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Ethyl Acetate Fraction of Adlay Bran Ethanolic Extract Inhibits Oncogene Expression and Suppresses DMH-Induced Preneoplastic Lesions of the Colon in F344 Rats through an Anti-inflammatory Pathway

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Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is a grass crop and was reported to possess anti-inflammatory activity and an antiproliferative effect in cancer cell lines. The purpose of this study was to evaluate the effects of the ethyl acetate fraction of an adlay bran ethanolic extract (ABE-Ea) on colon carcinogenesis in an animal model and investigate its mechanism. Male F344 rats received 1,2-dimethylhydrazine (DMH) and consumed different doses of ABE-Ea. The medium-dose group (17.28 mg of ABE-Ea/day) exhibited the best suppressive effect on colon carcinogenesis and prevented preneoplastic mucin-depleted foci (MDF) formation. Moreover, *RAS* and *Ets2* oncogenes were significantly down-regulated in this group compared to the negative control group, whereas *Wee1*, a gene involved in the cell cycle, was up-regulated. Cyclooxygenase-2 (COX-2) protein expression was significantly suppressed in all colons receiving the ABE-Ea, indicating that ABE-Ea delayed carcinogenesis by suppressing chronic inflammation. ABE-Ea included considerable a proportion of phenolic compounds, and ferulic acid was the major phenolic acid (5206 μg/g ABE-Ea) on the basis of HPLC analysis. Results from this study suggest that ABE-Ea suppressed DMH-indued preneoplastic lesions of the colon in F344 rats and that ferulic acid may be one of the active compounds.

KEYWORDS: Adlay bran; mucin-depleted foci (MDF); *RAS*; *Wee1*; cyclooxygenase-2 (COX-2)

INTRODUCTION

Colorectal cancer is one of the leading causes of cancer deaths in both men and women throughout the world. The etiology of colorectal cancer is complex and may be attributable to combined actions of environmental and inherited factors (*1*). Epidemiological studies indicated that colorectal cancer is strongly associated with the diet, and thus the occurrence of colorectal cancer may be prevented by dietary modifications (*2*). Aberrant crypt foci (ACF), preneoplastic lesions of colon cancer, are widely used as a biomarker in short-term tests to predict colon carcinogenesis (*3*). Changes in the type of mucin of ACF are related to the degree of dysplasia (*4*), whereas mucin-depleted foci (MDF) are cancer precursors and are used to predict colon cancer occurrence (*5*). Studies demonstrated that cyclooxygenase-2 (COX-2), which catalyzes the conversion of arachidonic acid to prostaglandins, and inducible nitric oxide synthase (iNOS), which stimulates nitric oxide (NO) synthesis, are up-regulated during inflammatory bowel disease (*6*) and colorectal carcinogenesis by the *RAS* oncogene family (*7*). COX-2 selective inhibitors are believed to suppress colonic ACF induced by azoxymethane (AOM), a carcinogenic metabolite of 1,2-dimethylhydrazine (DMH), and inhibit colon tumor formation (*8*).

Adlay or Job's tears (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is an annual crop belonging to the family Gramineae. The adlay seed consists of four parts from outside to inside: the hull, testa, bran, and endosperm. Dehulled adlay consists of the bran and endosperm, and it has long been used in food supplements and as a nourishing food. Adlay is believed to be beneficial to the human body, and recent studies reported that components in adlay seeds possess an anti-inflammatory activity and an antiproliferative effect on colorectal cancer cell lines. In an animal model, Shih et al. reported that a diet containing 20% dehulled adlay significantly suppressed the formation of preneoplastic ACF in colon carcinogenesis (*9*). Lee et al. pointed out that the ethyl acetate fraction of the adlay bran methanolic extract (ABM-Ea) exhibited antiproliferative effects against the HT-29 and COLO 205 human colon cancer cell lines via apoptosis (*10*). Furthermore, Huang et al. proved that phenolic compounds in adlay testa possess an anti-inflammatory effect, suggesting that phenolic acids may be some of the active compounds in adlay (*11*). These results suggest that adlay and its components may suppress cancer cell proliferation and have the potential to be developed as chemopreventive agents against colorectal cancer.

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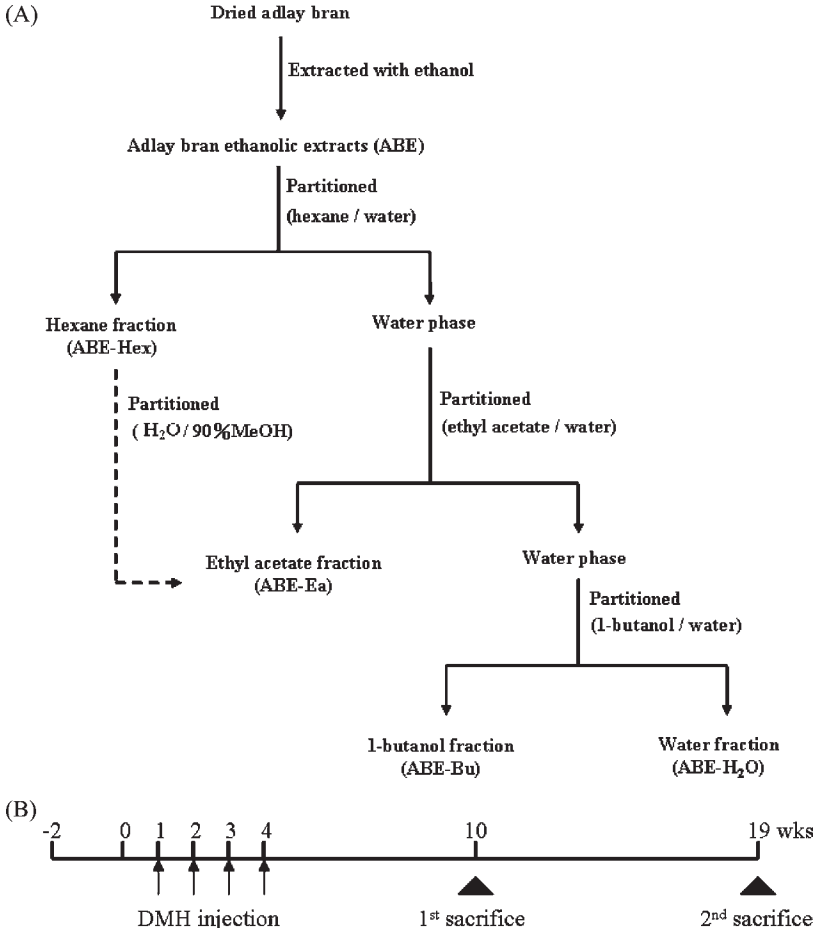

