ORIGINAL

Antimutagenicity of *Murdannia loriformis* in the *Salmonella* mutation assay and its inhibitory effects on azoxymethane-induced DNA methylation and aberrant crypt focus formation in male F344 rats

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Abstract : An 80% ethanol extract of *Murdannia loriformis*, a Thai medicinal plant, was examined for antimutagenic activity and cancer chemopreventive activity. In the *Salmonella* mutation assay, the extract showed antimutagenicity against 2-amino-3-methylimidazo [4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, 2-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole, 2-amino-6-methyldipyrido [1,2-*a*:3',2'-*d*] imidazole, 2-aminodipyrido[1,2-*a*:3',2'-*d*]imidazole, 2-aminoanthracene, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and methylazoxymethanol acetate and reduced their mutagenicities to 31.4〜67.9% at the dose of 10 mg/plate. However, it did not inhibit the mutagenicities of 2-amino-9*H*-pyrido[2,3-*b*]indole, 2-amino-3-methyl-9 *H*-pyrido[2,3-*b*]indole, benzo[*a*]pyrene, *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine and 1-nitropyrene. The extract itself showed no mutagenicity. The chemopreventive activity of *M. loriformis* was examined using azoxymethane (AOM)-induced aberrant crypt focus (ACF) formation in the colon of F344 rats. The extract at doses of 0.1-1.0 g/kg wt significantly inhibited ACF formation in the initiation stage (21-51%), although it was more effective at a lower dose. In the post-initiation stage, the extract also tended to inhibit ACF formation (12-27%) and significantly decreased the number of larger ACFs that have more than 3 aberrant crypts per focus. The extract inhibited the formation of O^6-methylguanine and N^7-methylguanine in the colonic mucosa and muscular layers but not or increased in the liver. These results indicate that *M. loriformis* extract has antimutagenic activity toward various known mutagens and that it inhibits AOM-induced ACF formation both in the initiation and post-initiation stages in the rat colon. J. Med. Invest. 49 : 25-34, 2002

Keywords **:** *Murdannia loriformis ; antimutagenicity ; azoxymethane-induced aberrant crypt foci ; O^6-methylguanine*

Received for publication August 9, 2001 ; accepted October 23, 2001.

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INTRODUCTION

Environmental factors, especially food, have been epidemiologically demonstrated to be closely associated with human colorectal cancer (1-3). While complete

removal of causative agents of cancer may not always be possible, chemoprevention of cancer using antimutagens and anticarcinogens present in foods and in the environment has been suggested to be most effective method to prevent human cancer. Large numbers of antimutagens and anticarcinogens exist in natural products, especially in the plant kingdom (4). These antimutagens and anticarcinogens may inhibit one or more stages of carcinogenesis and prevent or delay cancer development (5). Geographic variation in colon cancer may also be attributable to differences in consumption of protective factors in the diet such as fiber, antioxidants and antiprolifelative agents.

Various kinds of Thai medicinal plants have been shown to be antimutagenic and anticarcinogenic. Lemon grass, for example, has been shown to be antimutagenic against various mutagens (6) and to inhibit azoxymethane (AOM)-induced DNA adduct formation and aberrant crypt focus (ACF) formation in the rat colon (7). Roselle and bitter melon have been shown to inhibit colon carcinogen-induced ACF formation in the rat colon (8, 9). Tong Tak (10), *Acanthus ebracteatus* Vahl., *Plumbago indica* Linn. and *Rhinacanthus nasuthus* Kurz. (11) have also been found to be antimutagenic. *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy, a Thai medicinal plant, is a traditional drug in Thailand used for relief of bronchitis and cancer (12). Previous studies have shown that *M. loriformis* extract inhibits the mutagenicity of some kinds of mutagens in the *Salmonella* mutation assay and induces DT-diaphorase activity in a murine hepatoma cell line (Hepa 1c1c 7) (13). In addition, an active glycosphingolipid isolated from *M. loriformis* exerted cytotoxicity against human colon carcinoma and a human breast cancer cell line (14). However, *in vivo* effects of *M. loriformis* extract have not been reported.

ACFs are an early neoplastic lesions in the colonic mucosa and were first observed in colon carcinogen-treated mice and rats (15). They are morphologically distinguishable from normal crypts by their larger size and the more elliptical shape of the luminal opening, with thicker lining of epithelial cells. These lesions have also been observed in the human colon (16). AOM is a typical colon carcinogen, and AOM-induced ACFs in the rat colon have been used to identify chemopreventive agents for colon cancer (17). DNA adduct formation is also recognized as one of the common properties of most potent carcinogens and is the basis of current strategies in molecular epidemiology and biomonitoring (18). AOM-induced DNA adducts are N^7-methylguanine (N^7-meG) and O^6-methyguanine(O^6-meG). N^7-MeG is quantitatively the major alkylation product (19), but the level and persistence of O^6-meG in the target tissue is more closely correlated with the carcinogenicity (20).

In the present study, we investigated the inhibitory effects of *M. loriformis* extract on AOM-induced DNA adduct formation and ACF formation in the rat colon.

