

SUPPRESSION OF AZOXYMETHANE-INDUCED COLON CARCINOGENESIS IN MALE F344 RATS BY MANDARIN JUICES RICH IN β -CRYPTOXANTHIN AND HESPERIDIN

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We have reported protective effects of dietary administration of a powder “CHRP” containing high amounts of β -cryptoxanthin and hesperidin prepared from a Satsuma mandarin (*Citrus unshiu* Marc.) juice on azoxymethane (AOM)-induced rat aberrant crypt foci through suppression of crypt cell proliferation and/or induction of detoxifying enzymes. In the present study, we investigated the modifying effects of a commercial Satsuma mandarin (*Citrus unshiu* Marc.) juice (MJ) and those of MJ2 and MJ5, which were prepared from MJ and are richer in β -cryptoxanthin and hesperidin than MJ, on the occurrence of colonic tumors induced by AOM in male F344 rats. Rats were given 2 weekly s.c. injections of AOM (20 mg/kg body weight) to induce colonic neoplasms. They also received MJ, MJ2, or MJ5 as a drinking water at night for 36 weeks, starting 1 week after the last dosing of AOM. AOM exposure produced colonic adenocarcinoma with an incidence of 69% and a multiplicity of 0.76 ± 0.57 /rat at week 38. MJ, MJ2, and MJ5 administration significantly reduced the frequency of colonic carcinoma [MJ: 35% (49% reduction), $p < 0.02$; MJ2: 20% (64% reduction), $p = 0.0028$; and MJ5: 15% (78% reduction), $p < 0.00021$] and multiplicity [MJ: 0.40 ± 0.58 (47% reduction), $p < 0.05$; MJ2: 0.25 ± 0.43 (67% reduction), $p < 0.005$; and MJ5: 0.15 ± 0.36 (80% reduction), $p < 0.001$]. Also, the numbers of cancer cells positive for proliferative cell nuclear antigen (PCNA) and cyclin D1 in colonic tumors were lowered by these treatments. In addition, treatment with MJ, MJ2, or MJ5 significantly increased apoptotic index in colonic adenocarcinoma. These findings might suggest effective chemopreventive ability of MJ5, especially MJ5, in colon tumorigenesis. *Int. J. Cancer* 88:146–150, 2000.

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Dietary factors play an important role in prevention of human diseases including cancers (Rogers *et al.*, 1993). Colorectal cancer is the fourth most common malignant neoplasm in the world (Parkin *et al.*, 1999). In 1990, there were estimated to be a total of 8.1 million new cancer cases in the world (Parkin *et al.*, 1999). In developed countries, colorectal cancer was the second most common malignancy of both sexes in 1990 (Parkin *et al.*, 1999). Since an inverse relationship between the intake of fruits/vegetables and human colon cancer has been suggested (Block *et al.*, 1992; Dragsted, 1998), primary prevention, including chemoprevention utilizing the active compounds in edible plants, is important for reducing this malignancy (Greenwald *et al.*, 1995).

Carotenoids are a family of pigments with at least 600 members. They derive from lycopene after steps of cyclisation, dehydrogenation, and oxidation. They have several biological functions, such as provitamin A activity, scavenging of free radicals, enhancement of gap junctions, immunomodulation, and regulation of enzyme activity involved in carcinogenesis (Faure *et al.*, 1999). Certain carotenoids with or without provitamin A activity could inhibit chemical carcinogenesis in rodents (Tanaka *et al.*, 1995a,b, 1994a,b). Recently, β -cryptoxanthin, a carotenoid with nonsubstituted β -ionone cycles and provitamin A property, has been reported to have antipromoter action in *in vitro* study (Tsushima *et al.*, 1995). It also inhibits 2-stage mouse skin tumorigenesis in *in vivo* experiments (Onozuka *et al.*, 1998). Hesperidin, a type of