Figure 1. (**A**) Scheme of extracting and partitioning from adlay bran to obtain ABE-Hex, ABE-Ea, ABE-Bu, and ABE-H_2O, respectively. (**B**) Experimental schema. After 9 weeks of induction with 1,2-dimethylhydrazine (DMH), eight rats per group were sacrificed and their colons removed to examine for aberrant crypt foci (ACF) and mucin types on ACF and mucin-depleted foci (MDF). After 18 weeks of induction, the other rats were sacrificed, and their colons were preserved at −80 °C to carry out total RNA extraction and further analysis.

Inflammation-related pathways play critical roles in colorectal carcinogenesis, whereas in vivo investigations that use adlay bran extract as an experimental sample are insufficient. Our recent study found that the adlay bran ethanolic extract (ABE) inhibited ACF formation in a dose-dependent manner (unpublished data). To evaluate the chemopreventive effect of the ethyl acetate fraction of the ABE (ABE-Ea) against colorectal cancer, this study was designed to investigate the effects of various doses of the ABE-Ea on ACF and the mucin types of ACF and MDF using an animal model. To elucidate possible pathways of the ABE-Ea, total RNA of the colonic mucosa was extracted, a cDNA microarray was used as a comprehensive screening platform, and Western blotting was utilized to confirm the accuracy of the microarray experiment.

MATERIALS AND METHODS

Preparation of the ABE-Ea. Adlay seeds were purchased from local farmers who planted Taichung Shuenyu no. 4 (TCS4) of *C. lachryma-jobi* L. var. *ma-yuen* Stapf in Taichung, Taiwan. The adlay seeds were dried at 40 °C with ventilation and dehulled in a grinder. Samples were divided into the hull, testa, and dehulled adlay by gentle blowing using an electric fan. The dehulled adlay was separated into bran and polished adlay. Adlay bran was blended into powder form and screened through a 20-mesh sieve (with a 0.94 mm aperture). To prepare ABE-Ea, the method as mentioned in Lee et al. was modified (*10*). Adlay bran powder was protected from light and extracted with ethanol at room temperature for 24 h. The plant residue was filtered off, and the ethanolic extract was concentrated under reduced pressure by a rotary vacuum evaporator to obtain the ABE. The ABE was suspended in H_2O, followed by liquid partitioning with *n*-hexane, ethyl acetate, and *n*-butanol, which yielded four subfractions: ABE-Hex, ABE-Ea, ABE-Bu, and ABE-H_2O, respectively. The ABE-Hex was repartitioned with 90% MeOH/H_2O to extract the polar components, and the 90% MeOH/H_2O subfraction was combined into the ABE-Ea as shown in **Figure 1A**.

Animals and Diets. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University. Male F344 rats were obtained at an age of 3−5 weeks from the National Laboratory Animal Center (Taipei, Taiwan). Animals were housed in plastic cages in a room under controlled conditions of 23 ± 2 °C and 50 ± 10% relative humidity, with a 12 h light/dark cycle. Animals consumed a standard AIN-93G diet (*12*) and water ad libitum. Experimental samples for tube feeding were emulsified with a 0.5% carboxymethyl cellulose (CMC) solution. Piroxicam (Sigma, St. Louis, MO), a COX-2 selective inhibitor (*13*), was chosen as the positive control. After 2 weeks of acclimation, animals were divided into five groups and tube-fed. The experimental groups consisted of a negative control (NC, 0.5 mL of 0.5% CMC/day), a positive control (PC, 1.50 mg of piroxicam/day), low dose (L, 8.64 mg of ABE-Ea/day), medium dose (M, 17.28 mg of ABE-Ea/day), and high dose (H, 34.56 mg of ABE-Ea/day), respectively. One week later, animals were intraperitoneally injected with a colon-specific carcinogen, 1,2-dimethylhydrazine (DMH), purchased from Sigma, once weekly for 4 weeks at a dose of 40 mg/kg of body weight as shown in **Figure 1B**. Body weights of the animals and consumption of the experimental diets were recorded weekly. After 9 weeks of induction, eight rats per group were sacrificed, and their colons were removed to examine for ACF and mucin types on the ACF and MDF. After 18 weeks of induction, the other rats were sacrificed, and the colonic mucosa was preserved at −80 °C until total RNA was extracted and further analyses were carried out.

Determination of ACF and Mucin Types of ACF and MDF. The frequency of ACF was determined according to a previously described method (*14*). Colons were removed, cut along the longitudinal axis, and flushed with phosphate-buffered saline (PBS). Each colon was cut into three equal lengths (proximal, middle, and distal) and fixed flat between pieces of filter paper in 10% buffered formalin (Mallinckrodt Specialty Chemicals, Paris, KY) for at least 24 h. The fixed colon sections were stained with a 0.2% solution of methylene blue (Showa Chemicals, Tokyo, Japan) and placed on microscopic slides with the mucosal side up. ACF were examined under 40× magnification using a light microscope (Nikon, Tokyo, Japan) and were distinguished from normal crypts by their increased size, irregular and dilated luminal openings, and thicker epithelial lining and pericryptal zone. The number of ACF per colon, the number of aberrant crypts observed in each focus, and the location of each focus were recorded.

