JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Antimutagenic Constituents of Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) with Potential Cancer Chemopreventive Activity

Huang-Hui Chen,[†,||] Wenchang Chiang,[‡,||] Jang-Yang Chang,[†,§] Ya-Lin Chien,[‡] Ching-Kuo Lee,[#] Ko-Jiunn Liu,[†] Yen-Ting Cheng,[†] Ting-Fang Chen,[†] Yueh-Hsiung Kuo,[⊥,⊗,△,*] and Ching-Chuan Kuo[*,†,□]

[†] National Institute of Cancer Research, National Health Research Institutes, Tainan, Taiwan

[‡] Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan

[§] Department of Internal Medicine, National Cheng Kung University Hospital, Tainan, Taiwan

[#] School of Pharmacy, Taipei Medical University, Taipei, Taiwan

[⊥] Tsuzuki Institute for Traditional Medicine, China Medical University, Taichung, Taiwan

[⊗] Department of Chemistry, National Taiwan University, Taipei, Taiwan

[△] Agricultural Biotechnology Research Center, Academic Sinica, Taipei, Taiwan

[□] Institute of Biopharmaceutical Science, National Cheng Kung University Medical College, Tainan, Taiwan

ABSTRACT: Adlay has long been used in traditional Chinese medicine and as a nourishing food. The acetone extract of adlay hull had previously been demonstrated to possess potent antimutagenic activity. The aims of this study were to identify the antimutagenic constituents from adlay hull by using Ames antimutagenic activity-guide isolation procedures and to investigate their chemopreventive efficacies in cultured cells. The results demonstrated that six compounds showing great antimutagenic activity were identified by spectroscopic methods and by comparison with authentic samples to be *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, *trans*-coniferylaldehyde, sinapaldehyde, and coixol. Two of them, *trans*-coniferylaldehyde and sinapaldehyde, exhibit relatively potent scavenging of DPPH radicals, inhibit TPA stimulated superoxide anion generation in neutrophil-like leukocytes, and induce Nrf2/ARE-driven luciferase activity in HSC-3 cells. Moreover, *trans*-coniferylaldehyde possesses cytoprotective efficacy against *tert*-butyl hydroperoxide-induced DNA double-strand breaks in cultured cells, and the chemopreventive potency induced by *trans*-coniferylaldehyde may be through the activation of kinase signals, including p38, ERK1/2, JNK, MEK1/2, and MSK1/2. In summary, we first identified six antimutagenic constituents from adlay hull. Among them, *trans*-coniferylaldehyde would be a highly promising agent for cancer chemoprevention and merits further investigation.

KEYWORDS: traditional Chinese medicine, adlay, antimutagenic constituents, cancer chemoprevention, ARE, kinase signal

INTRODUCTION

Adlay ("soft-shelled Job's tears", *Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is a grass crop that has long been used in traditional Chinese medicine and as a nourishing food. According to the ancient Chinese medical book *Pen-Tsao-Kang-Mu*,[1] the seed of adlay was used in China for the treatment of warts, chapped skin, rheumatism, neuralgia, and inflammatory diseases. Indeed, the action of adlay against many kinds of disease can be attributed to various components that contain different pharmacological activities. Recently, a number of pharmacologically and physiologically interesting substances have been isolated from adlay, including antioxidant/free radical scavenging,[2,3] anti-inflammatory,[3−5] antitumor,[6−9] antiallergic,[10] hypoglycemic,[11] antimicrobial,[12] and ovulatory-active [13] agents.

It is well-documented that cancer begins after a mutational episode in a single cell and then it progressively transforms to malignancy in multiple stages through sequential acquisition of additional mutations.[14] In recent years, there have been considerable efforts to find cancer chemopreventive agents that can inhibit, reverse, or retard multistage carcinogenesis.[15] A wide array of phytochemicals have been studied for cancer chemoprevention, and many of these compounds exhibit antimutagenic activity.[16,17] Therefore, exploration of phytochemicals that contain chemopreventive potential through elimination of carcinogens/mutagens-induced mutagenicity draws scientist's attention in the area of cancer research.