MATERIALS AND METHODS

1) Chemicals

All heterocyclic amines (HCAs), including 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*b*]quinoline (MeIQ), 3-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-2), 2-amino-9*H*-pyrido[2,3-*b*] indole (AαC), and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*] indole (MeAαC) were kindly provided by Dr.K. Wakabayashi, National Cancer Center Research Institute. O^6-MeG was also a generous gift from Drs. K. Ishizaki and M. Ikenaga, Kyoto University. Aflatoxin B_1 (AFB_1), benzo[*a*]pyrene (B[*a*]P), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG), methylazoxymethanol acetate (MAM acetate), AOM, N^7-meG were purchased from Sigma Chemical Co., St. Louis, MO. 1-Nitropyrene (1-NP) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2), 2-aminoanthracene (2-AA) and other chemicals, reagent grade or higher, were from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

2) Extraction of Murdannia loriformis

M. loriformis was obtained from the garden at the Research Institute for Health Science, Chiang Mai University, Chiang Mai, Thailand. The whole fresh plant was washed with tap water, cut into small pieces, freeze-dried, and ground to a fine powder. The dry powder (100 g) was extracted with 1 liter of 80% ethanol for 24 h by stirring at room temperature. Then the extarct was filtered through a paper filter by suction, and the residue was extracted again with 80% ethanol. The filtrates were combined and evaporated to dryness in a rotary evaporator under reduced pressure at 50°C. The dried residue was used as *M. loriformis* extract that is abbreviated to ML in Tables and Figures. The extract was weighed,

dissolved in 25% dimethylsulfoxide (DMSO), and kept at 4°C until used. The yield of the extract was 0.12% from fresh plant and 1.2% from freeze-dried powder of the plant. For the *Salmonella* mutation assay, the extract was sterilized by filtration with a disposable filter unit (cellulose acetate membrane, pore size of 0.45 μm, Advantec) before being used.

3) Mutagenicity test

The mutagenicity and antimutagenicity of *M. loriformis* extract were assayed using *Salmonella typhimurium* strains TA98 and TA100 according to the procedure of Maron and Ames (21) with the modification of preincubation (22) in the presence or absence of 9,000×*g* supernatant fraction (S9) prepared from the liver of rats treated with drug-metabolizing enzyme inducers, benzoflavone and phenobarbital. The number of spontaneous revertants was 24±6 in strain TA98 without S9, 37±10 in strain TA98 with S9, 112±26 in strain TA100 without S9, and 105±18 in strain TA100 with S9, and they were subtracted in all data except for Table 1.

Table 1. Mutagenicity test on *M. loriformis* extract

Dose (mg/plate)	His^+ revertants/plate			
	TA98		TA100	
	−S9	+S9	−S9	+S9
0	24	36	108	114
0.01	29	32	95	102
0.10	30	40	99	105
1.00	24	38	100	100
10.0	22	36	106	92

4) Animals

Four-week-old male F344 rats (80-100 g) were purchased from SLC Japan (Hamamatsu, Japan). Animals were housed in plastic cages with sawdust bedding, maintained for 3 days and given food and water *ad libitum*. The room in which the rats were kept, in the Institute of Animal Experimentation, The University of Tokushima School of Medicine, was controlled at a temperature of 23±2°C, humidity of 55±10%, and a 13-h light/11-h dark cycle.

5) Analysis of aberrant crypt foci

After quarantine for one week, rats were divided into eight groups for ACF determination for the initiation stage as shown in Fig.1 (a). Rats in groups 1 through 5 were subcutaneously injected with AOM (15 mg/kg body weight) once a week for two weeks. Groups 6, 7 and 8 were injected with saline as vehicle controls. Groups 2, 3 and 4 were given *M. loriformis* extract (0.1, 0.5 or 1.0 g/kg body weight, respectively) once a day by intragastric gavage. Groups 1 and 6 received 25% DMSO as control. Group 5 was given 1.0 g/kg *M. loriformis* extract for only 1 week and on the next day they received the first AOM injection. All of the rats were sacrificed by cervical dislocation under anesthesia with lethal doses of diethyl ether.

The protocol for the post-initiation stage is shown in Fig.1 (b). The rats were divided into six groups. Two weeks after the second AOM injection, rats began to be given *M. loriformis* extracts (1.0 or 0.1 g/kg body weight) by intragastric gavage for 12 weeks. Animals were carefully observed daily and weighed weekly. The large intestines of sacrificed rats were removed, expanded with 10% formalin in a phosphate buffered saline solution (pH 7.4) on ice for 15 min, and then cut open longitudinally along the main axis. The intestines were cut into three portions: the first is the rectum to 2 cm from the anus, and the remaining colon was divided into two parts called the proximal and distal colon. Then each segment was placed between two pieces of filter paper, fixed in 10% buffered formalin for 24 h, and stained with 0.2% methylene blue in saline. ACF was examined under a microscope at a magnification of 40× according to the procedure of Bird (15).

6) Analysis of DNA adducts

Rats were treated and sacrified 12 h after the second AOM injection as described in Fig.1 (c). The perfused liver and colon were immediately removed. The colon was cut open longitudinally and washed with saline to remove colonic contents. Then the colon was laid flat on a glass plate and the mucosa was scraped off with a glass slide. The livers, colonic mucosa and colonic muscle layers were kept at -80°C until analysis of DNA adducts.

DNA was isolated by phenol extraction following treatment with RNaset A, T_1 and proteinase K (23). The two step procedure for detection of DNA adducts was based on the method of Beranek *et al*. (24). Purified DNA was dissolved in 10 mM sodium cacodylate (pH 7.0) at the concentration of 5 mg/ml and was subjected to neutral thermal hydrolysis by heating at 100°C for 30 min to release N^7-meG. The partially apurinic DNA was precipitated from the neutral thermal hydrolysate by addition of 0.1 volume of cold 1 N HCl and was collected by centrifugation at 3,000