flavonoid that is present in several vegetables and fruits, is known to have certain biological activities including antioxidant property, anti-inflammatory effect, and inhibition of prostaglandin biosynthesis (Tanaka *et al.*, 1997). Our previous studies demonstrated that hesperidin inhibits chemically induced carcinogenesis in several organs (Tanaka *et al.*, 1997). We have found that a powder “CHRP”, which was prepared from a commercial Satsuma mandarin (*Citrus unshiu* Marc.) juice (MJ) and contained high amounts of β -cryptoxanthin (0.67%) and hesperidin (3.58%) (Sumida *et al.*, 1999b), effectively suppressed the development of azoxymethane (AOM)-induced aberrant crypt foci (ACF) (Kohno *et al.*, 1999), which are considered to be precursor lesions for colon carcinoma (Bird, 1995). More recently, we have prepared citrus juices MJ2 and MJ5, which are rich in β -cryptoxanthin (Table I), from MJ (Sumida *et al.*, 1999a).

As the major events leading to cell proliferation occur in the G1 phase, altered expression of G1 cyclins and their Cdks might be an important step in carcinogenesis (Motokura and Arnold, 1993). Cyclin D1 overexpression was reported in human colon cancer (Sutter *et al.*, 1997) and in chemically induced colon carcinogenesis (Otori *et al.*, 1999; Wang *et al.*, 1998). Apoptosis is a type of cell death characterized by ultrastructural alterations distinct from necrosis. In colonic mucosa, there is a homeostatic relationship between cell proliferation, cell differentiation, and cell death. Alterations in these processes can lead to loss of growth control, thereby playing a significant role in colon carcinogenesis (Bedi *et al.*, 1995). Thus, cyclin D1 and apoptosis can be used as biomarkers for chemoprevention studies (Einspahr *et al.*, 1997; Weinstein *et al.*, 1997).

In the present study, modulatory effects of Satsuma mandarin (*Citrus unshiu* Marc.) juices (MJ, MJ2, and MJ5) on the occurrence of colonic neoplasms induced by AOM were examined in rats. Also, proliferating cell nuclear antigen (PCNA)-labeling index, apoptotic index, and positive rate of cyclin D1 expression in colonic neoplasms were determined immunohistochemically.

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TABLE I—COMPOSITION OF MJ, MJ2, AND MJ5

	Energy (kcal/100 g)	Water (g/100 g)	Protein (g/100 g)	Fat (g/100 g)	Fiber (g/100 g)	Ash (g/100 g)	β -cryptoxanthin (mg/100 g)	Hesperidin (mg/100 g)
MJ	37	89.3	0.5	Trace	0	0.3	0.8	79
MJ2	37	89.3	0.5	Trace	0	0.3	1.7	84
MJ5	40	88.8	0.6	0.1	0	0.3	3.9	100

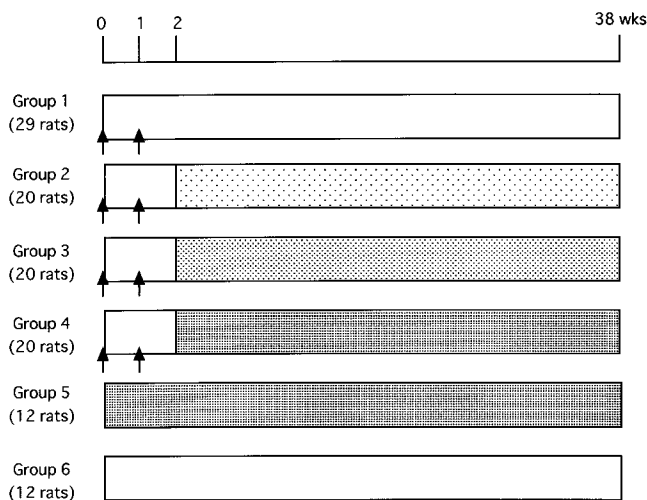


FIGURE 1—Experimental protocol. \uparrow : AOM (20 mg/kg bw), s.c. \square : Basal diet, CE-2 & tap water \square : MJ \square : MJ2 \square : MJ5

