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Cancer chemopreventive activity of carotenoids in the fruits of red paprika *Capsicum annuum* L.

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Abstract

Capsanthin (1) and related carotenoids (2–7) isolated from the fruits of red paprika *Capsicum annuum* L. showed potent in vitro anti-tumor-promoting activity with inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Among them, capsanthin diester (3) and capsorbin diester (5) showed strong inhibitory effects. Furthermore, capsanthin (1), capsanthin 3′-ester (2) and capsanthin 3,3′-diester (3), major carotenoids in paprika, exhibited potent anti-tumor-promoting activity in an in vivo mouse skin two-stage carcinogenesis assay using 7, 12-dimethylbenz[a]anthracene as an initiator and TPA as a promoter.

Keywords: *Capsicum annuum*; Carotenoid; Capsanthin; Cancer chemopreventive activity; Epstein–Barr virus; Early antigen two-stage mouse skin carcinogenesis

1. Introduction

Numerous epidemiological studies have demonstrated that carotenoids may be responsible for the beneficial effects associated with the intake of green and yellow vegetables and fruits for cancer prevention in humans [1,2]. It has been become clear that not only β-carotene [3–5] but also α-carotene [6–8], lycopene [8,9] and some xanthophylls such as lutein [9], canthaxanthin [10], fucoxanthin [11], halocynthiaxanthin [12], etc. possess significant cancer chemopreventive effects.

Ripe fruits of paprika (red pepper), which are used widely as vegetables and food colorants, are good source of carotenoid pigments. The red carotenoids in paprika (*Capsicum annuum* L.) are mainly capsanthin, capsorubin and capsanthin 3,6-epoxide. All three compounds possess a 3-hydroxy-κ-end group [13–17]. Thus, we examined the anti-tumor-promoting activities of carotenoids in fruits of red paprika. In this paper, we report the inhibitory effects of capsanthin and related carotenoids isolated from paprika (Fig. 1) on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and in

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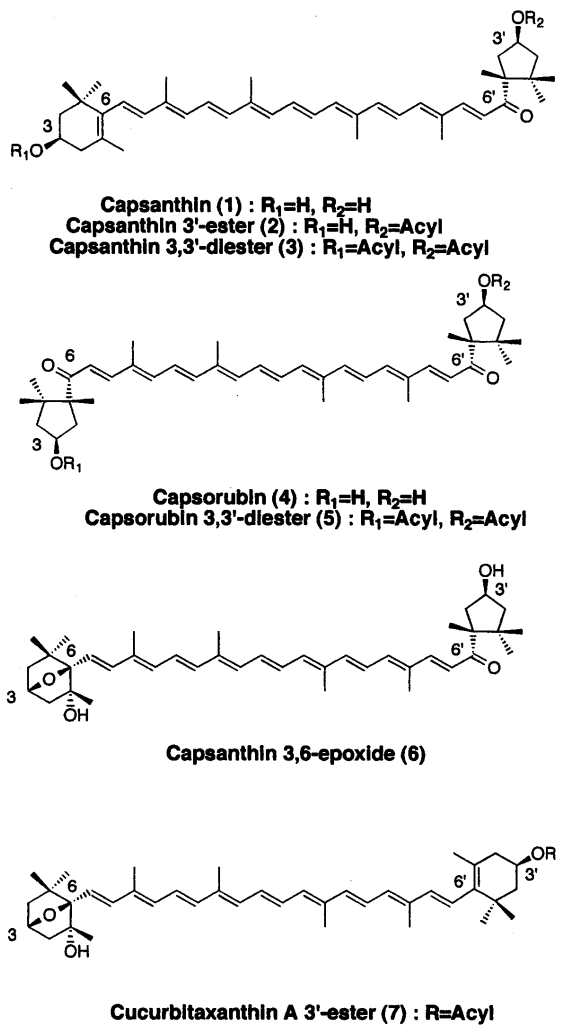

Fig. 1. Structures of capsanthin and related carotenoids isolated from paprika.

an in vitro two-stage carcinogenesis assay on mouse skin, using 7,12-dimethylbenz[a]anthracene as an initiator and TPA as a promoter.

