Luteolin: A Strong Antimutagen against Dietary Carcinogen, Trp-P-2, in Peppermint, Sage, and Thyme

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This study identified a strong antimutagen in peppermint, sage, and thyme against Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), one of the dietary carcinogens formed during cooking. The antimutagens in these herbs were purified by chromatographically monitoring the antimutagenicity with *Salmonella typhimurium* TA98 strain. Instrumental elucidation found that the active compound isolated from these herbs was 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one (luteolin), a common flavonoid. Luteolin was a desmutagen against Trp-P-2 but not a bioantimutagen. Chlorophyll was also found to be a desmutagen but required a larger dose. Other common phytochemicals, α-tocopherol, carotenoids, xanthophylls, sterols, and saponins, were almost nonactive against Trp-P-2. These results revealed that luteolin contributed greatly to the antimutagenicity against Trp-P-2 in these herbs.

Keywords: *Desmutagen; luteolin; herb; Trp-P-2; cancer prevention; dietary anticarcinogen*

INTRODUCTION

Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-b]indole) is considered to be a significant carcinogen in human cancer (Yamaizumi et al., 1980; Wakabayashi et al., 1992) because it is formed during cooking. An appreciable amount of Trp-P-2 occurs in cooked meat, and we may be at risk from dietary Trp-P-2.

The toxicity of Trp-P-2 can be evaluated with mutagenicity as an indicator for carcinogenesis, using *Salmonella typhimurium* TA98 strain in the presence of S9 mix (Matsukura et al., 1981). We have found that a small amount of herb extracts (water extract from 50 mg of fresh herbs) strongly suppressed the mutagenicity of 20 ng of Trp-P-2 (Natake et al., 1989). In particular, oregano, peppermint, sage, and thyme had the greatest activity. However, very little is known about the antimutagens in herbs. In our previous report (Kanazawa et al., 1995) we identified the active compounds in oregano to be galangin and quercetin, which were the specific desmutagens against Trp-P-2. The present study examined the active compounds in the other three herbs and found that luteolin was a strong desmutagen which neutralized Trp-P-2 before it could express mutagenicity.

MATERIALS AND METHODS

Materials. Dry powders of peppermint (*Mentha piperita*), sage (*Salvia officinalis*), and thyme (*Thymus valgaris*) were purchased from McCormick & Co. Inc. Trp-P-2 was purchased from Wako Pure Chem. Ind., Ltd. Luteolin was obtained from Funakoshi Co. Ltd. Carotenes and xanthophylls were a gift from F. Hoffmann-La Roche Ltd. Agar and extracts from beef and yeast (nutrient broth) for the cultivation of bacteria were purchased from Difco Laboratory. Organic solvent and water were distilled twice. All other chemicals were commercially available in high grade.

Extract from Herbs. The active compounds were extracted from the dry herb powder by a 20-min sonication in 10 volumes of six solvents used in order of the polarity: hexane, methylene chloride, ethyl acetate, acetone, methanol, and water, as shown previously (Kanazawa et al., 1995). The extracts were dried, weighed, and then dissolved in dimethyl sulfoxide (DMSO) for the following bioassay.

Determination of Antimutagenicity against Trp-P-2. The antimutagenicity of herb compounds was evaluated against 20 ng of Trp-P-2 as shown previously (Kanazawa et al., 1995). Essentially, the method of Ames et al. (1975) was used with some minor modifications and use of S9 mix (Danno et al., 1993). The S9 mix was prepared with the S9 fraction obtained from the liver of Sprague–Dawley rats given 500 mg/kg of body weight polychlorinated biphenyl 5 days before the sacrifice (Mizuno et al., 1987). Six different concentrations of the herb extracts in 0.1 mL of DMSO were incubated with Trp-P-2 in 0.1 mL of water at 37 °C for 30 min. After the addition of 0.5 mL of S9 mix and bacterial suspension of *S. typhimurium* TA98 (0.1 mL), the mixtures were incubated again at 37 °C for 20 min. The mixture was cultured for 2 days, and then the His^+ revertant colony number was counted.

To detect cytotoxicity of the herb extracts, the surviving bacterial number was measured simultaneously. The above incubation mixture was washed with 0.4 mL of 0.1 mM sodium phosphate buffer (pH 7.4). The bacteria were resuspended in the buffer and diluted to 10^6-fold with saline solution and cultured on the medium containing a 5 mM excess of histidine and nutrient broth to count the surviving colonies (His^-).