MATERIALS AND METHODS

Animals, carcinogen, diet, and MJs

Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), aged 5 weeks, were used. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (4 or 5 rats/cage) with free access to a drinking water and a basal diet, CE-2 (CLEA Japan, Tokyo, Japan), under controlled conditions of humidity ($50\% \pm 10\%$), lighting (12-hr light/dark cycle), and temperature ($23^\circ \pm 2^\circ\text{C}$). They were quarantined for 14 days and randomized by body weight into experimental and control groups. AOM was purchased from Sigma Chemical (St. Louis, MO). Pelleted CE-2 diet was used as a basal diet throughout the study. MJ is a commercial juice (Ehime brand made in December 1997 by Ehime Federation of Agricultural Cooperation Associations, Matsuyama City, Japan). MJ2 and MJ5 were prepared from MJ by centrifuging MJ twice at Ehime Citrus Research Institute (Matsuyama City, Japan) (Sumida *et al.*, 1999a). After freezing and thawing MJ pulp, a centrifugal separation was carried out, and then this procedure was done again with an addition of 0.01% pectin to increase β -cryptoxanthin content. The compositions of MJs are given in Table 1. The contents of β -cryptoxanthin and hesperidin in MJ2 and MJ5 were much higher than those of MJ. Quantitative analysis of β -cryptoxanthin in MJs was done by C_{30} stationary-phase high-performance liquid chromatography (Emenhiser *et al.*, 1995; Sumida *et al.*, 1999c). In this method, the 2 minor peaks of *cis*-isomers, which were identified as 13- and 13'-*cis*-geometric forms of β -cryptoxanthin, were found on the chromatogram. Concentration of hesperidin in MJs were measured by the method described by Fisher (1978). The contents of β -cryptoxanthin and hesperidin in MJs were monitored every 4 weeks by above methods during the study.

Experimental procedure

A total of 113 male F344 rats were divided into 6 groups as shown in Figure 1. Groups 1 through 4 were initiated with AOM by 2 weekly s.c. injections (20 mg/kg body weight). Rats in group

2 were given MJ as a drink for 36 weeks, starting 1 week after the last injection of AOM. Similarly, groups 3 and 4 were given MJ2 and MJ5, respectively. Group 5 was given MJ5 alone during the study (38 weeks). Group 6 served as an untreated control. MJ, MJ2, or MJ5 in black bottles was given to rats for 12 hr (between 8:00 p.m. and 8:00 a.m.) a day and tap water for the remaining 12 hr. MJs in black bottles were prepared every day and consumption of MJs/rat/day was recorded.

At week 38, all animals were sacrificed by an ether overdose to assess the incidences of neoplastic lesions in all organs including large bowel. At autopsy, the intestine was excised, opened longitudinally, flushed clean with saline, and examined for the presence of tumors. Colons, after fixed in 10% buffered formalin, were processed for histopathological examination by conventional methods. Intestinal neoplasms were diagnosed according to the criteria described by Ward (1974). Other organs, including liver and kidney, were also examined histopathologically.

Apoptosis in colonic tumors

Apoptosis is characterized by DNA fragmentation and cleavage into 180-bp to 200-bp internucleosomal-sized fragments. In general, the appearance of a "ladder" of nucleosomal-sized fragments on agarose gel electrophoresis has been used as a hallmark of apoptosis. However, a ladder of DNA fragments is also associated with necrosis in some types of cells (Fukuda *et al.*, 1993). The most commonly encountered histological manifestation of apoptosis is the presence of apoptotic bodies (Hall *et al.*, 1994). Therefore, in the present study, we have counted apoptotic cells with apoptotic bodies in colonic tumors. To identify apoptotic tumor cells, sections were stained with Feulgen/fast green and the number of apoptotic cells were quantified using a light microscopy ($\times 40$). Apoptotic cells with apoptotic bodies also showed cell shrinkage and nuclear condensation on hematoxylin- and eosin-stained histological sections. The apoptotic index, which represents the percentage of cells exhibiting apoptosis, was determined by counting at least 200 cells in randomly chosen fields of colonic tumors. All slides were blinded and scored by one investigator (T.T.).