The Ames *Salmonella typhimurium* mutagenicity assay (Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage leading to gene mutations.[18] It is interesting to note that the compound prevents mutagenesis caused by known carcinogens in the Ames test, indicating the possible role in chemoprevention.[19] We have previously investigated the antimutagenic activity of different portions of adlay, including adlay hull, adlay testa, adlay bran, dehulled adlay, and polished adlay. Among them, the acetone extract of adlay hull possesses the most potent antimutagenic activity against benzo(*a*)pyrene (B[a]P)-, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-, and 4-nitroquinoline-*N*-oxide (NQNO)-induced mutagenesis in *S. typhimurium* TA98.[20] However, the specific compounds responsible for the antimutagenic activity within this

Received: February 8, 2011
Revised: May 9, 2011
Accepted: May 11, 2011
Published: May 11, 2011

ACS Publications © 2011 American Chemical Society

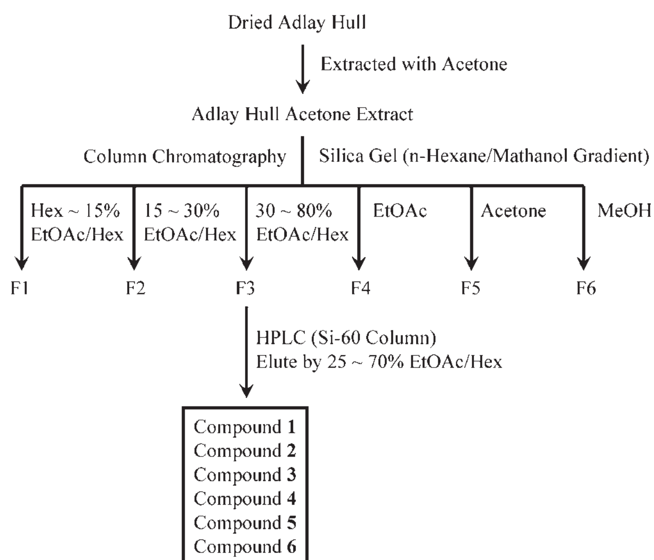

Figure 1. Scheme for the preparation of antimutagenic constituents from adlay hull.

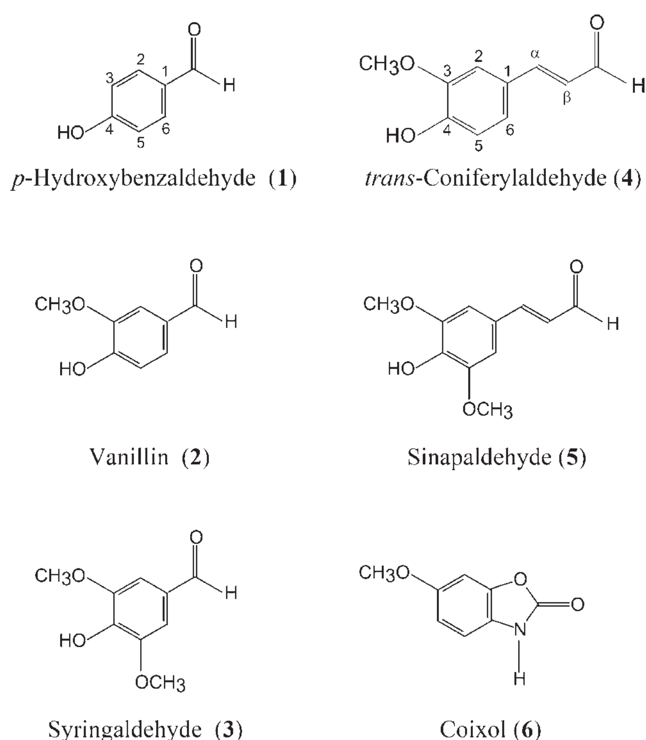

Figure 2. Chemical structures of antimutagenic constituents from adlay hull.

material remain unknown. The objectives of this work were designed to identify the antimutagenic constituents from the acetone extract of adlay hull by using the Ames test and also to investigate their chemopreventive efficacies in cellular models.

■ MATERIALS AND METHODS

Plant Material. Adlay was purchased from a local farmer who planted Taichung Shuenyu No. 4 (TCS4) of *C. lachryma-jobi* L. var. *ma-yuen* Stapf in Taichung, Taiwan. After the harvest, the seeds were dried at ambient temperature with ventilation and dehulled by a grinder. The samples were divided into hull, testa, and dehulled adlay by gentle blowing with an electric fan, and adlay hull was blended in powdered form and screened through a 20-mesh sieve (aperture = 0.94 mm).