2. Materials and methods

2.1. Apparatus

The UV-visible (vis) spectra were recorded with a Shimadzu UV-240 spectrophotometer in diethyl ether (Et_2O). The positive ion FAB MS spectra were recorded using a JEOL SX 102 mass spectrometer with *m*-nitrobenzyl alcohol as the matrix. The ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were measured with a Varian XL-300 spectrometer in $CDCl_3$ with TMS as an internal standard. HPLC was performed on a Shimadzu LC-6AD instrument with a Shimadzu SPD-6AV spectrophotometer set at 380 nm. The column used was a Lichrospher 100 RP-18 (Cica Merck, 20 × 250 mm, 10 μm) using dichloromethane (CH_2Cl_2)-acetonitrile (CH_3CN) (5:95) as the mobile phase, flow rate of 5 ml/min.

2.2. Plant material

The matured fruits were collected from paprika plants grown at the farm in Hitachinaka, Japan in September 1996.

2.3. Chemicals

The cell culture reagents, *n*-butyric acid and β-carotene were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 7,12-dimethylbenz[a]anthracene (DMBA) was obtained from Sigma Chemical Co. (St. Louis, MO).

2.4. Animals

Specific pathogen-free female ICR mice (6-weeks-old) were obtained from Japan SLC Inc. (Hamamatsu, Japan) and were housed five per polycarbonate cage, in a temperature-controlled room at 24 ± 2°C and given food and water ad libitum. All mice were fed Oriental MF (Oriental Yeast Co., Tokyo, Japan) ad libitum during the experiments.

2.5. In vitro EBV-EA induction effect

The EBV genome-carrying lymphoblastoid cells, Raji cells, derived from Burkitt's lymphoma, were cultivated in RPMI-1640 medium. The Raji cells were incubated for 48 h at 37°C in a medium containing *n*-butyric acid (4 mmol), TPA (32 pmol) and various amount of test compounds. Smears were made from the cell suspension, and the EBV-EA-inducing cell were stained by means of an indirect immunofluorescence technique, The details of the in vitro assay on EBV-EA induction have been reported previously [18].

2.6. *In vivo two-stage carcinogenesis assay on mouse skin papillomas promoted by TPA*

The animals were divided into four experimental groups, each with 15 mice. The backs of the mice were shaved with surgical clippers, and they were treated topically with DMBA (100 μg, 390 nmol) in acetone (0.1 ml) as the initiator. One week after initiation, papilloma formation was promoted twice a week by the application of TPA (1μg, 1.7 nmol) in acetone (0.1 ml) to the skin. Group I received TPA treatment alone, and groups II, III and IV received topical application of capsanthin (85 nmol), capsanthin 3′-ester (85 nmol) and capsanthin 3, 3′-diester (85 nmol) in acetone (0.1 ml), respectively, 1 h before the TPA treatment. The incidence and numbers of papillomas were monitored weekly for 20 weeks.

2.7. *Extraction, isolation and characterization of carotenoids*

The methanol (MeOH) extract of the fruits (800 g) of *C. annuum* L. was partitioned between *n*-hexane-Et_2O (1:1) and 10% aqueous NaCl. The organic layer was concentrated to dryness. The residue was subjected to silica gel column chromatography using hexane, hexane-ether (8:2), hexane-ether (7:3), hexane-ether (5:5), ether, ether-acetone (2:8), ether-acetone (5:5) and acetone, successively. Each fraction was further purified by HPLC on a C_{18} reversed phase column with CH_2Cl_2-CH_3CN (2:8) as the eluent. The structure and purity of carotenoids were confirmed by UV-vis, ^{1}H-NMR and FAB MS data and selected compounds were characterized by ^{13}C-NMR.

Capsanthin (1): yield 12 mg, 5.3 % of total carotenoid (eluted with ether-acetone (5:5) from silica gel column), UV-vis λ max (Et_2O) nm 468 and 496, FAB MS *m/z* 584 [M^+], ^{1}H-NMR and ^{13}C-NMR, the chemical shift and spin coupling constant values were in agreement with previously published values [16].