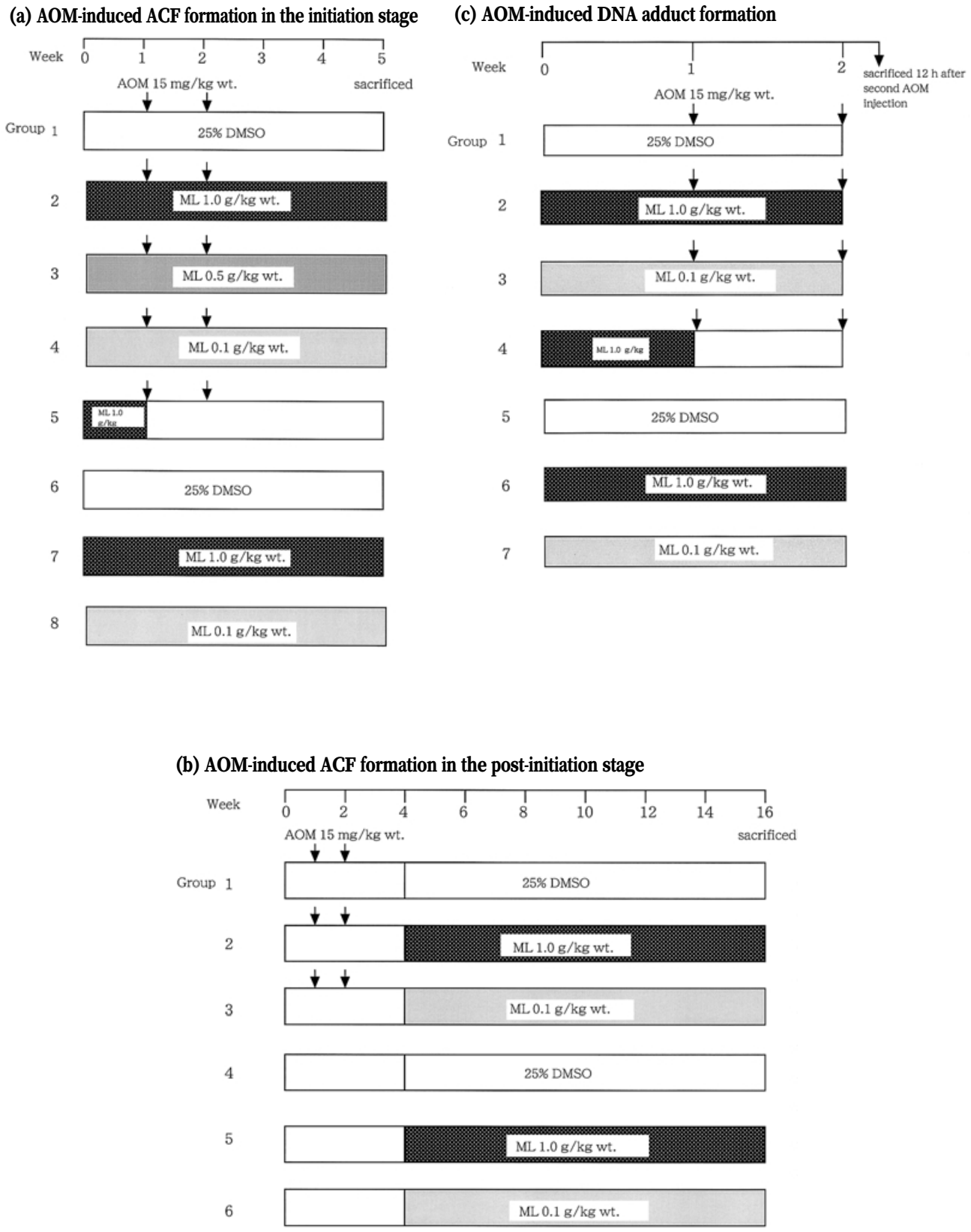

Figure 1. Experimental protocols for AOM-induced ACF formation in the initiation stage (a), in the post-initiation stage (b) and AOM-induced DNA adduct formation (c). Rats were treated with AOM and *Murdannia loriformis* extract as described in Materials and Methods.

rpm for 20 min at 0°C. The pellet was suspended in 50 mM Bis-Tris-1 mM $MgCl_2$ (pH 6.5) and completely hydrolyzed in 0.1 volume of 1 N HCl at 70°C for 30 min (acid hydrolysis). Methylated bases in neutral thermal hydrolysates (N^7-meG) and acid hydrolysates (O^6-meG) were analyzed by high-performance liquid chromatography (HPLC) using a Chemcosorb 7-SCX cation exchange column (4.6×250 mm, Chemco Scientific Co., Ltd., Osaka, Japan) and 4 mM ammonium formate (pH 3.0) as the mobile phase at a flow rate of 1.0 ml/min. Elution of the fluorescing base was monitored at 286 nm as an excitation wavelength and at 365 nm as an emission wavelength. Authentic N^7-meG and O^6-meG were used as standards.

7) Statistical analysis

The data were analyzed by one-way analysis of variance.

RESULTS

1) Antimutagenicity of M. loriformis

M. loriformis extract was not mutagenic for *Salmonella typhimurium* strains TA98 and TA100 in either the presence or absence of S9 mix (Table 1). Antimutagenic activities of the extract against various known mutagens are shown in Table 2. The extract showed antimutagenicity against tested heterocyclic amines, IQ, MeIQ, MeIQx, PhIP, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2, in dose-

Table 2. Antimutagenicity effects of 80% ethanol extract of *M. loriformis* on heterocyclic amines and known mutagens