The mucin types of ACF and MDF were identified according to a previously described method (*15*). For the high-iron diamine alcian blue (HIDAB) method, methylene blue-stained distal colon sections were dipped in a high-iron diamine solution (20 mg of *N*,*N*′-dimethyl-*p*-phenylenediamine and 120 mg of *N*,*N*′-dimethyl-*m*-phenylenediamine in 50 mL of distilled water plus 1.4 mL of a 60% ferric chloride solution) (Sigma) in a Petri dish protected against light for 50 min at room temperature. The colon sections were then rinsed in distilled water, stained with 1% alcian blue (Sigma) in 3% acetic acid for 30 min, rinsed in 80% ethanol followed by distilled water, and then observed microscopically. Using a light microscope (Nikon) at a magnification of 40×, dark brown staining of ACF implied sulfomucin (SUM) production, bright blue staining predominantly indicated sialomucin (SIM) production, and colorless or white staining of ACF implied the absence of mucin production. Using this HIDAB method, ACF producing >85% sialomucin were labeled SIM-ACF, those producing >85% sulfomucin were labeled SUM-ACF, and those in between determined to produce a combination of both SIM and SUM were labeled MIX-ACF, whereas those producing no or only a very small amount of mucin were labeled MDF.

cDNA Microarray Analysis. After 18 weeks of induction, total RNA of colonic mucosa from the medium-dose and negative control groups was isolated using TRIzol (Invitrogen) (*16*). The quality of RNA samples was monitored by the measurement of optical density at 260 and 280 nm and by 1.0% agarose formaldehyde gel electrophoresis. All samples had 260/280 ratios of about 1.7 and exhibited discrete 28S and 18S bands. The RNA was dissolved in 20 μL of diethylpyrocarbonate (DEPC) water, and the RNA obtained from the same group was pooled to eliminate individual differences (*17*). The total variance of gene expression was analyzed with a GeneChip Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA), and genes were considered to be up-regulated or down-regulated if their expression changed by at least 2-fold compared to the negative control. The NTUMAPS (National Taiwan University Microarray Analysis Platform and System) was used for data mining and pathway mapping. Values were considered to be positively expressed if the signal/background ratio was >100(*17*).

Western Blot Analysis. Total proteins were extracted from frozen colonic epithelium, and the protein concentration was determined using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA). Protein (60 μg) with NuPAGE LDS sample buffer and β-mercaptoethanol (Sigma) were heated to 100 °C for 10 min. After cooling at room temperature for 5 min, proteins were fractioned by 4−12% NuPAGE Novex Tris-acetate mini gel electrophoresis (Invitrogen). Proteins were then transferred to Millipore polyvinylidene difluoride membranes. Membranes were blocked with blocking buffer (1% albumin solution; Sigma) at room temperature for 1 h. Blots were probed with COX-2 and iNOS (1:800, Abcam) polyclonal antibodies at 4 °C overnight. After being extensively washed to eliminate nonspecific binding, the membranes were incubated with a goat anti-rabbit secondary antibody labeled with alkaline phosphatase at room temperature for 1 h. The Western blots were visualized using BCIP/NBT alkaline phosphatase substrate (Sigma). β-Actin (1:10000; Sigma) was detected in the same sample to ensure equal protein loading. The expression level of each protein relative to β-actin was calculated by ImageJ 1.40q (*11*).

High-Performance Liquid Chromatographic (HPLC) Analysis. Phenolic compounds in the ABE-Ea were analyzed by HPLC according to the method of Huang et al. (*11*). A Hitachi L-6200 intelligent pump and Hitachi L-7455 photodiode array detector were used. Gradient elution was performed with a 2% acetic acid aqueous solution (v/v, A) and 0.5% acetic acid in acetonitrile (v/v, B) at a constant rate of 1 mL/min through a C18 (150 × 4.6 mm, 5 μM) reverse-phase column (Kanto Chemical). Initial starting conditions were 5% B; 0−10 min, B increased from 5 to 10%; 10−40 min, B increased from 10 to 40%; 40−55 min, B increased from 55 to 80%; 60−65 min, B increased from 80 to 100%; 65−70 min, B decreased from 100 to 50%; 70−75 min, B decreased from 50 to 30%; 75−80 min, B decreased from 30 to 10%; and finally 80−85 min, B decreased from 10 to 5% at original condition. The absorbencies at 280 nm were measured, and the concentration of each analyzed compound was calculated by calibration curves from reference standards. The phenolic compounds were isolated from adlay, identified with spectral analysis, and analyzed by the HPLC method as mentioned above (*11*). The retention times of isolated compounds and reference standards purchased from Sigma were similar.

Statistical Analysis. ACF and MDF incidences among groups were compared by a Chi-squared test. Other results among groups were analyzed by analysis of variance (ANOVA) and Duncan's multiple-range test. p values of <0.05 were considered to significantly differ.

RESULTS AND DISCUSSION

Effects of ABE-Ea on Preneoplastic Lesions of the Colon. The ABE-Ea did not affect the normal growth of the experimental animals; there were no significant differences between the negative control and experimental groups in terms of the final body weight and daily feed intake (**Table 1**). Also, no obvious pathological changes in the organs and tissues were observed. As shown in **Table 1**, the ABE-Ea did not affect the incidence, number, or crypt multiplicity of ACF. Piroxicam significantly reduced the numbers of ACF and aberrant crypts compared to group NC ($p < 0.05$).