Capsanthin 3′-ester (2): yield 24 mg, 10.6% of total carotenoid (eluted with hexane-ether (1:1) from silica gel column), UV-vis λ max (Et_2O) nm 468 and 496, FAB MS *m/z* 822 [M^+] (3′-*O*-palmitoyl capsanthin), 794 (3′-*O*-myristoyl capsanthin), 766 (3′-*O*-lauroyl capsanthin), the relative ratio of intensity of each molecular ions, *m/z* 822: *m/z* 794: *m/z* 766 (12:70:18), ^{1}H-NMR ($CDCl_3$), the ^{1}H-NMR data were in agreement with 3′-*O*-esterified capsanthin reported by Goda et al. [19], ^{13}C-NMR ($CDCl_3$) δ 12.74 (C-19,20),12.80 (C-20′), 12.87 (C-19′), 14.13 (CH_3 fatty acid), 20.78 (C-18′), 21.63 (C-18), 22.69 (CH_2 fatty acid), 24.77 (CH_2 fatty acid), 25.05 (C-17′), 25.61 (C-16′), 28.72 (C-16), 29.14, 29.27, 29.35, 29.47, 29.60, 29.64 (CH_2 fatty acid), 30.26 (C-17), 31.91, 34.65 (CH_2 fatty acid), 37.12 (C-1), 42.20 (C-4′), 42.54 (C-4), 43.73 (C-1′), 47.66 (C-2′), 48.40 (C-2), 58.51 (C-5′), 65.08 (C-3), 73.24 (C-3′), 120.80 (C-7′), 124.05 (C-11), 125.51 (C-7), 125.84 (C-5), 124.60 (C-11′), 131.20 (C-10), 131.21 (C-15′), 132.35 (C-13), 133.95 (C-9′), 134.99 (C-14′), 135.87 (C-9), 136.11 (C-14), 136.94 (C-13′), 137.60 (C-12), 137.71 (C-6), 138.41 (C-8), 140.70 (C-10′), 141.82 (C-12′), 147.02 (C-8′), 173.63 (C = O fatty acid), 202.51 (C-6′).

Capsanthin 3,3′-diester (3): yield 40 mg, 17.8 % of total carotenoid (eluted with hexane-ether (8:2) from silica gel column); UV-vis λ max (Et_2O) nm 468 and 496; FAB MS *m/z* 1060 [M^+] (dipalmitoyl capsanthin), 1032 (palmitoyl myristoyl capsanthin), 1004 (dimyristoyl capsanthin or palmitoyl lauroyl capasnthin), 976 (lauroyl myristoyl capsanthin), 948 (dilauroyl capsanthin), the relative ratio of intensity of each molecular ion, *m/z* 1060: *m/z* 1032: *m/z* 1004: *m/z* 976:948 (4:14:35:36:11); ^{1}H-NMR ($CDCl_3$), the ^{1}H-NMR data were in agreement with 3′-*O*-esterified capsanthin reported by Goda et al. [19], ^{13}C-NMR ($CDCl_3$) δ 12.74 (C-19,20),12.80 (C-20′), 12.87 (C-19′), 14.13 (CH_3 fatty acid), 20.78 (C-18′), 21.53 (C-18), 22.69 (CH_2 fatty acid), 24.77 (CH_2 fatty acid), 25.05 (C-17′), 25.51 (C-16′), 28.62 (C-16), 29.14, 29.27, 29.35, 29.47, 29.60, 29.64 (CH_2 fatty acid), 30.16 (C-17), 31.91, 34.65 (CH_2 fatty acid), 36.82 (C-1), 38.60 (C-4), 42.20 (C-4'), 43.73 (C-1′), 44.11 (C-2), 47.66 (C-2′), 58.51 (C-5′), 68.50 (C-3), 73.24 (C-3′, The signals in the olefinic region are the same as that of 2.