Mutagen	Dose (μg/plate)	Strain	S9	His⁺ revertant/plate (% Mutagenicity)						
				M. loriformis extract (mg/plate)						
				0	0.313	0.625	1.25	2.5	5.0	10.0
IQ	0.004	TA98	+	882 (100)	811 (92.0)	812 (92.1)	861 (97.7)	675 (76.5)	645 (73.1)	412 (46.7)
MeIQ	0.0025	TA98	+	1922 (100)	1827 (95.0)	1826 (95.0)	1717 (89.3)	1628 (84.7)	1349 (70.2)	1076 (56.0)
MeIQx	0.01	TA98	+	1293 (100)	1446 (111.8)	1299 (100.5)	1263 (97.7)	1219 (94.3)	994 (76.9)	842 (62.7)
PhIP	1.0	TA98	+	1069 (100)	1014 (94.8)	863 (80.7)	963 (90.1)	835 (78.1)	845 (79.0)	613 (57.3)
Trp-P-1	0.05	TA98	+	1523 (100)	1339 (87.8)	1434 (94.2)	1368 (89.8)	1357 (89.1)	1104 (72.5)	941 (61.8)
Trp-P-2	0.1	TA98	+	1437 (100)	1421 (98.9)	1339 (93.2)	1409 (98.0)	1120 (77.9)	994 (69.2)	734 (51.1)
Glu-P-1	0.05	TA98	+	2407 (100)	2149 (89.3)	1978 (82.2)	1769 (73.5)	1683 (69.1)	1557 (64.7)	914 (38.0)
Glu-P-2	4.0	TA98	+	1030 (100)	854 (82.9)	905 (87.9)	829 (80.5)	866 (84.1)	540 (52.4)	365 (35.4)
AαC	5.0	TA98	+	1244 (100)	1271 (102.2)	1271 (102.2)	1262 (101.4)	1282 (103.0)	1324 (106.4)	1305 (104.9)
MeAαC	5.0	TA98	+	816 (100)	767 (94.0)	748 (91.7)	904 (110.8)	790 (96.8)	827 (101.3)	924 (113.2)
AFB_1	0.1	TA98	+	1632 (100)	1592 (97.5)	1653 (101.3)	1686 (103.3)	1707 (104.6)	1756 (107.6)	1364 (83.6)
B[*a*]P	2.5	TA100	+	494 (100)	591 (119.6)	591 (119.6)	548 (110.9)	489 (99.0)	417 (84.4)	477 (96.5)
2-AA	1.0	TA98	+	3626 (100)	2350 (64.8)	2139 (59.0)	2099 (57.9)	2108 (58.1)	2086 (57.5)	1967 (52.3)
AF-2	0.025	TA98	—	1905 (100)	1327 (69.7)	1354 (71.1)	1256 (65.9)	1245 (65.4)	1300 (68.2)	1294 (67.9)
MNNG	0.5	TA100	—	2646 (100)	2783 (105.2)	2113 (79.8)	2176 (82.2)	1715 (64.8)	1143 (43.2)	1251 (47.3)
ENNG	0.25	TA100	—	455 (100)	457 (100.4)	618 (135.8)	547 (120.2)	392 (86.1)	473 (103.9)	507 (111.4)
1-NP	0.1	TA98	—	546 (100)	468 (85.7)	499 (91.4)	503 (92.2)	569 (104.2)	490 (89.8)	587 (107.5)
MAM	4.00*	TA100	—	668 (100)	245 (36.7)	180 (26.9)	162 (24.2)	196 (29.3)	198 (29.6)	210 (31.4)

*mg/plate

dependent manner except for AαC and MeAαC. About 40-60% of their mutagenicities was decreased at 10 mg/plate. The extract also decreased the mutagenicities of 2-AA, AF-2, MNNG and MAM, and the mutagenicity of MAM was decreased to 36.7% even at the lowest dose (0.31 mg/plate). The extract had no effect on the mutagenicities of B[*a*]P, ENNG and 1-NP.

2) Inhibitory effect of M. loriformis extract on AOM-induced DNA methylation

The inhibitory effects of *M. loriformis* extract on AOM-induced DNA methylation are shown in Table 3. Methylated DNA adducts were not detected in the solvent control group or in the extract treated-group. The extract inhibited the formation of O^6-meG by about 10-20% and 30-60% in the colonic mucosa and muscular layers of the AOM-treated rats, respectively, but not in a dose-dependent manner, and a significant decrease was observed only in the muscular layers of rats treated with AOM and 1.0 g/kg of the extract. N^7-MeG was not decreased by continuous feeding. Pretreatment at a dose of 1.0 g/kg for 1 week before AOM administration was most effective, but the level of O^6-meG in the colonic mucosa was not significantly lower than that in the AOM-treated rats. In the pretreatment group, the extract also significantly inhibited the formation of N^7-meG both in the colonic mucosa and muscular layer. Methylguanines in the liver tended to be increased by the extract. The levels of O^6-meG and N^7-meG in the liver of pretreatment group (group 4) were increased by 2.2 and 1.5 fold, respectively, as compared with those of the AOM-treated group.

3) Inhibitory effect of M. loriformis extract on AOM-induced ACF formation

The body weights of rats in the control and *M. loriformis*-treated groups were not significantly different in either the initiation or the post-initiation stage during the experiments. No abnormality was seen with the naked eye in any group both in the initiation and post-initiation stages. The effects of *M. loriformis* extract on AOM-induced ACF formation in the initiation stage are shown in Table 4. Pretreatment with the extract only for 1 week before AOM injection at a dose of 1.0 g/kg most effectively inhibited the ACF formation, and the number of ACFs significantly

Table 3. Effects of *Murdannia loriformis* extract on the levels of O^6-methylguanine and N^7-methylguanine in AOM-induced rats