Numbers of MIX-ACF in groups PC, L, M, and H were significantly reduced, and the number of SIM-ACF in group H was also reduced compared to group NC ($p < 0.05$) as shown in **Table 1**. Previous studies reported that changes in the type of mucin on ACF are related to the degree of dysplasia, and the degrees of advancement are in an increasing order of SUM-ACF, MIX-ACF, and SIM-ACF (*4*). In this study, group NC showed a low SUM-ACF proportion and a high MIX-ACF proportion. In contrast, groups L, M, and H showed similar proportions of SUM-ACF and MIX-ACF. The ABE-Ea seemed to retard ACF at a lower degree of advancement of the mucin type, which means that the ABE-Ea suppressed mucin alteration of ACF. There were no significant differences in crypt multiplicity of SUM-ACF, MIX-ACF, and SIM-ACF among all groups. Additionally, there were no MDF in group PC or M. Also, the MDF incidence in groups PC and M was significantly reduced compared to that of group NC ($p < 0.05$), whereas there were no significant differences in the number or crypt multiplicity of MDF among the groups. Previous studies reported that MDF are cancer precursors and can be used to predict colon cancer (*15*). The results of the present study suggest that the ABE-Ea at a medium dosage may have the potential to suppress the formation of tumors.

As shown in **Table 2A**, there were no ACF with seven or eight crypts in groups L, M, and H, which means the formation of ACF with seven and eight crypts in L, M, and H was significantly inhibited by the ABE-Ea. Numbers of ACF with one crypt in groups PC and M were significantly reduced, and the number of ACF with three crypts in group PC was also significantly reduced compared to group NC ($p < 0.05$). Numbers of small ACF in group PC were significantly reduced compared to group NC ($p < 0.05$). Also, the distribution of ACF changed when given the ABE-Ea. As shown in **Table 2B**, there were no significant differences among groups in the numbers of ACF in the proximal and middle colon sections, but the numbers of ACF in the distal

Table 1. Effects of ABE-Ea on Body Weight, Feed Intake, and DMH-Induced Preneoplastic Lesions of the Colon in F344 Rats [a]

	group[b]				
	NC	PC	L	M	H
initial body wt (g)	139 ± 27	139 ± 35	139 ± 34	139 ± 35	139 ± 33
final body wt (g)	358 ± 23	349 ± 44	369 ± 18	350 ± 16	356 ± 23
av feed intake (g/day)	15.6 ± 0.5	15.0 ± 0.1	15.6 ± 0.4	15.0 ± 0.5	15.4 ± 0.6
ACF incidence (no. of rats with ACF/total rats)	100% (8/8)	100% (8/8)	100% (8/8)	100% (8/8)	100% (8/8)
no. of aberrant crypt foci (ACF)/colon	260 ± 95 a	171 ± 55 b	208 ± 68 ab	198 ± 51 ab	200 ± 59 ab
no. of aberrant crypts/colon	501 ± 181 a	342 ± 115 b	410 ± 139 ab	399 ± 97 ab	394 ± 123 ab
crypt multiplicity (no. of aberrant crypts/focus)	1.9 ± 0.1	2.0 ± 0.2	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
no. of ACF producing[c]					
SUM	31 ± 15 (27%)	33 ± 19 (48%)	42 ± 26 (46%)	34 ± 19 (41%)	40 ± 11 (49%)
MIX	73 ± 23 a (63%)	30 ± 8 b (43%)	44 ± 21 b (48%)	43 ± 19 b (52%)	38 ± 16 b (46%)
SIM	12 ± 9 a (10%)	6 ± 4 ab (9%)	6 ± 4 ab (6%)	6 ± 7 ab (7%)	4 ± 2 b (5%)
mucin-depleted foci (MDF) incidence (no. of rats with MDF/total rats)	50.0% (4/8)	0%[d] (0/8)	37.5% (3/8)	0%[d] (0/8)	25.0% (2/8)
no. of MDF/MDF-bearing rats	1.8 ± 1.0		1.0 ± 0.1		1.5 ± 0.7
crypt multiplicity (no. of aberrant crypts/no. of MDF)	8.5 ± 3.9		4.7 ± 0.6		6.3 ± 1.1

[a] All values are presented as the mean ± SD ($n = 8$). Values with different letters in a row show significant difference from one another as determined by Duncan's multiple-range test, $p < 0.05$. [b] NC, negative control group, 0.5 mL of 0.5% CMC/day; PC, positive control group, 1.50 mg of piroxicam/day; L, low dosage of the ethyl acetate fraction of adlay bran ethanolic extract (ABE-Ea) group, 8.64 mg of ABE-Ea/day; M, medium dosage of ABE-Ea group, 17.28 mg of ABE-Ea/day; H, high dosage of ABE-Ea group, 34.56 mg of ABE-Ea/day. [c] SUM, sulfomucin; MIX, mixed sulfomucin and sialomucin; SIM, sialomucin. [d] Significantly differs from group NC as determined by Chi-squared test, $p < 0.05$.

Table 2. Effects of ABE-Ea on the Size and Distribution of ACF in F344 Rats[a]

(**A**) ACF Size

	no. of foci containing								
group[b]	1 crypt	2 crypts	3 crypts	4 crypts	5 crypts	6 crypts	≥7 crypts	small ACF[c]	large ACF[d]
NC	91 ± 38 a	118 ± 41	39 ± 14 a	10 ± 5	2 ± 1	0.9 ± 0.8	0.4 ± 0.7 a	247 ± 90 a	14 ± 7
PC	48 ± 19 b	88 ± 26	25 ± 11 b	8 ± 4	2 ± 2	0.4 ± 0.5	0 b	161 ± 50 b	11 ± 6
L	64 ± 25 ab	100 ± 23	32 ± 15 ab	9 ± 5	3 ± 3	0.1 ± 0.4	0 b	196 ± 62 ab	12 ± 7
M	58 ± 25 b	97 ± 19	31 ± 11 ab	11 ± 4	1 ± 1	0.5 ± 0.8	0 b	185 ± 49 ab	13 ± 5
H	67 ± 22 ab	91 ± 25	29 ± 10 ab	10 ± 6	3 ± 2	0.6 ± 1.2	0 b	187 ± 53 ab	14 ± 8

(**B**) ACF Distribution

	ACF distribution		
group[b]	proximal colon	middle colon	distal colon
NC	39 ± 39 (13%)	106 ± 47 (40%)	116 ± 35 a (47%)
PC	26 ± 12 (15%)	76 ± 31 (44%)	69 ± 22 b (41%)
L	37 ± 22 (18%)	80 ± 26 (38%)	91 ± 45 ab (44%)
M	34 ± 36 (17%)	82 ± 37 (41%)	83 ± 39 ab (42%)
H	33 ± 22 (16%)	86 ± 39 (43%)	81 ± 20 ab (41%)