PCNA and cyclin D1

Formalin-fixed colonic tumor tissues embedded in paraffin were cut (3 μm) and mounted onto gelatin-coated glass slides for immunohistochemical staining of PCNA and cyclin D1. Immunohistochemistry was performed with a monoclonal mouse anti-PCNA (dilution 1:300; Sigma) and a rabbit polyclonal anti-cyclin D1 antibody (dilution 1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA) (Otori *et al.*, 1999) using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The number of PCNA- or cyclin D1-positive nuclei in colonic tumors was determined by counting the number of positive cells among at least 500 cells in the tumor and was indicated as percentages.

Statistics

Where applicable, data were analyzed using χ^2 test, Fisher exact probability test, Student *t*-test or Welch *t*-test with $p < 0.05$ as the criterion of significance.

RESULTS

General observation

All animals remained healthy throughout the experimental period. Body weight gain is given in Table II. Consumption of MJ,

TABLE II – BODY, LIVER, AND RELATIVE LIVER WEIGHTS AT THE END OF THE STUDY

Group no.	Treatment	No. of rats examined	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM	29	374 ± 27 ^{1,2}	13.2 ± 2.2	3.53 ± 0.15
2	AOM → MJ	20	390 ± 28	13.9 ± 1.0	3.57 ± 0.22
3	AOM → MJ2	20	385 ± 49	13.7 ± 1.7	3.58 ± 0.30
4	AOM → MJ5	20	374 ± 39	13.8 ± 1.6	3.72 ± 0.58
5	MJ5	12	405 ± 12	14.0 ± 1.0	3.47 ± 0.21
6	None	12	394 ± 17	13.8 ± 1.1	3.49 ± 0.18

¹Mean ± SD. ²Significantly different from group 6 by Student *t*-test ($p < 0.05$).

TABLE III – CONSUMPTION OF MJ, MJ2, MJ5, AND TAP WATER

Group no.	Treatment	MJ (ml/day/rat)	MJ2 (ml/day/rat)	MJ5 (ml/day/rat)	Tap water (ml/day/rat)	Mean intake of drinking water (ml/day/rat)
1	AOM	—	—	—	14.5 ± 1.5 ¹	14.5
2	AOM → MJ	7.8 ± 1.3	—	—	7.5 ± 1.4	15.3
3	AOM → MJ2	—	6.8 ± 1.2	—	9.0 ± 1.7	15.8
4	AOM → MJ5	—	—	6.9 ± 1.9	9.7 ± 1.1	16.6
5	MJ5	—	—	6.3 ± 1.5	10.0 ± 1.0	16.3
6	None	—	—	—	13.4 ± 0.6	13.4

¹Mean ± SD.

TABLE IV – INCIDENCE OF LARGE BOWEL TUMORS IN EACH GROUP

Group no.	Treatment (no. of rats examined)	No. of rats with tumors at:								
		Entire intestine			Small intestine			Large intestine		
		Total	AD ¹	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM (29)	20 (69%)	5 (17%)	20 (69%)	9 (31%)	3 (10%)	7 (24%)	20 (69%)	2 (7%)	20 (69%)
2	AOM → MJ (20)	14 (70%)	5 (25%)	11 (55%)	6 (30%)	0	6 (30%)	11 (55%)	5 (25%)	7 ² (35%)
3	AOM → MJ2 (20)	10 (50%)	5 (25%)	6 ³ (30%)	5 (25%)	2 (10%)	3 (15%)	7 ² (35%)	3 (15%)	5 ⁴ (25%)
4	AOM → MJ5 (20)	7 ² (35%)	5 (25%)	6 ³ (30%)	3 (15%)	0	3 (15%)	7 ² (35%)	5 (25%)	3 ⁵ (15%)
5	MJ5 (12)	0	0	0	0	0	0	0	0	0
6	None (12)	0	0	0	0	0	0	0	0	0