Calculation of the Antimutagenicity. The antimutagenicity was determined by three separate tests with three plates per the experimental points and calculated as follows: $\{(A - B) - (C - D)\}/(A - B) \times 100$; A, revertant number given by 20 ng of Trp-P-2 (mean ± SD is 2650 ± 78, n = 792); B, spontaneous revertants (22 ± 1, n = 792); C, revertants by both of the extract and 20 ng of Trp-P-2; and D, revertants by each extract. The IC_{50} value, which is the amount to require for 50% inhibition of the mutagenicity of 20 ng of Trp-P-2, was determined by plotting the antimutagenicity with six different doses versus log of the dosed amount in three independent series (Kanazawa et al., 1995).

Bioantimutagenicity Test. The bioantimutagenicity of luteolin was examined as mentioned previously (Kanazawa et al., 1995). Briefly, Trp-P-2 was activated by S9 mix and given to the bacteria for 15 min at 37 °C before luteolin was added to the bacteria. Then, the decrease of His^+ colony number by luteolin was counted.

Purification and Identification of the Antimutagen. The antimutagens were separated from the ethyl acetate extracts of herbs with a Sephadex LH-20 column (diameter 2.5 × 55 cm or diameter 1.6 × 60 cm) and a silica gel column

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Table 1. Antimutagenicity of the Herb Extracts against 20 ng of Trp-P-2

	peppermint		sage		thyme	
solvent	anti.[a] (%)	yield[b] (g)	anti.[a] (%)	yield[b] (g)	anti.[a] (%)	yield[b] (g)
hexane	39	1.8	76	1.9	68	4.3
methylene chloride	65	1.5	91	2.2	70	5.3
ethyl acetate	73	1.0	93	0.9	78	1.0
acetone	37	1.0	85	1.0	74	2.6
methanol	15	21.5	7	20.5	42	19.0
water	16	12.4	nonactive	13.0	enhanced[c]	11.1

[a] The antimutagenicity of each extract was tested with 50 μg from peppermint, 10 μg from sage, and 25 μg from thyme (by dry weight). [b] Yield (g) from 100 g of herbs. [c] Water extract from thyme increased the revertant number given by Trp-P-2 to 2-fold.

(Wakogel C-100, diameter 1.4 × 30 cm) chromatographies (Kanazawa et al., 1995). The fractions obtained were dried under a nitrogen stream and weighed, and then the antimutagenicity was measured. The antimutagens purified by recrystallization three times were identified by instrumental analyses: ultraviolet, infrared (with a Shimadzu IR-408), nuclear magnetic resonance (NMR, Bruker AC-250), and electron ionization mass (EI/MS, JEOL DX-500) spectra.

RESULTS

Purification of the Antimutagens in Peppermint, Sage, and Thyme. We had preliminarily found that dry herbs (commercial food) had similar antimutagenic activity against Trp-P-2 in a dose-dependent manner as fresh herbs without affecting the survival bacterial number. Thus, the present study examined the antimutagens in a commercial powder of dry herbs.

Peppermint, sage, and thyme were extracted with six solvents in order of polarity one after another three times each. Table 1 shows the antimutagenicity and yield of each extract. The ethyl acetate extracts from every herb had the strongest antimutagenicity dose-dependently without affecting the survival cell number. The methylene chloride extracts also showed a dose-dependent activity and gave higher yields, but the major component was chlorophyll. Chlorophyll is known to be an antimutagen (Negishi et al., 1990; Arimoto et al., 1993). Then, we proceeded to purify the active compounds in the ethyl acetate extracts.