[a] All values are presented as the mean ± SD ($n = 8$). Values with different letters in a row show significant difference from one another as determined by Duncan's multiple-range test, $p < 0.05$. [b] NC, negative control group, 0.5 mL of 0.5% CMC/day; PC, positive control group, 1.50 mg of piroxicam/day; L, low dosage of the ethyl acetate fraction of adlay bran ethanolic extract (ABE-Ea) group, 8.64 mg of ABE-Ea/day; M, medium dosage of ABE-Ea group, 17.28 mg of ABE-Ea/day; H, high dosage of ABE-Ea group, 34.56 mg of ABE-Ea/day. [c] Small aberrant crypt foci (ACF), 1−3 crypts. [d] Large aberrant crypt foci (ACF), ≥4 crypts.

colon was significantly lower in group PC compared to group NC. ACF develop as early as 2−4 weeks after carcinogen administration and predominantly appear in the distal colon during the early stage; then some of them begin to expand as time passes (*18*). Numbers of ACF in groups L, M, and H did not significantly change compared to group NC, but there was a trend that the ABE-Ea lowered the number of ACF in the distal colon, indicating that the ABE-Ea may suppress the development of ACF in the early stage.

Significantly Changed Genes of Colonic Mucosa in Group M Compared to Group NC. The composition of ABE-Ea was complicated, and there may be some active components within it, whereas some components may be antagonistic. Because there was no MDF, regarded as a tumor precursor, in the colon of the M group, the RNA of colonic mucosa from the M group was chosen to conduct a cDNA microarray experiment. Total RNA was extracted from colonic mucosa removed from groups M and NC, and significantly changed genes are listed in **Table 3**. There were 23 down-regulated genes and 16 up-regulated genes. Four oncogenes (*Ets2*, *Rap2b*, *Rad23b*, and *Timm17a*) were down-regulated. Many functional genes are involved in colorectal carcinogenesis, such as tumor suppressor genes and DNA mismatch repair genes (*19*). Their expressions were not influenced in the colonic mucosa of group M compared to group NC (data not shown). *Ets2* belongs to the V-ets erythroblastosis virus E26 oncogene, whereas the latter two (*Rap2b* and *Rad23b*) belong to the *RAS* oncogene family, and *Timm17a* is regarded as a potential oncogene related to xenobiotic degradation pathways. The *RAS* oncogene family is strongly related to colorectal carcinogenesis, especially *K-Ras*, but the family contains numerous members (*20*).

Table 3. Significantly Changed Genes in Colon Mucosa of Group M Compared to Group NC

gene	function	ratio
down-regulated		
Timm17a	translocator of inner mitochondrial membrane 17a	0.4952
Ets2	V-ets erythroblastosis virus E26 oncogene homologue 2 (avian) (mapped)	0.4925
Hnf4a	hepatocyte nuclear factor 4, α	0.4886
Srxn1	sulfiredoxin 1 homologue (*S. cerevisiae*)	0.4879
Mobk1b	MOB1, Mps one binder kinase activator-like 1B (yeast)	0.4838
Casp3	caspase 3, apoptosis-related cysteine protease	0.4817
Nr4a3	nuclear receptor subfamily 4, group A, member 3	0.4795
Napsa	napsin A aspartic peptidase	0.4774
Aplp2	amyloid β (A4) precursor-like protein 2	0.4772
Atrx	α thalassemia/mental retardation syndrome X-linked homologue (human)	0.4745
Rap2b	RAP2B, member of RAS oncogene family	0.4668
Bdnf	brain derived neurotrophic factor	0.4598
Tm9sf4	transmembrane 9 superfamily protein member 4	0.4528
Eprs	glutamyl-prolyl-tRNA synthetase	0.4447
Tjp2	tight junction protein 2	0.4440
Kidins220	kinase D-interacting substance 220	0.4428
Zfp655	zinc finger protein 655	0.4364
Camk2d	calcium/calmodulin-dependent protein kinase II, δ	0.4319
Birc4	baculoviral IAP repeat-containing 4	0.4160
Crym	crystallin, μ	0.4157
Rad23b	Rad23b homologue (*S. cerevisiae*)	0.3964
Clca6	chloride channel calcium activated 6	0.3624
Cask	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	0.3555
up-regulated		
Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	2.0419
Cyp1a1	cytochrome P450, family 1, subfamily a, polypeptide 1	2.0758
Oas1b	2−5 oligoadenylate synthetase 1B	2.0998
Trim28	tripartite motif protein 28	2.1510
Cda08	T-cell immunomodulatory protein	2.2601
Plcb4	phospholipase C, β 4	2.2828
Otof	otoferlin	2.2902
Wee1	Wee1 homologue (*S. pombe*)	2.3573
Ivl	involucrin	2.4355
Cd79b	CD79B antigen	2.4389
Megf10	Megf10 protein	2.4465
Fgf23	fibroblast growth factor 23	2.4813
Igha_mapped	immunoglobulin heavy chain (α polypeptide) (mapped)	2.5125
Tas2r1	taste receptor, type 2, member 1	2.5606
Prkwnk1	protein kinase, lysine deficient 1	2.7530
Gch	GTP cyclohydrolase 1	2.8026

All *RAS* oncogenes were investigated in the microarray experiment, and most of them were down-regulated but not significantlt so compared to group NC (data not shown). In colon carcinogenesis, *RAS* oncogenes play key roles in promoting expressions of iNOS and COX-2, which are related to inflammation (*19*). We suggest that anti-inflammation is one of the possible pathways through which the ABE-Ea acts in DMH-treated F344 rats. Among all members of the *RAS* oncogene superfamily, two significantly down-regulated genes were *RAP2b* and *RAD23b*. *RAP2b* is a *RAS*-related GTP-binding protein that regulates cell proliferation, differentiation, intracellular vesicular trafficking, cytoskeletal rearrangement, cell cycle events, and glucose transport. Studies showed that *RAP2b* activates and interacts with phospholipase C-epsilon (PLC-ε), which in turn facilitates cell growth by activating the *Ras*−*Raf*−MAPK/ERK kinase−ERK pathway after stimulation of G-protein-coupled receptors and/or the epithelial growth factor receptor (EGFR), increasing intracellular calcium (*21*). *RAD23b* (*hHR23b*), which is specifically involved in nucleotide excision repair (NER) of DNA lesions, is down-regulated during early apoptosis of hepatoma cells (*22*).