Capsorubin (4): yield 12 mg, 5.3 % of total carotenoid (eluted with ether-acetone (2:8) from silica gel column), UV-vis λ max (Et_2O) nm 445, 479 and 510, FAB-MS *m/z* 600 [M^+], ^{1}H-NMR and ^{13}C-NMR, the chemical shift and spin coupling constant values were in agreement with previously published values *[16].*

Capsorubin 3,3′-diester (5): yield 12 mg, 5.3 % of total carotenoid (eluted with hexane-ether (7:3) from

silica gel column), UV-vis λ max (Et_2O) nm 445, 479 and 510, FAB-MS *m/z* 1076 [M^+] (dipalmitoyl capsorubin), 1048 (palmitoyl myristoyl capsorubin), 1020 (dimyristoyl capsorubin), 992 (myristoyl lauroyl capsorubin), 964 (dilauroyl capsorubin), the relative ratio of intensity of each molecular ion, *m/z* 1076: *m/z* 1048: *m/z* 922: *m/z* 964 (6:18:41:24:11), ^{1}H-NMR ($CDCl_3$) δ 0.86 (6H, s, H-16, 16′), 0.88 (6H, t, J = 7 Hz, CH_3 fatty acid), 1.18 (6H, s, H-17, 17′), 1.25 (s, CH_2 fatty acid), 1.32 (6H, s, H-18, 18′), 1.57 (2H, dd, J = 15, 3.5 Hz, H-4β, 4′β), 1.74 (2H, dd, J = 13.5, 4 Hz, H-2β, 2′β), 1.96 (6H, s, H-19, 19′), 1.99 (6H, s, H-20, 20′), 2.09 (2H, dd, J = 13.5, 8 Hz, H-2α, 2′α), 2.27 (4H, t, 7 Hz, CH_2 fatty acid), 2.99 (2H, dd, J = 15, 9Hz, H-4α, 4′a), 5.24 (2H, m, H-3, 3′), The signals in the olefinic region are the same as that of (4) [16], ^{13}C-NMR ($CDCl_3$) δ 12.80 (C-20, 20′), 12.87 (C-19, 19′), 14.13 (CH_3 fatty acid), 20.78 (C-18, 18′), 22.69 (CH_2 fatty acid), 24.77 (CH_2 fatty acid), 25.05 (C-17, 17′), 25.61 (C-16, 16′), 29.14, 29.27, 29.35, 29.47, 29.60, 29.64, 31.91, 34.65 (CH_2 fatty acid), 42.20 (C-4, 4′), 43.73 (C-1, 1′), 47.66 (C-2, 2′), 58.51 (C-5, 5′), 73.24 (C-3, 3′), 173.63 (C = O fatty acid), The signals in the olefinic region are the same as that of (4) [16].

Capsanthin 3,6-epoxide (6): yield 12 mg, 5.3% of total carotenoid (eluted with ether-acetone (2:8) from silica gel column), UV-vis λ max (Et_2O) nm 468, FAB MS *m/z* [M^+] 600, ^{1}H-NMR and ^{13}C-NMR, the chemical shift and spin coupling constant values were in agreement with previously published values [17].

Cucurbitaxanthin A 3′-ester (7): yield 12 mg, 5.3 % of total carotenoid (eluted with ether-hexane (3:7) from silica gel column), UV-vis λ max (Et_2O) nm 425, 444 and 472 nm, FAB-MS *m/z* 822 [M^+] (3′-*O*-palmitoyl cucurbitaxanthin A), 794 (3′-*O*-myristoyl cucurbitaxanthin A), 766 (3′-*O*-lauroyl cucurbitaxanthin A), the relative ratio of intensity of each molecular ion, *m/z* 822: *m/z* 794: *m/z* 766 (20:54:26), ^{1}H-NMR ($CDCl_3$) δ 0.88 (3H, s, H-17), 0.88 (3H, t, J = 7 Hz, CH_3 fatty acid), 1.08 (3H, s, H-16′), 1.11 (3H, s, H-17′), 1.21 (3H, H-18), 1.25 (s, CH_2 fatty acid), 1.44 (3H, s, H-16), 1.61 (1H, d, J = 11.5 Hz, H-2β), 1.72 (3H, s, H-18′), 1.84 (2H, m, H-2α, 2′ax), 1.95 (3H, s, H-19), 1.97 (9H, s, H-20, 19′, 20′), 2.29 (2H, t, J = 7, CH_2 fatty acid), 2.44 (1H, dd, J = 16, 6 Hz, H-4′eq), 4.40 (1H, t-like, J = 7 Hz, H-3), 5.07 (1H, m, H-3′), 5.74 (1H, d, J = 16 Hz, H-7), 6.10 (2H, m, H-7′,8′), 6.16 (1H, d, J = 11 Hz, H-10′), 6.20 (1H, d, J = 11 Hz, H-10), 6.25 (2H, m, H-14, 14′), 6.36 (2H, d, J = 15, H-12, 12′), 6.37 (1H, d, J = 16 Hz, H-8), 6.62 (4H, m H-11, 11′, 15, 15′). The following additional carotenoids were isolated from *C. annuum* L.: β-carotene (10 mg, 4.4% of total carotenoid), β-cryptoxanthin (1 mg, 0.4 %), cryptocapsin (2 mg, 0.8 %), cycloviolaxanthin (4 mg, 1.6 %), cucurbitaxanthin A (2 mg, 0.8 %), cucurbitachrome (10 mg, 4.4 %), zeaxanthin (50 mg, 22.0 %), 9-*Z*-