Treatment	n	O^6-Methylguanine (μmol/mol guanine)[b]			N^7-Methylguanine (mmol/mol guanine)		
		Colonic mucosa	Muscular layer	Liver	Colonic mucosa	Muscular layer	Liver
25% DMSO+AOM	5	91.4±27.2	41.0±30.9	508.6±112.2	1.67±0.50	1.69±0.75	7.37±2.49
ML[a] 1.0 g/kg wt+AOM	5	79.2±12.4	16.8±9.3[c]	471.5±33.3	1.45±0.34	1.85±1.11	8.47±3.63
ML 0.1 g/kg wt+AOM	5	82.8±18.1	27.9±9.0	695.6±294.1	1.59±0.72	1.13±0.22	16.77±12.82[c]
ML 1.0 g/kg wt(l wk) +AOM	5	73.0±14.6	23.0±12.3	1119.1±239.4[f]	0.43±0.26[f]	0.29±0.21[e]	10.80±5.13
25% DMSO	4	<3.9±1.4[g]	<5.9±4.3[d]	<5.4±0.9[f]	<0.13±0.05[f]	<0.20±0.14[c]	<0.18±0.03[g]
ML 1.0 g/kg wt	4	<4.8±1.2[g]	<3.9±0.5[e]	<5.9±0.8[f]	<0.16±0.04[f]	<0.13±0.02[s]	<0.20±0.06[g]
ML 0.1 g/kg wt	3	<4.2±0.9[g]	<4.1±1.6[s]	<4.6±1.3[f]	<0.14±0.03[f]	<0.14±0.05[e]	<0.15±0.04[g]

[a]*Murdannia loriformis* extract [b]Mean±SD [c]Significantly different from 25% DMSO+AOM at $p<0.05$ [d]$p<0.005$ [e]$p<0.001$ [f]$p<0.0005$ [g]$p<0.0001$

Table 4. Inhibitory effect of *M. loriformis* AOM-induced aberrant crypt foci in male F344 rats in the initiation stage

Treatment	n	Colon		Rectum		Total	
		ACF[b]	Crypt/focus	ACF	Crypt/focus	ACF (% inhibition)	Crypt/focus
25% DMSO+AOM	8	115.4±29.6	1.32±0.11	14.4±5.8	1.49±0.41	129.8±31.6(0)	1.35±0.15
ML[a] 1.0 g/kg wt+AOM	6	94.2±25.2	1.28±0.09	8.5±4.0[c]	1.08±0.10[d]	102.7±27.4(20.9)	1.27±0.09
ML 0.5g/kg wt+AOM	7	75.8±36.6[c]	1.33±0.12	10.1±2.8[c]	1.23±0.09[c]	86.0±35.9[d](33.8)	1.32±0.11
ML 0.1g/kg wt+AOM	8	70.5±24.5[c]	1.31±0.08	8.8±4.4[c]	1.23±0.19[c]	79.2±24.8[c](39.0)	1.30±0.08
ML 1.0 g/kg wt (l wk) +AOM	8	48.9±13.6[e]	1.27±0.11	8.2±2.3[d]	1.18±0.15[c]	57.1±14.9[r](56.0)	1.25±0.11
25% DMSO	6	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]
ML 1.0 g/kg wt	6	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]
ML 0.1 g/kg wt	6	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]

[a]*Murdannia loriformis* extract [b]Mean number of ACFs/rat±SD [c]Significantly different from 25% DMSO+AOM at $p<0.05$ [d]$p<0.005$ [e]$p<0.0005$ [f]$p<0.0001$

Table 5. Inhibitiory effects of *M. loriformis* extract on AOM-induced aberrant crypt foci in male F344 rats in the post-initiation stage

Treatment	n	Colon			Rectum			Total		
		ACF[b]	Crypt/focus	Multiple ACF[c]	Focus	Crypt/focus	Multiple ACF	Focus	Crypt/focus	Multiple ACF
25% DMSO+AOM	10	257.4±65.6	2.71±0.27	52.1±12.2	57.4±23.0	2.34±0.25	7.8±3.5	324.8±78.0	2.64±0.26	59.9±12.3
ML[a]1.0 g/kg wt+AOM	5	236.0±38.3	2.33±0.09[e]	43.2±8.3	51.6±11.1	2.39±0.40	9.4±6.6	287.6±47.1	2.34±0.11[e]	52.6±11.6
ML 0.1 g/kg wt+AOM	7	223.0±35.6	2.35±0.25[e]	38.3±11.9[d]	45.8±11.0	2.16±0.15	5.4±4.2	268.8±44.2	2.32±0.22[d]	43.7±14.5[d]
25% DMSO	6	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]
ML 1.0 g/kg wt	4	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]
ML 0.1 g/kg wt	5	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]	0±0[f]

[a]*M. loriformis* extract [b]Mean number of ACF/rat±SD [c]Multiple ACFs have more than 3 aberrant crypts/focus.
[d]Significantly different from 25% DMSO+AOM at $p<0.005$ [e]$p<0.001$ [f]$p<0.0001$

(p<0.0001) decreased to 44% of that in the positive control group (25% DMSO+AOM). When the extract was administered throughout the experimental period at doses of 1.0, 0.5 and 0.1 g/kg, it inhibited ACF formation by about 21%, 34% and 39%, respectively. The number of ACFs was significantly decreased only at 0.5 g/kg wt (p<0.005) and at 0.1 g/kg wt(p<0.0005). However, the extract was more effective at a lower dose. Multiplicity of ACF (crypt/focus) was decreased by *M. loriformis* treatment only in the rectum.

In the post-initiation stage, the extract did not significantly decrease ACF formation either in the colon or rectum (Table 5). However, it significantly inhibited the formation of larger ACFs (more than 3 crypts/focus) by 27.0% in the total colon at 0.1 g/kg. Multiplicity of ACF was significantly decreased by treatment with the extract both at 0.1 and 1.0 g/kg.

4) Correlation between the number of ACFs and DNA adduct level

A significant correlation between the number of ACFs and the level of O^6-meG in the colonic mucosa was observed (r=0.808, p=0.0382, Fig.2), but not between the number of ACFs and the level of N^7-meG in the colonic mucosa (data not shown).