Besides *RAS* genes, many cancer-related genes were down-regulated, such as *Ets2* (ratio = 0.4925). *Ets2* is a member of the *Ets* oncogene family, which promotes cell proliferation, cancer metastasis, and angiogenesis. Our data showed that there were no MDF in group M and that the numbers of total ACF and small ACF slightly decreased in ABE-Ea-fed groups. The results suggest that the ABE-Ea might retard ACF fission through suppressing abnormal cell proliferation due to lower *Ets* oncogene expression. Also, *Timm17a* was down-regulated (ratio = 0.4952). *Timm17a* (on chromosome 1q) is considered to be an oncogene and is highly expressed in *RAS*, MAPK, PIK3, and xenobiotic degradation pathways (*23*). This result proves that the ABE-Ea can mediate *RAS*-related signal transduction pathways. On the other hand, RNA modification is often higher in abnormal cells than in normal cells, and it is related to tRNA (tRNA) flexibility. Glutamyl-propyl-tRNA synthetase (*Eprs*) is an enzyme that can combine with dihydrouridine synthase (hDUS) to increase the flexibility of tRNA, followed by increasing the cell proliferation rate (*24*). In our study, *Eprs* was significantly down-regulated (ratio = 0.4447), which means that the proliferation rate of abnormal cells decreased, which brought about a retardation in ACF fission in this experiment.

Wee1 was up-regulated (ratio = 2.3573) in the colon of group M compared to group NC. *Wee1* is related to the cell cycle and is involved in the cell division cycle 25 (CDC25) phosphatase system, which may be a new target to control the cell cycle (*25*). In the G_2/M phase, the cyclin B/cyclin-dependent kinase 1 (CDK1) complex is dephosphorylated by CDC25 to help cells undergo

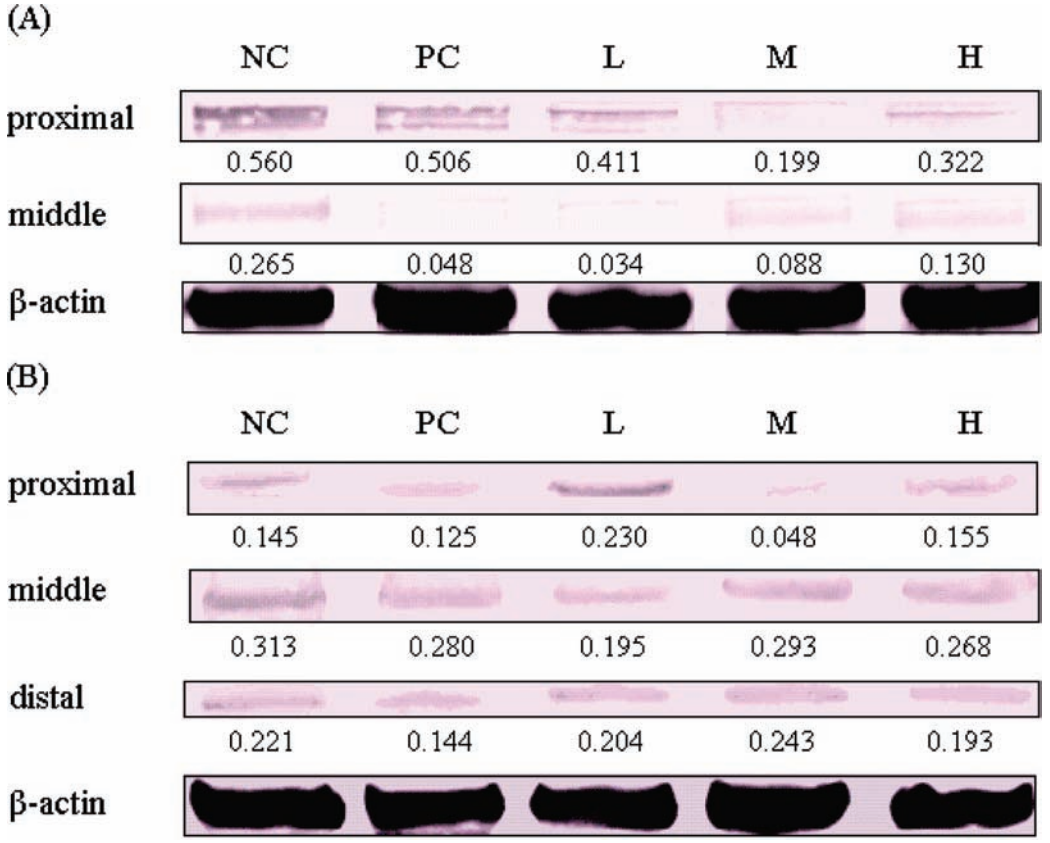

Figure 2. Effect of ethyl acetate fraction of adlay bran ethanolic extract on iNOS (**A**) and COX-2 (**B**) expression of colonic mucosa in DMH-treated male F344 rats (β-actin was used as an internal control). The numbers within the NC, PC, L, M, and H columns represent the amount of each protein in each sample relative to the amount of β-actin found in each sample. NC, negative control group, 0.5 mL of 0.5% CMC/day; PC, positive control group, 1.50 mg of piroxicam/day; L, low dosage of ethyl acetate fraction of adlay bran ethanolic extract (ABE-Ea) group, 8.64 mg of ABE-Ea/day; M, medium dosage of ABE-Ea group, 17.28 mg of ABE-Ea/day; H, high dosage of ABE-Ea group, 34.56 mg of ABE-Ea/day.