Table 1
Relative ratio of EBV-EA activation with respect to positive control (100%) in the presence of capsanthin (1) and related compounds (2–7).

	Relative ratio of EBV-EA activation[a]			
	Concentration (mol ratio/TPA)[b]			
Samples	1000	500	100	10
Capsanthin (**1**)	8.6 (70)[c]	40.3 (>80)	82.1 (>80)	100.0 (>80)
Capsanthin 3'-ester (**2**)	3.2 (70)	31.6 (>80)	73.4 (>80)	95.9 (>80)
Capsanthin diester (**3**)	0.0 (70)	24.5 (>80)	69.3 (>80)	90.4 (>80)
Capsorubin (**4**)	0.0 (70)	35.9 (>80)	80.1 (>80)	100.0 (>80)
Capsorubin diester (**5**)	0.0 (70)	26.1 (>80)	72.0 (>80)	92.8 (>80)
Capsanthin 3,6-epoxide (**6**)	0.0 (70)	32.8 (>80)	79.2 (>80)	94.6 (>80)
Cucurbitaxanthin A-3'ester (**7**)	0.0 (70)	39.0 (>80)	86.2 (>80)	100.0 (>80)
β-Carotene	2.5 (70)	25.0 (>80)	89.4 (>80)	100.0 (>80)

[a] Values represent relative percentage of the positive control value (100%).
[b] TPA concentration was 20 ng (32 pmol)/ml.
[c] Values in parentheses are viability percentages of Raji cells.

capsanthin (5 mg, 2.2 %), capsanthone (2 mg, 0.8 %), antheraxanthin (5 mg, 2.2 %), mutatoxanthin (2 mg, 0.8 %), violaxanthin (3 mg, 1.3 %), luteoxanthin (2 mg, 0.8 %), auroxanthin (2 mg, 0.8 %), neoxanthin (1 mg, 0.4 %). They were identified by UV-Vis, EI-MS, ^{1}H NMR and CD spectral data.

3. Results and discussion

In vitro anti-tumor promoting activities of capsanthin (1) and related carotenoids (2–7) isolated from paprika and of β-carotene, which is well known as a strong anti-tumor-promoter [3–5], were examined by using the Epstein–Barr virus (EBV) activation assay in Raji cells. Their inhibitory effects on the activation and viability of the Raji cells are shown in Table 1. Capsanthin (1) and related compounds (2-7) showed inhibitory effects on EBV-EA induction without significant cytotoxcity on Raji cells in the assay. Among them, capsanthin diester (3) and capsorubin diester (5) showed remarkable inhibitory effects at 1000 and 500 mol/TPA ratio. Furthermore, at 1000 mol/TPA ratio, capsorubin (4), capsanthin 3,6-epoxide (6) and cucurbitaxanthin A 3′-ester (7) were more active than β-carotene. Capsanthin 3′-ester (2) and capsanthin (1) showed equivalent and slightly weaker activity, respectively, than that of β-carotene. On the other hand, inhibitory activity of capsanthin derivatives increased in the order of capsanthin (1), capsanthin 3′-ester (2) and capsanthin diester (3). Capsorubin diester (5) was also more active than capsanthin (4). These results indicated that esterification of the hydroxy groups with fatty acids enhanced the inhibitory activity toward EBV-EA activation.