Figure 1 shows the purification process of the active compounds in peppermint. The ethyl acetate extract was dried and dissolved in methanol. Most of the active compounds were soluble in methanol, and then the methanol-soluble products were separated into 10 fractions on Sephadex LH-20. The active fractions 6–9 were collected and gel-filtrated again on the Sephadex column. When the fractions 11 and 12 were further separated on Sephadex, fractions 20, 21, and 24 had strong activity with dose-dependency and were present in high yields. Comparing their IC_{50} values against 20 ng of Trp-P-2, fraction 24 gave the lowest IC_{50} (0.15 μg) and was purified 39-fold over the ethyl acetate extract (Table 2). Also, fractions 20, 21, and 24 were analyzed by high-performance liquid chromatography using a column of Inertsil ODS (diameter 4.6 × 250 mm) maintained at 45 °C with gradient elution from 55% to 70% methanol in 50 mM phosphate buffer (pH 3.3) within 5 min for the mobile phase. Fraction 24 gave a single peak at t_R 8.57 min, but fractions 20 and 21 gave 10 and 11 peaks, respectively (data not shown). So, the active compound in fraction 24 was purified by recrystallization with methanol three times and submitted for instrumental elucidation of the chemical structure.

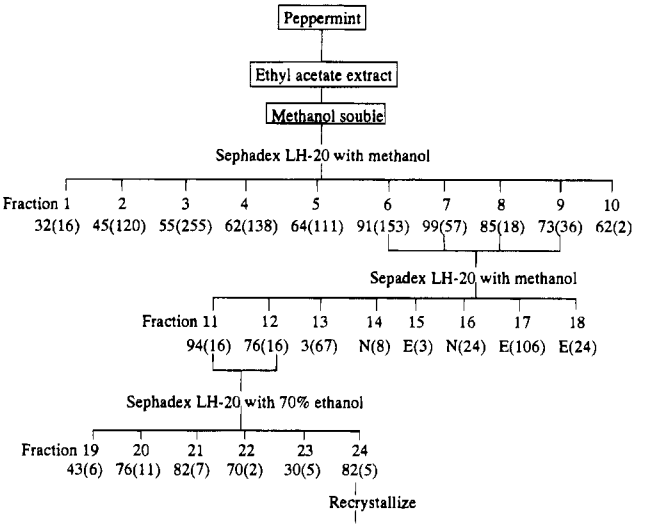

Figure 1. Purification of the antimutagen from peppermint. Ethyl acetate extract from peppermint in Table 1 was dried and dissolved in methanol. The methanol soluble products were gel filtrated through a Sephadex LH-20 column (diameter 2.5 × 55 cm) with methanol. Fractions 6–9 were collected and filtered again on a Sephadex column (diameter 1.6 x 60 cm). Active fractions 11 and 12 were further chromatographed on the Sephadex column (diameter 1.6 × 60 cm) with 70% ethanol in water. Figures before the parentheses are the antimutagenicity (%) against 20 ng of Trp-P-2 and the figures in parentheses are yields in milligrams from 100 g of peppermint. The antimutagenicity was evaluated with 10 μg each (by dry weight) of fractions 1–10 and with 5 μg each (by dry weight) of fractions 11–24. "N" means nonactive and "E" means enhancing the mutagenicity of Trp-P-2.

Table 2. IC_{50} (Micrograms) of the Separate Fractions from Peppermint, Sage, and Thyme against the Mutagenicity of 20 ng of Trp-P-2

peppermint	sage	thyme
ethyl acetate extract, 5.8	ethyl acetate, 5.3	ethyl acetate, 11
fractions 6–9, 2.4	fraction 6, 0.60	fraction 3, 1.3
fractions 11 + 12, 1.1	fraction 8, 0.30	fraction 5, 0.95
fraction 20, 1.7	fraction 10, 1.0	fraction 6, 0.17
fraction 21, 0.87	fraction 11, 0.19	
fraction 24, 0.15		
recrystallized 24, 0.14	recrystallized 11, 0.15	recrystallized 6, 0.15
commercial luteolin, 0.14		

Figure 2 shows the purification of antimutagen in sage. The first Sephadex gel filtration for the methanol-soluble products gave six fractions, and fraction 6 had the strongest activity. The second gel filtration for fraction 6 gave the active fraction 8, and the third gel filtration separated the fraction 8 into two active fractions, 10 and 11. Fraction 11 showed 5-fold lower IC_{50} (0.19 μg) than fraction 10 (1.0 μg) (Table 2). Then, fraction 11 was purified by the recrystallization with methanol three times.