mitosis. The reaction is reversible and driven by the *Wee1*/*Myt1* (a membrane-associated Tyr/Thr CDC2-inhibiting kinase) complex. When the *Wee1*/*Myt1* complex interferes with CDC25, apoptosis occurs due to retarded mitosis. Lee et al. pointed out that apoptosis of COLO 205 colon cancer cells was induced by the ABM-Ea (*10*). In our results, the *Wee1* gene was significantly up-regulated, and *Myt1* was slightly but not significantly up-regulated (data not shown). ACF consist of abnormal cells, so the apoptosis pathway may occur in the colons of experimental animals. Otherwise, gene expression of caspase 3 (*Casp3*), which leads to cell cycle arrest at the G2/M phase, was significantly down-regulated (ratio = 0.4817), indicating that the ABE-Ea did not affect the G2/M phase of the cell cycle. Therefore, we suppose that the ABE-Ea led to apoptosis through suppressing mitosis but not arresting the cell cycle at the G2/M phase. *RAD23b* is down-regulated during early apoptosis of hepatoma cells (*22*) and may be a target to predict apoptosis. From these results, we suggest that apoptosis induction is another possible pathway to inhibit ACF fission in the colon of animals administered the ABE-Ea.

Green and Hudson demonstrated that many genes are involved in colorectal carcinogenesis (*19*). At the beginning, loss of *APC* accompanied by increasing EGFR and COX-2 promotes adenoma formation. Both the increases in *RAS* oncogene expressions and loss of *SMAD3*, *SMAD4*, *DCC*, *TGFβ*, *TGFβII*, and *TP53* promote adenocarcinoma formation. When *PRL3* is highly expressed, cancer cells possess a metastatic ability. In all stages of carcinogenesis, *MSH2*, *MSH3*, *MSH6*, *MLH1*, and *MLH2*, which are related to DNA mismatch repair (MMR), are lost. In the present study, *RAP23b* and *RAD2b*, two members of the *RAS* oncogene family, were significantly down-regulated (ratios = 0.4668 and 0.3964, respectively). Also, most of the other members of the *RAS* oncogene superfamily were slightly down-regulated, indicating that the ABE-Ea may inhibit inflammation-related protein expression to suppress carcinogenesis.

iNOS and COX-2 Proteins Expression in Colonic Mucosa. All *RAS* oncogenes were investigated in the microarray experiment, and most of them were down-regulated but not significantly so (data not shown). To confirm the results from the microarray experiment, the *Ras*-related downstream proteins, iNOS and COX-2, were investigated. **Figure 2** shows the expressions of the *RAS*-mediated inflammation-related proteins, iNOS and COX-2, in colonic mucosa. In the proximal and middle colon, iNOS expression was significantly reduced in ABE-Ea-fed groups compared to group NC. iNOS was not detected in the distal colon. In view of COX-2 expression, groups PC and M showed significant inhibitory effects in the proximal colon. In the middle colon, only group L showed a more significant inhibitory effect compared to group NC. There were no obvious among-group differences in COX-2 expression in the distal colon. iNOS and COX-2 are pro-inflammatory enzymes, which mediate many biological pathways such as carcinogenesis and inflammation. iNOS and COX-2 expressions were significantly suppressed as shown in **Figure 2** in the proximal colon of group M. Overall, the ABE-Ea could suppress procarcinogenesis protein expression through down-regulating oncogene expressions. In this animal model, carcinogenesis should develop near the adenoma phase, and iNOS and COX-2 expressions were truly high in group NC. In addition to *Rap2b* and *Rad23b*, all expression sequence tags (ESTs) of the *RAS* oncogene superfamily on the array chip were screened. Most of these members showed a down-regulated trend,

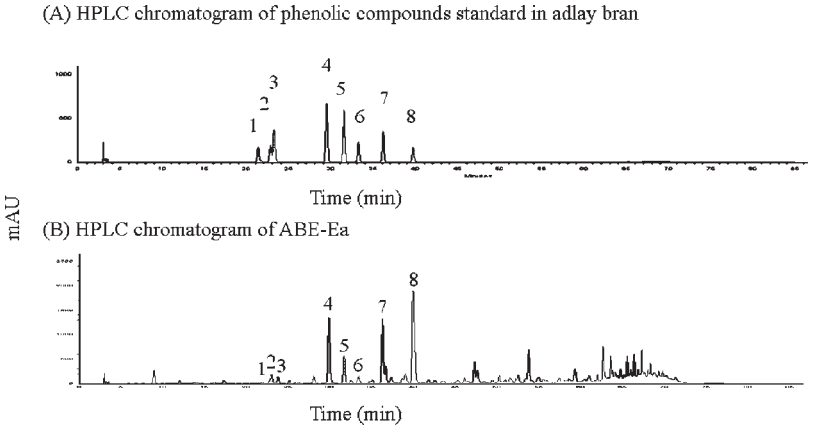

Figure 3. HPLC chromatogram and phenolic compound contents of ABE-Ea: (**A**) HPLC chromatogram of phenolic compounds standard; (**B**) HPLC chromatogram of the ABE-Ea. Peaks containing phenolic compounds are labeled 1−7, and 6-methoxy-2-benzoxazolinine is labeled 8.