¹AD, adenoma; ADC, adenocarcinoma. ^{2,3}Significantly different from group 1 by χ^2 test (² $p < 0.02$, ³ $p < 0.01$). ^{4,5}Significantly different from group 1 by Fisher exact probability test (⁴ $p = 0.0028$, ⁵ $p = 0.00021$).

MJ2, MJ5, and tap water are summarized in Table III. Intakes (ml/day/rat) of MJ2 and MJ5 were slightly lower than those of MJ. Mean tap water intakes in groups 3 and 4 were slightly larger than those in group 2. Rats tolerated the treatments with MJ, MJ2, or MJ5 well, supporting normal growth in rats without any adverse effects.

Incidence and multiplicity of colonic neoplasms

Macroscopically, most tumors developed in the large intestine (mainly in the middle and distal colon) and some in the small intestine of rats in groups 1 through 4. They were sessile or pedunculated tumors, and histologically diagnosed as tubular adenomas, adenocarcinomas with a higher incidence of adenocarcinoma. A few rats in groups 1 through 4 had renal mesenchymal tumors and/or altered hepatocellular foci, but these lesions were not found in any other groups. Animals in groups 5 and 6 did not have any neoplasms in examined organs including the intestines. The incidence and multiplicity of intestinal neoplasms are shown in Tables IV and V. AOM administration induced large intestinal adenocarcinomas with an incidence of 69% (20 of 29 rats) and a multiplicity of 0.76 ± 0.57 . The incidences and multiplicities of groups 2 through 4 were significantly smaller than those of group 1 ($p = 0.05$ or $p = 0.00021$) as shown in Tables IV and V.

PCNA- and cyclin D1-labeling indices

The PCNA- and cyclin D1-labeling indices in colonic neoplasms of each group are shown in Table VI. The mean PCNA-labeling indices of adenomas and adenocarcinomas in rats of group 1 were $54.0\% \pm 5.0\%$ ($n = 2$) and $55.1\% \pm 6.9\%$ ($n = 20$), respectively. The mean cyclin D1-positive rates of these neoplasms were $30.0\% \pm 4.0\%$ ($n = 2$) and $34.1\% \pm 8.5\%$ ($n = 20$), respectively. The mean PCNA-labeling indices of adenomas found in groups 2 through 4 were significantly smaller than that of group 1 ($p < 0.005$ or $p < 0.05$). Similarly, the mean PCNA-labeling indices of adenocarcinomas present in groups 3 and 4 were significantly lower than that of group 1 ($p < 0.05$ or $p < 0.005$). The mean cyclin-positive indices of adenomas in groups 3 and 4 were significantly smaller than that of group 1 ($p < 0.05$ or $p < 0.001$). The mean cyclin-positive rate of adenocarcinomas of group 4 was significantly lower than that of group 1 ($p < 0.05$).

Apoptosis

As shown in Figure 2, the apoptotic index of colonic adenomas did not significantly differ among the groups. However, colonic adenocarcinomas of animals administered MJ, MJ2, or MJ5 exhibited a significantly increased apoptotic index as compared with those given tap water ($p < 0.01$ or $p < 0.02$).