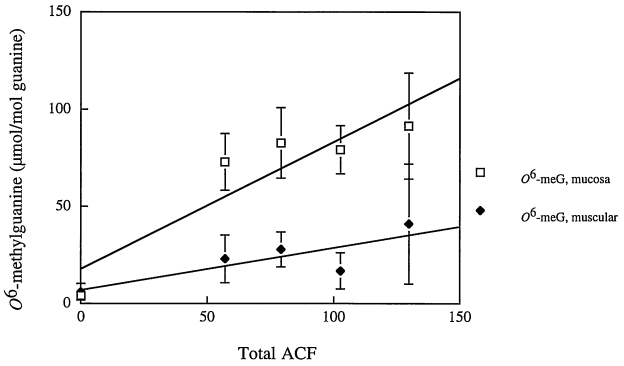

Figure 2. Correlation between the number of ACFs/rat and O^6-methylguanine levels in the rat colon. Correlation coefficients were 0.808 in the colonic mucosa (p=0.0382) and 0.680 in the muscular layer (p=0.0979).

DISCUSSION

Colorectal cancer has a high mortality rate in Western countries and this may also soon be the case in Asian countries because of rapid Westernization of life style. However, the mortality rate of colorectal cancer in Thailand is still low compared with that in Japan (25, 26). This may be partially due to greater consumption of spices, herbs and medicinal plants in Thailand. Many kinds of chemical compounds present in natural dietary products have been shown to be protective against chemically induced toxicity and carcinogenesis (4, 27-29). Some Thai medicinal plants have been reported to be antimutagenic (6, 8-11, 13) and to inhibit AOM-induced ACF formation in the rat colon (7-9). *M. loriformis* is a Thai medicinal plant that has been used for relief of bronchitis and cancer (12). In the present study, we examined 80% ethanol extract of *M. loriformis* for its antimutagenic activity and inhibitory effects on AOM-induced ACFs formation.

Antimutagenicity of *M. loriformis* against various known mutagens was measured by the *Salmonella* mutation assay in both strains TA98 and TA100 in the presence or absence of S9 mix. The extract inhibited the mutagenicity of tested heterocyclic amines except for AαC derivatives. These results are similar to those of a previous report (13). Since *M. loriformis* inhibited almost all of the HCA-induced mutations, it may possibly inhibit the metabolic activation of HCAs by cytochrome P450 1A2. Factors affecting the metabolic activation and DNA adduct formation of HCAs were reviewed by Kato (30). Unsaturated fatty acids such as oleic and linoleic acids inhibited the mutagenicity of Trp-P-2 mainly

through the inhibition of hepatic mixed function oxidase (31), and microsomal lipids or lecithin diminished the amount of *N*-OH Trp-P-2 without inhibiting the metabolism of Trp-P-2. Compound lipids such as glycosphingolipid in *M. loriformis* possibly inhibit P450 1A2-mediated activation or interact with activated HCAs. Mutagenicity of methylating agents was also inhibited, especially in the case of MAM, whose mutagenicity was reduced by about 70% with maximum inhibition at the lowest dose. The interactions of some components of *M. loriformis* with MAM or its ultimate form might contribute to the decrease of mutagenicity.

Since *M. loriformis* extract efficiently inhibited MAM-induced mutation (Table 2), we examined whether the extract inhibits AOM-induced DNA methylation and ACF formation in the rat colon. Formation of ACF in the rat colon in the initiation stage was inhibited by feeding rats the extract, while the formation of O^6-meG in the rat colon was weakly inhibited by *M. loriformis* treatment. Since the level of DNA adducts correlated well with the number of ACFs in the colon (Fig.2), inhibition of ACF formation was thought to be partially due to the decrease in the level of adducts.

AOM was oxidatively metabolized to MAM by P 450 2E1 and transported to the colon via the blood stream (32). Alternatively, MAM was conjugated with glucuronic acid and transported via bile to the colon (33). Conjugated MAM is hydrolyzed by bacterial β-glucuronidase to free MAM in the intestine (34), and released MAM is further metabolized and methylates DNA in the colon (35). Although N^7-meG is a major adduct, O^6-meG in the target tissue is more closely correlated with carcinogenicity than is N^7-meG because thymine can be incorporated opposite O^6-meG resulting in GC→AT transition mutations (19), and such alterations are frequently observed in tumor-associated genes (36).

In the present study, *M. loriformis* extract inhibited AOM-induced ACF formation partially due to the decrease of O^6-meG level. Although mechanisms of inhibition of DNA adducts formation by the extract are still unclear, current evidence suggests that the components of *M. loriformis* can induce chemoprotective enzymes such as glutathione *S*-transferase, UDP-glucuronyl transferase and DT-diaphorase (37). However, the level of both O^6-meG and N^7-meG in the liver were rather increased by the extract. Modification of AOM metabolism in the liver and lowered distribution of the metabolites to the colon might contribute to the decrease of O^6-meG in the colon. Other possible mechanisms of inhibition of ACF formation are enhanced DNA repair or modification of cell proliferation as do curcumin and other plant components (38).

The number of ACFs was more decreased at a lower dose in the continuously fed group and most strongly inhibited in the pretreatment group (Table 4). It is not known why a lower dose was more effective in inhibition of ACF formation.

In the post-initiation stage, multiplicity of ACF in the total colon was significantly decreased by the extract (Table 5). The number of ACFs that have more than 3 crypts/focus, which is highly correlated with tumor formation (17), was also significantly decreased. These results suggest that *M. loriformis* inhibits the growth of ACFs. Recent studies have shown that dietary sphingolipids inhibit dimethylhydrazine-induced colon cancer in CF1 mice (39-41), presumably because they are digested to the lipid backbones ceramide and sphingosine that inhibit cell growth and induce differentiation and apoptosis (42). Glycosphingolipids, constituents of *M. loriformis*, could contribute to the inhibition of ACF formation by the above mechanisms.