Isolation and Identification of Antimutagenic Constituents from Adlay Hull. IR spectra were recorded on a Perkin-Elmer 983 G infrared spectrometer. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker AM-300 and Bruker AMX-500 instruments; COSY and HMBC spectra were obtained on a Bruker AMX-500 instrument and recorded using standard pulse sequences. MS analysis was taken on a JEOL JMS-HX300 mass spectrometer. The measurement of melting points was performed with a Yanaco MP-S3 micro melting point apparatus (Yanagimoto Co., Kyoto, Japan). Ultraviolet absorption spectra of the purified active fractions were recorded on a U-2000 spectrophotometer (Hitachi) in methanol. Thin-layer chromatography was performed on silica gel 60F$_{254}$ TLC plates (Merck, Darmstadt, Germany), with compounds visualized by spraying with 10% (v/v) H_2SO_4 in an ethanol solution. High-performance liquid chromatography (HPLC) was performed with a GBCLC-1440 instrument and GBCLC-1240 RI detector (GBC Scientific Equipment, Australia). A 10 × 250 mm i.d., 7 μm, Lichrosorb Si-60 column (Merck) was used for analysis. All solvents used for chromatographic isolation were of analytical grade and purchased from Tedia Co. (Fairfield, OH).

Figure 1 shows the scheme for the preparation of antimutagenic constituents from adlay hulls. The powder of adlay hull was extracted with acetone (w/v = 1:10) and stirred on a stirring plate at room temperature for 24 h. Contents were filtered through no. 1 filter paper (Whatman Inc., Hillsboro, OR). The filtrate was concentrated to dryness in vacuo to obtain acetone extract. Acetone extract was chromatographed on a silica gel column using an *n*-hexane (Hex)−methanol gradient system to afford six subfractions, including F1 (100% Hex−15% ethyl acetate (EtOAc)/Hex), F2 (15−30% EtOAc/Hex), F3 (30−80% EtOAc/Hex), F4 (100% EtOAc), F5 (100% acetone), and F6 (100% methanol). F3 was further purified by HPLC on a Lichrosorb Si-60 column at 2 mL/min, using different proportions of EtOAc/Hex as the eluent to yield compounds **1**−**6** (Figure 2): *p*-hydroxybenzaldehyde (**1**), amorphous powder (identical with the literature values);[21] vanillin (**2**), colorless crystals (identical with the literature values);[22] syringaldehyde (**3**), colorless crystals (identical with the literature values);[22] *trans*-coniferylaldehyde (**4**), colorless crystals (identical with the literature values);[23] sinapaldehyde (**5**), colorless crystals (identical with the literature values);[24] coixol (**6**), colorless crystals (identical with the literature values).[25]

Antimutagenic Activity Assay. The histidine-requiring strains of *S. typhimurium* TA98 were kindly supplied by Dr. Bruce N. Ames (University of California, Berkeley). The antimutagenic effect of test sample was analyzed according to the Ames test.[26] Indirect-acting mutagen, IQ (5 ng/plate), was used in this study, which required S9 mix (ICN-Cappel, Aurora, OH) for metabolic activation. In brief, 0.5 mL of S9 mix was mixed with IQ and then added to the mixture of test sample and *S. typhimurium* TA98. After gentle vortexing and preincubation at 37 °C for 20 min, 2 mL of the top agar supplemented with L-histidine and D-biotin kept at 45 °C was added and vortexed for 3 s. The resulting entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37 °C for 48 h and then the revertant bacterial colonies on each plate were counted. Percent inhibition (%) = [1 − (no. of His$^+$ revertants in the presence of samples − Nno. of spontaneous revertants)/(no. of His$^+$ revertants in the absence of samples − no. of spontaneous revertants)] × 100. The extent of antimutagenicity was categorized as not antimutagenic when the percentage of inhibition was <25%, moderately antimutagenic when the percentage of inhibition was 25−50%, and strongly antimutagenic when the percentage of inhibition is >50%.

Determination of the Scavenging Effect on 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) Radicals. This method was taken from

that of Kuo et al.[2] Test compound was mixed with 300 μM DPPH radicals and kept in the dark for 90 min. The absorbency of the samples was measured using an Optimax automated microplate reader (Molecular Devices, Sunnyvale, CA) at 517 nm against methanol without DPPH as the blank reference. Each sample was quadruplicated in the test, and the values were averaged.

Cell Culture. Human promyelocytic leukemia cell line HL-60 and squamous cell carcinoma lines HSC-3 (originated from tongue cancer) were obtained from American Type Culture Collection (ATCC) and JCR Bank, respectively. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. HSC-3 cells were cultured in Eagle's MEM with 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate. All cell lines were grown in a humidified atmosphere at 37 °C in a 5% CO_2 atmosphere.