TABLE V – MULTIPLICITY OF LARGE BOWEL TUMORS IN EACH GROUP

Group no.	Treatment (no. of rats examined)	Multiplicity (no. of tumors/rat) of intestinal tumors at:								
		Entire intestine			Small intestine			Large intestine		
		Total	AD ¹	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM (29)	1.17 ± 0.99 ²	0.17 ± 0.38	1.00 ± 0.79	0.34 ± 0.54	0.10 ± 0.30	0.24 ± 0.43	0.83 ± 0.65	0.07 ± 0.25	0.76 ± 0.57
2	AOM → MJ (20)	1.00 ± 0.89	0.25 ± 0.43	0.75 ± 0.83	0.35 ± 0.57	0 ± 0.57	0.35 ± 0.57	0.65 ± 0.65	0.25 ± 0.43	0.40 ± 0.58 ³
3	AOM → MJC2 (20)	0.70 ± 0.84	0.30 ± 0.56	0.40 ± 0.66 ⁴	0.25 ± 0.43	0.10 ± 0.30	0.15 ± 0.36	0.45 ± 0.74	0.20 ± 0.51	0.25 ± 0.43 ⁵
4	AOM → MJC5 (20)	0.55 ± 0.80 ³	0.25 ± 0.43	0.30 ± 0.46 ⁶	0.15 ± 0.36	0 ± 0.36	0.15 ± 0.36	0.40 ± 0.58 ³	0.25 ± 0.43	0.15 ± 0.36 ⁶
5	MJC5 (12)	0	0	0	0	0	0	0	0	0
6	None (12)	0	0	0	0	0	0	0	0	0

¹AD, adenoma; ADC, adenocarcinoma. ²Mean ± SD. ³⁻⁶Significantly different from group 1 by Student *t*-test or Welch *t*-test (³*p* < 0.05, ⁴*p* < 0.01, ⁵*p* < 0.005, ⁶*p* < 0.001).

TABLE VI – PCNA- AND CYCLIN D1-POSITIVE INDICES OF LARGE BOWEL TUMORS IN EACH GROUP

Group no.	Treatment	Adenoma		Adenocarcinoma	
		PCNA-positive index (%) / no. of tumors examined	Cyclin D1-positive index (%) / no. of tumors examined	PCNA-positive index (%) / no. of tumors examined	Cyclin D1-positive index (%) / no. of tumors examined
1	AOM	54.0 ± 5.0 ¹ /2	30.0 ± 4.0/2	55.1 ± 6.9/20	34.1 ± 8.5/20
2	AOM → MJ	40.4 ± 2.6 ² /5	23.6 ± 4.8/5	51.7 ± 7.6/7	31.7 ± 6.7/7
3	AOM → MJC2	39.3 ± 3.4 ³ /3	21.7 ± 1.7 ³ /3	47.8 ± 7.0 ³ /5	30.0 ± 4.5/5
4	AOM → MJC5	28.0 ± 6.0 ² /5	13.8 ± 1.9 ⁴ /5	41.7 ± 6.0 ² /3	22.0 ± 3.3 ³ /3

¹Mean SD. ²⁻⁴Significantly different from group 1 by Student *t*-test (²*p* < 0.005, ³*p* < 0.05, ⁴*p* < 0.001).

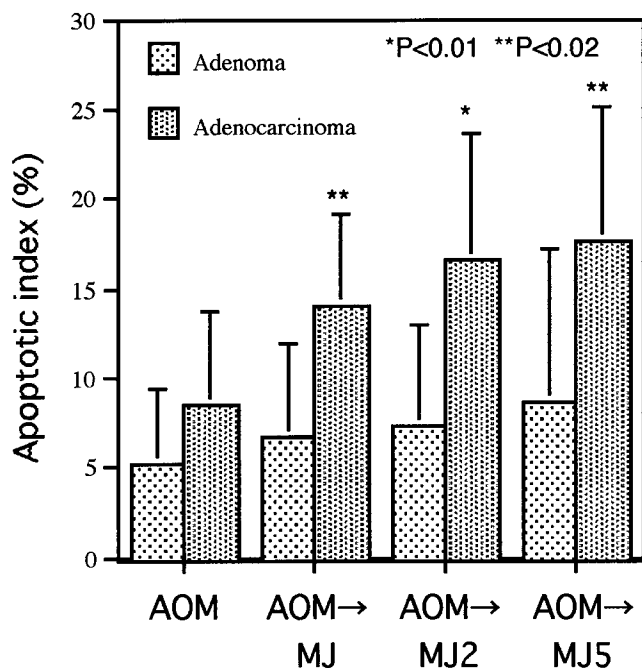


FIGURE 2 – Apoptotic index in colonic tumors found in rats given AOM alone or MJs after AOM exposure.