We have investigated antimutagenicity of Thai medicinal plants and their inhibitory effects on AOM-induced ACF formation. Among tested plants, *M. loriformis* and roselle (8) decreased the mutagenicity of heterocyclic amines such as PhIP and IQ, which are colon carcinogens in rodents. Lemon grass was the strongest inhibitor against the ACF formation in the initiation stage by enhancing detoxification enzyme activity (7). Inhibitory effect of *M. loriformis* on the ACF formation was similar to those of roselle (8) and bitter melon (9).

In conclusion, *M. loriformis* extract showed antimutagenicity against various known mutagens and inhibited the formation of AOM-induced ACF and DNA adduct formation in the rat colon. Chemopreventive agents have been classified into three categories based on the time period that the agents exert their inhibitory activity in animal carcinogenesis models : inhibitors of carcinogen formation, blocking agents that are inhibitors of tumor initiation, and suppressing agents that are inhibitors of tumor promotion and/or progression (5). *M. loriformis* extract may act as either a blocking or suppressing agent. However, Yamada *et al.* (43) currently suggested that β-catenine-accumulated crypts, which are independent of ACFs, are truly premalignant lesions for colon cancer. Usefulness of *M. loriformis* extract for colon cancer chemoprevention should be further investigated.

ACKNOWLEDGEMENTS

We thank Drs. K. Ishizaki and M. Ikenaga, Kyoto University, for supplying the standard O^6-meG, and we also thank Dr. K. Wakabayashi, National Cancer Center Research Institute, for supplying HCAs.

REFERENCES

1. Wynder EL, Kajitani T, Ishikawa S, Dodo H, Takano A : Environmental factors of cancer of the colon and rectum. II Japanese epidemiological data. Cancer 23 : 1210-1220, 1969
2. Haenszel W, Locke FB, Segi M : A case-control study of large bowel cancer in Japan. J Natl Cancer Inst 64 : 17-22, 1980
3. Henderson BE, Ross RK, Shibata A, Paganini-Hill A, Yu MC : Environmental carcinogens and anticarcinogens. In : Wattenberg, L, Lipkin M, Boone CW, Kelloff GJ, eds. Cancer Chemoprevention. CRC Press, Inc., Boca Raton, 1992, pp.3-17
4. Wall ME, Wani MC, Hughes TJ, Taylor H : Plant antimutagens. In : Kuroda Y, Shankel D M, Waters MD, eds. Antimutagenesis and Anticarcinogenesis Mechanisms II. Plenum Press, New York and London, 1990, pp.61-78.
5. Wattenberg LW : Chemoprevention of cancer. Cancer Res 45 : 1-8, 1985
6. Vinitketkumnuen U, Puatanachokchai R, Kongtawelert P, Lertprasertsuke N, Matsushima T : Antimutagenicity of lemon grass (*Cymbopogon citratus* Stapf) to various known mutagens in *Salmonella* mutation assay. Mutation Res 341 : 71-75, 1994
7. Suaeyun R, Kinouchi T, Arimochi H, Vinitketkumnuen U, Ohnishi Y : Inhibitory effects of lemon grass (*Cymbopogon citratus* Stapf) on formation of azoxymethane-induced DNA adducts and aberrant crypt foci in the rat colon. Carcinogenesis 18 : 949-955, 1997
8. Chewonarin T, Kinouchi T, Kataoka K, Arimochi H, Kuwahara T, Vinitketkumnuen U, Ohnishi Y : Effects of roselle (*Hibiscus sabdariffa* Linn.), a Thai medicinal plant, on the mutagenicity of various known mutagens in *Salmonella typhimurium* and on formation of aberrant crypt foci induced by the colon carcinogens azoxymethane and 2-amino-1-methyl-6-phenylimidazo[4, 5-*b*]pyridine in F344 rats. Food Chem Toxicol 37 : 591-601, 1999
9. Chiampanichayakul S, Kataoka K, Arimochi H, Thumvijit S, Kuwahara T, Nakayama H, Vinitketkumnuen U, Ohnishi Y : Inhibitory effects of bitter melon (*Momordica charantia* Linn.) on bacterial mutagenesis and aberrant crypt focus formation in the rat colon. J Med Invest 48 : 88-96, 2001
10. Higashimoto M, Purintrapiban J, Kataoka K, Kinouchi T, Vinitketkumnuen U, Akimoto S, Matsumoto H, Ohnishi Y : Mutagenicity and antimutagenicity of extracts of three spices and a medicinal plant in Thailand. Mutation Res 303 : 135-142, 1993
11. Rojanapo W, Tepsuwan A, Siripong P : Mutagenicity and antimutagenicity of Thai medicinal plants. In : Kuroda Y, Shankel DM, Waters MD, eds. Antimutagenesis and Anticarcinogenesis Mechanisms II. Plenum Press, New York and London, 1990, pp.447-452
12. Amarin Printing Group : Medicinal Plants in Siri Rukhachati Garden. Mahidol University, (Bangkok), Thailand, 1992, p.257
13. Vinitketkumnuen U, Charoenkunathum W, Kongtawelert P, Lertprasertsuke N, Picha P, Matsushima T : Antimutagenicity and DT-diaphorase inducer activity of the Thai medicinal plant, *Murdannia loriformis*. J Herbs Spices & Med Plants 4 : 45-52, 1996
14. Jiratchariyakul W, Okabe H, MoonKarndi P, Picha P, Frahm AW : Cytotoxicity principles of *Murdannia loriformis* (Hassk) Rolla Rao et Kammathy. The 3rd National Cancer Conference, Bangkok, Thailand, 1-42, 1995
15. Bird RP : Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen : preliminary findings. Cancer Lett 37 : 147-151, 1987
16. Pretlow TP, Barrow BJ, Ashton WS, O' Riordan MA, Pretlow TG, Jurcisek JA, Stellato TA : Aberrant crypts : putative preneoplastic foci in human colonic mucosa. Cancer Res 51 : 1564-1567, 1991
17. Bird RP : Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett 93 : 55-71, 1995
18. Dipple A : DNA adducts of chemical carcinogens. Carcinogenesis 16 : 437-441, 1995
19. Saffhill R, Margison GP, and O' Connor PJ : Mechanisms of carcinogenesis induced by alkylating agents. Biochim Biophys Acta 823 : 111-145, 1985
20. Margison GP, Kleihues P : Chemical carcinogenesis in the nervous system. Preferential accumulation of O^6-methylguanine in rat brain deoxyribonucleic acid during repetitive administration of *N*-methyl-