Figure 3 is the purification process of the antimutagen in thyme. The active fraction 3 obtained from the methanol-soluble products was dried and dissolved in acetone and then separated by the silica gel column chromatography. Fraction 6 had the strongest activity, and fraction 5 gave the highest yield. Comparing their IC_{50}, fraction 6 showed 5-fold stronger antimutagenicity than fraction 5 (Table 2). When these fractions were allowed to stand on ice, fraction 6 gave yellow crystalline needles. Then, these crystals were recrystallized from methanol three times for the instrumental analysis.

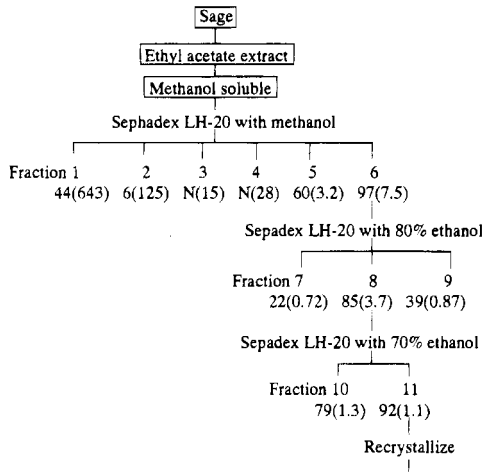

Figure 2. Purification of the antimutagen from sage. Ethyl acetate extract from sage in Table 1 was gel filtrated through the Sephadex LH-20 column (diameter 2.5 × 55 cm) by the same method as that from peppermint. Fraction 6 was dried and dissolved in 80% ethanol in water and then filtered again through the Sephadex column (diameter 1.6 × 60 cm) with 80% ethanol. Active fraction 8 was further chromatographed on the Sephadex column (diameter 1.6 × 60 cm) with 70% ethanol in water. Figures before parentheses are antimutagenicity (%) against 20 ng of Trp-P-2 ("N" means nonactive) and the figures in parentheses are yields in milligrams from 100 g of sage. The antimutagenicity was evaluated with 25 μg each (by dry weight) of fractions 1–6 and with 2.5 μg each (by dry weight) of fractions 7–11.

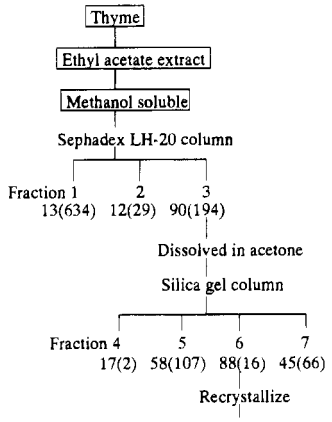

Figure 3. Purification of the antimutagen from thyme. Ethyl acetate extract from thyme in Table 1 was gel filtrated through a Sephadex LH-20 column (diameter 2.5 × 55 cm) by the same method as that from peppermint. Active fraction 3 was dried and dissolved in acetone and then chromatographed on the silica gel column (diameter 1.4 × 30 cm), eluting with hexane/water-saturated ethyl acetate (6:4). Figures before parentheses are antimutagenicity (%) against 20 ng of Trp-P-2 and the figures in parentheses are yields in milligrams from 100 g of thyme. The antimutagenicity was evaluated with 25 μg each (by dry weight) of fractions 1–3 and with 1 μg each (by dry weight) of fractions 4–7.

Identification of the Antimutagens. All three crystalline materials obtained from peppermint, sage, and thyme gave the same ultraviolet spectrum in methanol (λ_{max} at 207, 254, and 350 nm). The spectrum shifted to longer wavelength upon addition of aluminum chloride, representative of compounds having a flavonoid skeleton (Mabry et al., 1970). Also, all the isolated materials gave exactly the same infrared spectra, EI/MS and NMR which indicated that they had the same chemical structure. The infrared spectrum in KBr showed an aromatic conjugated ketone (ν_{max} 1655 cm^{-1}) and phenolic OH (ν_{max} 3000 cm^{-1}). The EI/MS spectrum

Table 3. Desmutagenicity of Luteolin

	colony number[a]	
	revertants	survival ($\times 10^6$)
desmutagenicity[b]		
without luteolin	2685 ± 240	460 ± 44
with 2 μg of luteolin	483 ± 86	494 ± 64
bioantimutagenicity		
without luteolin	1168 ± 235	229 ± 29
with 10 μg luteolin	844 ± 135	220 ± 27

[a] Mean ± SD (n = 6). [b] The desmutagenicity was evaluated with the same test as the antimutagenicity.

