophage by TUNEL assays as against control cells. Apoptotic nuclei were stained dark brown. This figure is reproduced in colour in *Free Radical Research* online.

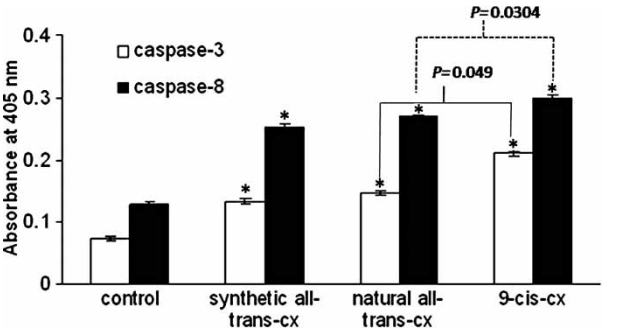

Figure 4. Induction of caspase-3 and caspase-8 activities in canthaxanthin (cx) isomer treated THP-1 macrophage. Cells without canthaxanthin treatment are referred as control. Each value is mean ± SD ($n = 3$). *Values significantly different from the respective controls ($p < 0.05$, student's *t*-test).

crystallize or aggregate [7]. Higher solubility and cellular uptake of 9-*cis*-canthaxanthin than all-*trans* isomer (Supplementary Figure 1) may also contribute to the higher apoptotic activity of 9-*cis* isomer. The 9-*cis*-canthaxanthin appeared to generate more distinct metabolites than that from all-*trans* isomers when the culture media were analysed by RP-HPLC (results not shown). Further studies including the identification of 9-*cis* and all-*trans*-canthaxanthin metabolites are necessary to elucidate the mechanism of canthaxanthin-mediated apoptosis and higher apoptotic activity of 9-*cis*-canthaxanthin.

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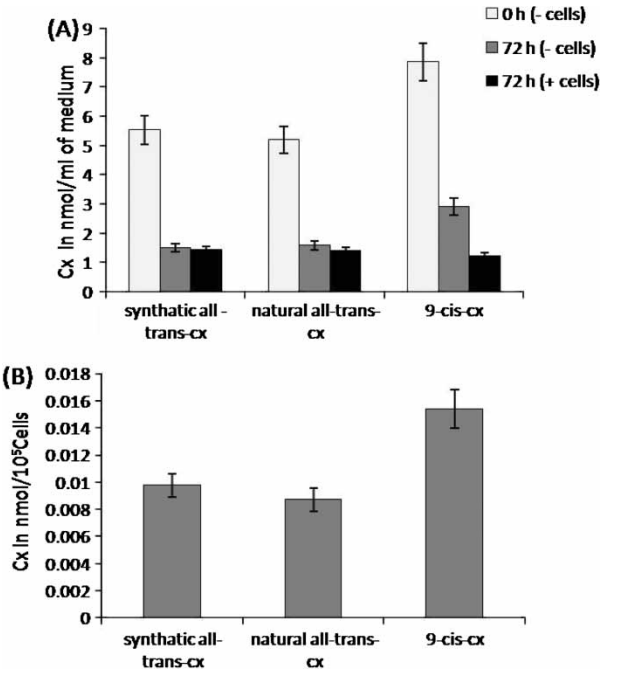

Supplementary Figure 1. (A) Soluble canthaxanthin (cx) in RPMI 1640 containing 10% bovine calf serum after incubation with or without THP-1 cells. (B) Intracellular canthaxanthin after incubation with cells. Aliquots from 8mM cx stock solution were added to culture medium with or without 1×10^5 THP-1 cells to give the final concentration of 30μM and incubated for 0 or 72 h. After the incubation medium was aspirated and filtered through 0.2μ syringe filter (to remove the insoluble cx crystals) and the filtrate was lyophilised to extract canthaxanthin. To estimate intracellular cx content after 72 h of incubation the cells were washed twice with 0.5 ml of PBS and pelleted. The carotenoid was extracted from lyophilized medium or cells using 1:3 (v/v) methanol and hexane:diethylether (1:1, v/v). Amount of extracted carotenoid was estimated spectrophotometrically. The results are shown as means of two independent sets of experiment. The variation was less than 5% ($p < 0.05$, student's t-test).