In vivo anti-tumor-promoting activity of capsanthin (1), capsanthin 3′-ester (2) and capsanthin diester (3) was elucidated by a two-stage carcinogenesis method for mouse skin papillomas promoted by TPA. The incidence (%) of papilloma-bearing mice and the average number of papillomas per mouse are presented and compared with a positive control in Fig. 2. When capsanthin (85 nmol), capsanthin 3′-ester (85 nmol) and capsanthin diester (85 nmol) were applied before each TPA treatment, they remarkably delayed the formation of papillomas and reduced the number of papillomas per mouse as shown in Fig. 2. In the positive control group (initiated with 390 nmol of DMBA and promoted with TPA), the first papillomas appeared within 6 weeks of promotion, and in the groups treated with (1), (2) and (3), the first papillomas appeared at 7, 9 and 9 weeks, respectively. After 9 weeks promotion, the positive control group showed a 100% incidence of papillomas, while in groups (1), (2) and 3, only 40, 20 and 0.6% of mice bore papillomas, respectively. After 20 weeks of promotion, more than nine papillomas were found per mouse in the control group. whereas only seven, six point three and five papillomas per mouse were found in groups (1), (2) and (3) respectively. Esterifi-

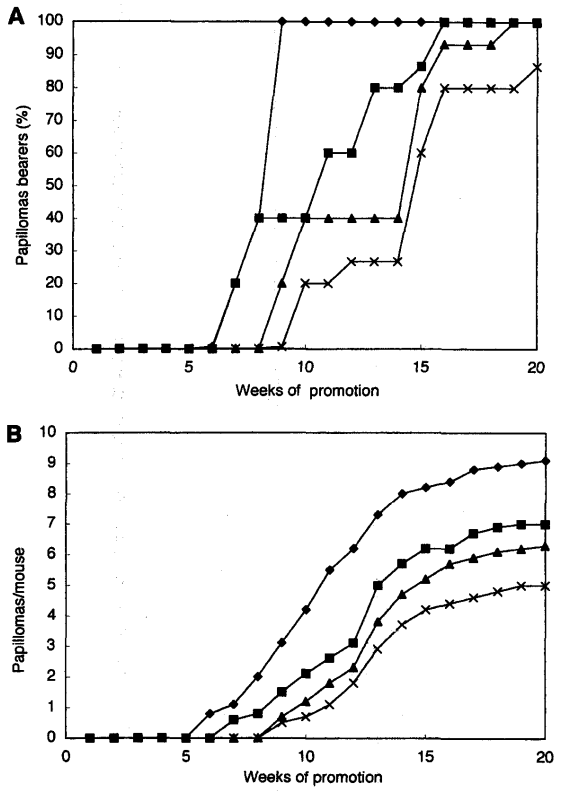

Fig. 2. Inhibition of TPA-induced tumor promoting by multiple application of capsanthin (1), capsanthin 3′-ester (2) and capsanthin diester (3). All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. (A) Percentage of mice bearing papillomas; (B) Average number of papillomas per mouse. (●), Control TPA alone; (■), TPA + 85 nmol of capsanthin (1): (▲), TPA + 85 nmol of capsanthin 3′-ester (2); (X), TPA + 85 nmol of capsanthin 3, 3′-diester (3).

cation of the hydroxy groups with fatty acids increased the anti-tumor promoting activity for two-stage carcinogenesis on mouse skin using DMBA as a initiator and TPA as a promoter. It is well known that esterification enhances the liposolubility and stability of xanthophylls. Thus, esterification may increase absorption of capsanthin into mouse skin tissue. This is the first report on in vivo anti-tumor promoting activity of capsanthin (1) and its esters (2) and (3), which are major carotenoids in paprika.

Capsanthin and capsorubin have been reported to show antioxidative activities for including quenching singlet oxygen [20], suppressing free radical induced lipid peroxidation [21,22] and suppressing generation of superoxide and nitric oxide [23]. On the basis of these results and the present results described above, paprika carotenoids might be valuable as anti-tumor-promoter and chemopreventive agents in chemical carcinogenesis.

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