Determination of the Scavenging Effect on Superoxide Anion Radicals on Cultured Cells. An inhibitory test of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced superoxide generation was conducted as previously reported.[27] Briefly, HL-60 cells were preincubated in complete culture medium with 1.3% (v/v) dimethyl sulfoxide (DMSO) at 37 °C in a 5% CO_2 incubator for 5 days to differentiate cells into neutrophil-like leukocytes. Morphological evaluation of the differentiation cells revealed mostly band and segmented cells. The nitroblue tetrazolium (NBT) test was use to screen cells for their capacity to undergo oxidative metabolism. More than 65% of the differentiation HL-60 cells were routinely found to reduce NBT after expose to TPA. Differentiated cells (5×10^5/mL) were plated in a 96-well microplate suspended in Hank's balanced salt solution. Free radical formation was induced by the addition of TPA (8.1 μM), followed by the addition of samples and 60 μM cytochrome *c* and incubation at 37 °C for 60 min. Cytochrome *c* reduction was measured at 550 nm. Using induction by TPA alone as a measure of maximum free radical formation, the percentage of radical formation in TPA plus agent-treated samples was determined.

Reporter Plasmids and Expression Constructs. The antioxidant responsive element-luciferase reporter plasmids were generated using the pGL3 promoter vector (Promega U.K.) containing an SV40 promoter upstream of the firefly luciferase gene. The DNA fragment containing nine copies of the ARE sequence (5′-GTGACAAAGCA-3′) was cloned into the *Nhe*I and *Xho*I restriction sites upstream of the promoter-luc$^+$ transcriptional unit. After the plasmids were generated, the DNA sequence of the inserts was verified.

Transient Transfection and Analysis of Luciferase Reporter Gene Activity. The Dual-luciferase Reporter Assay System (Promega U.K.) was used to determine reporter gene activity in transiently transfected cells. Transient transfection was performed in 24-well plates at a cell density of 2×10^5 per well. The 9×ARE pGL3 plasmid was cotransfected with pRL-TK plasmid, encoding Renilla luciferase as an internal control for transfection efficiency, for 24 h by using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Following transfection, cells were treated with 50 μM levels of test samples and *tert*-butylhydroquinone (*t*BHQ; positive control) for another 24 h, and then cell lysates were prepared for luciferase activity determination. The firefly and Renilla luciferase activities were measured using a luminometer according to the manufacturer's instructions. The relative firefly lucifrease activity was normalized by Renilla luciferase activity.

Cell Viability Assay. Cells in logarithmic phase were seeded into 96-well plates to adhere overnight. They were then exposed to 50 μM levels of test drugs in quadruplicate for 24 h and then incubated with a serum-free medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL for 2 h. The conversion of MTT to formazan by metabolically viable cells was measured by the absorbance at 570 nm in a 96-well microtiter plate reader.

Histone H_2AX Phosphorylation. Histone H_2AX phosphorylation was measured by flow cytometry.[28] Cells (1×10^6) were treated with 50 μM *trans*-coniferylaldehyde for the indicated times, then washed with PBS, and fixed by 70% ethanol at −20 °C for at least 2 h. The fixed cells were incubated for 5 min in PBS containing 0.1% Triton X-100 at 0 °C, followed by incubation for 1 h at room temperature with antiphosphohistone H_2AX (Ser139) murine monoclonal antibody (Upstate Biotechnology, Charlottesville, VA), and finally incubated for 1 h with rhodamine-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Cellular fluorescence was measured using flow cytometry.

Phosphokinase Array. The expression and activation of signaling pathways by test sample was analyzed using the Human Phospho-Kinase Array Kit (Proteome ProfilerTM, R&D System, Abingdon, U.K.) according to the manufacturer's instructions. Briefly, cells were treated with 50 μM *trans*-coniferylaldehyde for 3 h. Protein was quantitated using a BCA Protein Assay (Thermo Scientific, Rockford, IL). Three hundred micromolar levels of protein lysates were employed per array. The relative expression of phosphorylated proteins was estimated following quantification of scanned images by Image J software. The average signal was calculated after subtraction of background values from negative control.

Western Blot Analysis. Cells were initially seeded at a density of 1×10^6 in 100 mm^2 dishes. After treatment with 50 μM *trans*-coniferylaldehyde for 3 h, adherent cells were washed twice with PBS, gently scraped from the dishes, centrifuged, and lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 0.8 M NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM phenylmethanesulfonyl fluoride, and proteinase inhibitor aprotinin, leupeptin, and pepstatin, 20 mg/mL each). Lysates were centrifuged at 12000*g* for 15 min, and the supernatants were collected and quantified. Equal amounts of lysate (on a protein basis) were then differentiated by SDS-PAGE, transferred on PVDF membranes, conjugated with various specific primary antibodies (antiphosphorylated ERK, JNK, p-38, MEK, and MSK antibodies were purchased from Cell Signaling Technology, Beverly, MA), and then probed with appropriate secondary antibodies. The immunoreactive bands were detected with the ECL method and visualized on Kodak Bio-MAX MR film.