detected a molecular ion peak at m/z 286 (M^+) as a base ion peak and fragment ion peaks at m/z 153 ($[C_7H_5O_4]^+$) as the secondary peak and at m/z 258 (CO) as the tertiary peak. The spectrum of ^{1}H-NMR in DMSO-d_6 gave signals at δ 6.19 (1H, d, J = 2.0 Hz), 6.44 (1H, d, J = 2.0 Hz), 6.67 (1H, s), 6.89 (1H, d, J = 8.6 Hz), 7.42 (1H, m), 7.69 (1H, d, J = 3.0 Hz), 9.89 (3H, OH), and 13.0 (1H, perie OH). These signals were assigned to protons on C-6; C-8; C-3; C-5′; C-6′; C-2′; C-7, C-3′, and C-4′; and C-5 on the flavone ring, respectively. These analysis data completely coincided with those of authentic luteolin. Thus, all the crystalline materials from peppermint, sage, and thyme was identified as luteolin, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one.

Desmutagenicity of Luteolin against Trp-P-2. Antimutagens can be classified into desmutagens and bio-antimutagens according to their modes of action (Kada and Shimoi, 1987). The desmutagenicity is an activity to neutralize a mutagen before it damages the DNA and is evaluated with the same test as antimutagenicity. The bioantimutagenicity means suppressing activity in the process of mutagenesis (mutation fixation) after DNA has been damaged by the mutagen. The fixed His^+ revertant, which has been induced by activated Trp-P-2 (Kato and Yamazoe, 1987), can be evaluated by counting the number of His^+ revertant colonies. Table 3 shows the comparison between the desmutagenicity and bioantimutagenicity of commercial luteolin. In the desmutagenicity test, 2 μg/plate of luteolin suppressed the mutagenicity of 20 ng of Trp-P-2 by 82%. On the contrary, in the bioantimutagenicity test, the larger amount of luteolin (10 μg/plate) decreased the His^+ revertants by 27%. Therefore, the antimutagenicity of luteolin against Trp-P-2 was due to the desmutagenicity which neutralized Trp-P-2 before mutating the bacteria. Then, the IC_{50} of commercial luteolin for the desmutagenicity against 20 ng of Trp-P-2 was determined (Table 2). Commercial luteolin gave 0.14 μg, which coincided with the IC_{50} of each active compound purified from peppermint, sage, and thyme.

On the other hand, in the both tests of desmutagenicity and bioantimutagenicity, luteolin did not decrease the surviving bacterial numbers, indicating that luteolin had no cytotoxicity.

Antimutagenicity of Other Phytochemicals. Herbs include many phytochemicals, and also some of them may have antimutagenicity. Several common phytochemicals were examined for antimutagenicity against 20 ng of Trp-P-2 (Table 4). However, of phytochemicals tested, only chlorophyll exhibited a dose-dependent antimutagenicity and gave 230 μg as the IC_{50} value. Ascorbic acid suppressed partly the mutagenicity of Trp-P-2 but not in a dose-dependent fashion. Ascorbic acid at more than 10 μg/plate strongly enhanced the mutagenicity. α-Tocopherol also showed partly sup-

Table 4. Antimutagenicity of Common Phytochemicals against the Mutagenicity of 20 ng of Trp-P-2

phytochemicals	suppressing activity[a]	phytochemicals	suppressing activity[a]
chlorophyll[b]	active (IC_{50} = 230 μg)	canthaxanthin	partly (25%)
		lutein	partly (26%)
ascorbic acid	partly (18%)	stigmasterol	partly (15%)
α-tocopherol	partly (30%)	campesterol	nonactive
α-carotene	nonactive	β-sitosterol	nonactive
β-carotene	nonactive	chromosaponin I[c]	nonactive
astaxanthin	partly (7%)	soyasaponin I[c]	nonactive
zeaxanthin	partly (16%)		