N-nitrosourea. Biochemical J 148 : 521-525, 1975
21. Maron DM, Ames BN : Revised methods for the *Salmonella* mutagenicity test. Mutation Res 113 : 173-215, 1983
22. Yahagi T, Nagao M, Seino Y, Matsushima T, Sugimura T, Okada M : Mutagenicities of *N*-nitrosamines on *Salmonella*. Mutation Res 48 : 121-130, 1977
23. Gupta RC : Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. Proc Natl Acad Sci USA 81 : 6943-6947, 1984
24. Beranek DT, Weis CC, Swenson DH : A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. Carcinogenesis 1:595-606, 1980
25. IARC:Cancer in Thailand 1988-1991. In : Vatanasapt V, Martin N, Sriplung H, Chindavijak K, Sontipong S, Sriamporn S, Parkin D M, Ferlay J,.eds. IARC Technical Report No.16, Lyon, 1993, p.64
26. Utsunomiya J, Yamamura B, Kusunoki M, Yanagi H, Shoji Y, Noda M, Ikeuchi H, Taniguchi H : Comparison of colorectal cancer in Japan and USA. Karkinos 6 : 839-844, 1993
27. Hayatsu H, Arimoto S, Negishi T : Dietary inhibitors of mutagenesis and carcinogenesis. Mutation Res 202 : 429-446, 1988
28. Hocman G : Prevention of cancer : vegetables and plants. Comp Biochemi Physio, P B, Comp Biochemi : an International. 93 B, 201-212, 1989
29. Morse ME, Stoner GD : Cancer chemoprevention : Principles and Prospects. Carcinogenesis 14, 1737-1746, 1993
30. Kato R : Metabolic activation of mutagenic heterocyclic aromatic amines from protein pyrolysates. CRC Crit Rev Toxicol 16 : 307-348, 1986
31. Saito K, Yamazoe Y, Kamataki T, Kato R : Interactions between the active metabolite of tryptophan pyrolysate mutagen, *N*-hydroxy-Trp-P-2, and lipids : the role of lipid peroxides in the conversion of *N*-hydroxy-Trp-P-2 to non-reactive forms. Chem-Biol Interact 45 : 295-304, 1983
32. Sohn OS, Ishizaki H, Yang CS, Fiala ES : Metabolism of azoxymethane, methylazoxymethanol and *N*-nitrosodimethylamine by cytocrome P450 IIE1. Carcinogenesis 12 : 127-131, 1991
33. Weisburger JH : Colon carcinogens : their metabolism and mode of action. Cancer 28 : 60-69, 1971
34. Matsumoto H, Takata RH, Komeiji DY : Synthesis of the glucuronic acid conjugate of methylazoxymethanol. Cancer Res 39 : 3070-3073,1979
35. Craven PA, Neidig M, Derubertis FR : Fatty acid stimulated oxidation of methylazoxymethanol in rat colonic mucosa. Cancer Res 45 : 1115-1121, 1985
36. Fearon ER, Jones PA : Processing toward a molecular description of colorectal cancer development. Fed Am Soc Exp Biol : Fed Proc 6 : 2783-2790, 1992
37. Talalay P : Mechanisms of induction of enzymes that protect against chemical carcinogenesis. Adv Enz Regulation 28 : 237-250, 1989
38. Kelloff GJ, Boone CW, Crowell JA, Steele V E, Lubet RA, Doody LA, Malone WF, Hawk ET, Sigman CC : New agents for cancer chemoprevention. J Cell Biochem 26 S : 1-28, 1996
39. Dillehay DL, Webb SK, Schmelz EM, Merrill AH Jr. : Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF 1 mice. J Nutr 124 : 615-620, 1994
40. Schmelz EM, Dillehay DL, Webb SK, Reiter A, Adams J, Merrill AH Jr. : Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1,2-dimethylhydrazine : implications for dietary sphingolipids and colon carcinogenesis. Cancer Res 56 : 4936-4941, 1996
41. Schmelz EM, Bushnev AS, Dillehay DL, Liotta DC, Merrill AH Jr. : Suppression of aberrant colonic crypt foci by synthetic sphingomyelins with saturated or unsaturated sphingoid base backbone. Nutr Cancer 28 : 81-85, 1997
42. Vesper H, Schmelz EM, Dillehay DL, Lynch D, Merrill AH Jr. : Sphingolipids in food and the emerging importance of sphingolipids in nutrition. J Nutr 129 : 1239-1250, 1999
43. Yamada Y, Yoshimi N, Hirose Y, Matsunaga K, Katayama M, Sakata K, Shimizu M, Kuno T, Mori H : Sequential analysis of morphological and biological properties of β-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. Cancer Res 61 : 1874-1878, 2001