Table 4. Phenolic Compound and 6-Methoxy-2-benzoxazolinine Contents in ABE-Ea[a]

no.	phenolic compound standard	retention time (min)	content (μg/g ABE-Ea)
1	chlorogenic acid	21.3	194 ± 13
2	vanillic acid	22.4	1127 ± 52
3	caffeic acid	23.1	386 ± 25
4	4-hydroxyacetophenone	29.1	2058 ± 118
5	*p*-coumaric acid	31.1	1047 ± 67
6	syringaldehyde	32.7	670 ± 47
7	ferulic acid	35.6	5206 ± 368
8	6-methoxy-2-benzoxazolinine	38.8	13627 ± 599

[a] ABE-Ea, ethyl acetate fraction of the adlay bran ethanolic extract. Values are the mean of three determinations ± standard deviation (SD).

although they were not significant. The results prove that suppression of *RAS* oncogene expressions is one of the anticarcinogenesis pathways affected by ABE-Ea administration.

Analysis of the Phenolic Compounds in the ABE-Ea by HPLC. Analytical plots of phenolic compounds in the ABE-Ea are shown as **Figure 3**. Eight compounds were contained in the standard chromatogram (**Figure 3A**): (1) chlorogenic acid, (2) vanillic acid, (3) caffeic acid, (4) 4-hydroxyacetophenone, (5) *p*-coumaric acid, (6) syringaldehyde, (7) ferulic acid, and (8) 6-methoxy-2-benzoxazolinine, all purchased from Sigma. These phenolic compounds were reported by Huang et al. (*11*). **Figure 3B** shows the HPLC spectrum of the ABE-Ea, with eight peaks corresponding to the compounds listed above, whereas ferulic acid and 6-methoxy-2-benzoxazolinine seemed to be major components in the ABE-Ea (**Table 4**). 6-Methoxy-2-benzoxazolinine was one of the major components in ABE-Ea, but it did not express anti-inflammatory effects in our previous study(*11*), and there was no literature to report that the compound related to retarding colon cancer carcinogenesis. Ferulic acid is one of the effective components in adlay bran that retards preneoplastic lesions of colon cancer. According to the observations of Kawabata et al. (*26*), administration of 250 ppm ferulic acid for 5 weeks suppressed ACF formation in the AOM-treated F344 rat colon, whereas there was no obvious difference between the 250 and 500 ppm groups until 35 weeks. Han et al. demonstrated that ethyl 3-(4′-geranyloxy-3-methoxyphenyl)-2-propenoate (a ferulic acid derivative) might be a chemopreventive agent for colon carcinogenesis when provided in animals' diets at 0.1% (*27*). Wargovich et al. demonstrated that ferulic acid at a dosage of 10 g/kg of diet suppressed ACF formation in experimental animals' colons after initiation protocol handling (*28*). According to these results, ferulic acid may be one of the active compounds against colon carcinogenesis in the ABE-Ea.

The results above show that administration of the ABE-Ea retarded colorectal carcinogenesis. Many in vitro studies pointed out that the ethyl acetate fraction of adlay bran extracts inhibited proliferation of cancer cells. Lee et al. found that the ABM-Ea induced apoptosis in human colon cancer COLO 205 cells (*11*). Three new lactams, coixspirolactam A, coixspirolactam B, and coixspirolactam C, were isolated and showed an antiproliferative effect in human cancer HT-29 cells (*29*). Bao et al. demonstrated that a neutral lipid isolated from adlay inhibited the growth of human pancreatic cancer cells via apoptosis, G_2/M arrest, and regulation of gene expression (*30*). Dietary fiber is regarded as a component that suppresses colorectal carcinogenesis, whereas according to these studies there are other active components in adlay bran in addition to fiber. Our previous study showed that administration of adlay bran caused similar effects in suppressing colon carcinogenesis (*9*), and our recent study proved that both the adlay ethanolic extract and residue had the same activity (unpublished data). According to the present study, the ABE-Ea at a level of 17.28 mg/day exhibited the best preventive effect on colon carcinogenesis. The ABE-Ea reduced different preneoplastic lesions and reduced colonic inflammation and thus delayed colon carcinogenesis. In group M, MIX-ACF significantly decreased compared to group NC, and no MDF existed in colons of group M. MDF are regarded as cancer precursors, which can be used to predict cancer occurrence (*15*). This means that rats in group M had a lower probability of developing colon cancer.

In conclusion, we demonstrated that the ABE-Ea possesses anticarcinogenic activity, and the mechanisms may be related to inhibition of *RAS*-promoted pro-cancer protein overexpressions, *Ets*-promoted cell proliferation, and promotion of *Wee1*-mediated apoptosis. Although the overall anticarcinogenic mechanism of the ABE-Ea needs further investigations, our data suggest that the ABE-Ea may be beneficial in delaying colon carcinogenesis. The anti-inflammatory activity of the ABE is clear, and one of the major active compounds within it is ferulic acid.

ABBREVIATIONS USED

ACF, aberrant crypt foci; MDF, mucin-depleted foci; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; AOM, azoxymethane; DMH, 1,2-dimethylhydrazine; ABM, adlay bran methanolic extract; ABM-Ea, ethyl acetate fraction of adlay bran methanolic extract; ABE, adlay bran ethanolic extract; ABE-Ea, ethyl acetate fraction of adlay bran ethanolic extract; cDNA, cDNA; TCS4, Taichung Shuenyu no. 4; ABE-Hex, hexane fraction of adlay bran ethanolic extract; ABE-Bu, butanol fraction of adlay bran ethanolic extract; ABE-H_2O, H_2O fraction of

adlay bran ethanolic extract; IACUC, Institutional Animal Care and Use Committee; CMC, carboxymethyl cellulose; PBS, phosphate-buffered saline; HIDAB, high-iron diamine alcian blue; SUM, sulfomucin; SIM, sialomucin; DEPC, diethylpyrocarbonate; NTUMAPS, National Taiwan University Microarray Analysis Platform and System; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance; PLC-ε, phospholipase C-epsilon; EGFR, epithelial growth factor receptor; NER, nucleotide excision repair; Eprs, glutamyl-propyl-tRNA synthetase; hDUS, dihydrouridine synthase; CDC25, cell division cycle 25; CDK1, cyclin-dependent kinase 1; Myt1, membrane-associated Tyr/Thr CDC2 inhibiting kinase; Casp3, caspase 3; ESTs, expression sequence tags.

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