[a] Phytochemicals with the concentration between 10 ng/plate and 1 mg/plate were tested toward 20 ng/plate of Trp-P-2. "Active" means that the compound exhibited a dose-dependent activity and gave the represented IC_{50} value. "Partly" means that they suppressed the mutagenicity in part but not in a dose-dependent manner. Figures in the parentheses are antimutagenicity % against Trp-P-2 when 5 μg/plate of the phytochemicals were dosed. [b] Chlorophyll was prepared from methylene chloride extract of fresh sage grown in our garden by thin-layer chromatography on Merck Kieselgel PF_{254} using toluene/diethyl ether (7:3) as the developing solvent. [c] Saponins were a kind gift from Prof. T. Hashimoto (Tsurumi et al., 1992).

pressive activity without dose-dependency. Two carotenes were nonactive, and four xanthophylls were partly suppressive. Stigmasterol was partly suppressive, and the other sterols and saponins were nonactive. The phytochemicals tested did not show an antimutagenicity against Trp-P-2 as strong as that of luteolin (Table 2). Thus, luteolin was considered to be responsible for the antimutagenicity in peppermint, sage, and thyme.

DISCUSSION

This study demonstrated that the popular herbs peppermint, sage, and thyme included strongly desmutagenic luteolin against Trp-P-2. The contents of luteolin in these herbs could be estimated from the yields of active fractions 24 in peppermint (Figure 1), 11 in sage (Figure 2), and 6 in thyme (Figure 3). The major components in these active fractions were luteolin, because their IC_{50} values remained almost unchanged after the recrystallizations (Table 2). Therefore, the luteolin contents in 100 g of dry peppermint, sage, and thyme were estimated to be around 5, 1.1, and 16 mg, respectively. The IC_{50} of luteolin against 20 ng of Trp-P-2 was 0.14 μg (Table 2). Assuming that 1 g of cooked meat had mutagenicity as strong as 20 ng of Trp-P-2 (Yamaizumi et al., 1980), it was considered that the mutagenicity from 1 g of broiled meat could be mitigated by half with a very small amount of these herbs: 2.8 mg of peppermint, 13 mg of sage or 0.9 mg of thyme.

On the other hand, chlorophyll was also a desmutagen (Table 4), and its content in these herbs was much larger than that of luteolin, assuming that the methylene chloride extracts were composed mainly of chlorophyll (Table 1). However, the IC_{50} of chlorophyll was 230 μg (Table 4), which was 1640-fold weaker than that of luteolin. These results strongly suggested that luteolin contributed greatly to the desmutagenicity in these herbs.

The effect of luteolin on the mutagenicity of Trp-P-2 was desmutagenic (Table 3). Galangin and quercetin found previously in oregano were also desmutagens (Kanazawa et al., 1995). The IC_{50} of galangin and quercetin were 0.12 and 0.81 μg, respectively. The desmutagenicity of luteolin (IC_{50}, 0.14 μg) was similar to that of galangin and stronger than that of quercetin. Luteolin lacks one hydroxy group at the C3 position of quercetin, and galangin lacks two hydroxy groups at the C3′ and C4′ positions of quercetin. These are flavonoids, which are known to be strong antioxidants. Cholbi et al. (1991) showed that luteolin was a stronger antioxidant than quercetin and that the presence of hydroxyls at C3′ and C4′ was not necessary for the antioxidative potency. Thus, the desmutagenic ability of these flavonoids coincides with the antioxidative potency. On the other hand, the mutagenicity of Trp-P-2 is known to be due to active oxygen radicals generating from its activated forms (Wakata et al., 1985; Wataya et al., 1988). One possible desmutagenic mechanism of flavonoids is thought to be scavenging of the radicals before they damage the DNA.

These flavonoids are commonly included in various edible plants, herbs, vegetables, fruits, and tea (Hertog et al., 1993a,b). Edenharder et al. (1993) have also reported that flavonoids suppressed the mutagenicity of the other dietary carcinogens formed during the cooking process. Although an appreciable amount of dietary carcinogens occurs in cooked meat (Yamaizumi et al., 1980; Wakabayashi et al., 1992), the cancer risk may be mitigated by having the edible plants at the same meal.

It is well-known that flavonoids can inhibit carcinogenesis by benzo[*a*]pyrene, dimethylbenz[*a*]anthracene, *N*-nitorosomethylurea, and methylcholanthrene (Huang et al., 1981; Verma et al., 1988; Mukhtar et al., 1988; Bon et al, 1992). Flavonoids in the diet are considered to be one of the most significant anticarcinogens; their anticarcinogenic mechanisms are being studied in our laboratory.

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