DISCUSSION

The results described here clearly indicate that MJs containing various amounts of β-cryptoxanthin and hesperidin inhibited AOM-induced rat colon carcinogenesis. The potency of MJ2 and MJ5, which contained higher amounts of β-cryptoxanthin and hesperidin, was superior to that of a commercial citrus juice, MJ. These results confirmed our previous data on the effects of

“CHRP” on ACF formation (Kohno *et al.*, 1999). Since β-cryptoxanthin (Onozuka *et al.*, 1998; Tsushima *et al.*, 1995) and hesperidin (Tanaka *et al.*, 1997) were reported to inhibit chemically induced carcinogenesis and the other components are almost similar among MJs, these 2 compounds might be responsible for the observed results in this study. However, other components in the mandarin juice than the 2 compounds may also have protective effects on carcinogenesis, although their potencies may be less strong than the 2 compounds. It would be important in future studies to examine synergistic chemopreventive effects of these pure compounds.

Whereas genetic and environmental factors have been recognized as the causatives in the etiology of colonic carcinoma, it is the carcinoma most related to diet and nutritional habits. International dietary guidelines for the prevention of chronic diseases recommend increased consumption of plant foods, including fruits, vegetables, and cereal. Such plant foods contain the traditional macronutrients and a wide variety of physiologically active phytochemicals, including flavonoids. In the present study, rats given MJs including MJ2 and MJ5, which are rich in β-cryptoxanthin and hesperidin, showed no adverse effects on food consumption and growth rate. Previous studies have also shown β-cryptoxanthin (Onozuka *et al.*, 1998) and hesperidin (Tanaka *et al.*, 1997) to be nontoxic in long-term in vivo experiments.

Several explanations for inhibitory effects of MJs on AOM-induced colon tumorigenesis were considered. In this study, treatment with MJs, especially MJ5, significantly lowered the PCNA- and cyclin D1-labeling indices in colonic neoplasms induced by AOM. Therefore, it is likely that such a control of cell proliferation in colonic neoplasms is responsible for observed suppression of colon carcinogenesis, because cell proliferation plays an important role in multistage carcinogenesis with multiple genetic changes (Cohen, 1998). In the present study, cyclin D1 overexpression was found in colonic adenomas and adenocarcinomas induced by AOM, as reported by other investigators (Otori *et al.*, 1999; Wang *et al.*, 1998). The results in the present study may indicate that MJ2 and MJ5, which are rich in β-cryptoxanthin and hesperidin, suppress cyclin D1 overexpression in late stages of colon carcinogen-

esis. In the present study, MJ treatments induced apoptosis in colonic adenocarcinomas but not in adenomas. Similar findings have shown that a monoterpene perillyl alcohol in cherry and spearmint induced apoptosis in colonic tumors and inhibited AOM-induced colon carcinogenesis (Reddy *et al.*, 1997). Similarly, a nonsteroidal anti-inflammatory drug, sulindac, which inhibits colon carcinogenesis, induces apoptosis (Boolbol *et al.*, 1996). Thus cellular responses like apoptosis to these substances may contribute to chemopreventive effect against colon carcinogenesis processes, particularly at later stage.

In conclusion, the results of this study suggest that MJ's, particularly MJ5, have a beneficial effect on chemically induced colonic tumorigenesis in rats. The findings provide an effective chemopreventive approach to management of colonic malignancy.

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