Statistical Analysis. All assays were carried out at least in triplicate. Data were expressed as a mean with standard deviation (SD). Student's *t* test was used to compare the mean of each group with that of the control group. A *p* value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Isolation and Identification of Antimutagenic Constituents from Acetone Extract of Adlay Hull. Our previous study showed that the acetone extract of adlay hull possesses potent antimutagenic activity against B[a]P-, IQ-, and NQNO-induced mutagenesis in *S. typhimurium* TA98.[20] To isolate the component responsible for the antimutagenic activity, the acetone extract of adlay hull was further fractionated by using silica gel column chromatography to obtain six subfractions (F1−F6) (Figure 1). As shown in Table 1, subfractions F1, F5, and F6 in the concentration of 500 μg/plate were shown to have 50.2, 34.7, and 36.6%, respectively, inhibition on the mutagenicity of IQ to *S. typhimurium* TA98 in the presence of S9 metabolic activation, suggesting that these three fractionated extracts are moderate antimutagenic agents (Table 1). In addition, subfractions F2, F3, and F4 in concentrations of 300 and 500 μg/plate exhibited >90% inhibition on the mutagenicity of IQ to *S. typhimurium* TA98. Among them, F3 showed the most potent antimutagenicity in our experimental system (Table 1).

In the search for active components, subfraction F3 was chromatographed over silica gel and HPLC to yield six compounds.

Table 1. Antimutagenic Activity of Various Subfractions Obtained from Acetone Extract of Adlay Hull on IQ-Induced Mutagenicity in *Salmonella typhimurium* TA98

	His$^+$ revertants at dose of			
	300 μg/plate		500 μg/plate	
sample	revertant no.[a]	% inhibition[b]	revertant no.[a]	% inhibition[b]
spontaneous[c]	28 ± 3		29 ± 2	
control[d]	1820 ± 172	0	1293 ± 6	0
F1	nd[e]		659 ± 8	50.2
F2	160 ± 13	92.6	72 ± 6	96.5
F3	61 ± 5	98.2	32 ± 6	100
F4	222 ± 5	89.2	45 ± 7	98.7
F5	nd		854 ± 11	34.7
F6	nd		831 ± 10	36.6

[a] Values are the mean ± SD of histidine revertants minus the number of spontaneous revertants of three independent experiments carried out in triplicate. [b] Inhibition (%) = [1 − (no. of His$^+$ revertants in the presence of samples − no. of spontaneous revertants)/(no. of His$^+$ revertants in the absence of samples − no. of spontaneous revertants)] × 100. [c] The number of spontaneous revertants was determined in the absence of test samples and mutagen (5 ng/plate IQ). [d] The control represented experiments in the presence of 5 ng/plate IQ but without test samples. [e] Not done.

Table 2. Antimutagenic Effect of Constituents Purified from Subfraction F3 of the Acetone Extract of Adlay Hull toward IQ in *Salmonella typhimurium* TA98

	His$^+$ revertants at dose of 300 μg/plate	
sample	revertant no.[a]	% inhibition[b]
spontaneous[c]	38 ± 4	
control[d]	1469 ± 71	0
p-hydroxybenzaldehyde (**1**)	1288 ± 75	12.6
vanillin (**2**)	1041 ± 82	29.9
syringaldehyde (**3**)	501 ± 50	67.6
trans-coniferylaldehyde (**4**)	428 ± 53	72.7
sinapaldehyde (**5**)	171 ± 12	90.7
coixol (**6**)	711 ± 49	53.0

[a] Data are the mean ± SD of three independent experiments carried out in triplicate. [b] Inhibition (%) = [1 − (no. of His$^+$ revertants in the presence of samples − no. of spontaneous revertants)/(no. of His$^+$ revertants in the absence of samples − no. of spontaneous revertants)] × 100. [c] The number of spontaneous revertants was determined in the absence of test samples and mutagen (5 ng/plate IQ). [d] The control represented experiments in the presence of 5 ng/plate IQ but without test samples.

These compounds were identified to be *p*-hydroxybenzaldehyde (**1**), vanillin (**2**), syringaldehyde (**3**), *trans*-coniferylaldehyde (**4**), sinapaldehyde (**5**), and coixol (**6**) by spectroscopic methods (Figure 2). These purified compounds were then subjected to the Ames test; the result demonstrated that these compounds exhibit various antimutagenic activities against IQ in the presence of S9. Among them, *p*-hydroxybenzaldehyde (**1**) showed no antimutagenic activity, vanillin (**2**) showed moderate antimutagenic activity, and the rest of the four compounds showed strong antimutagenic activity. The compounds with strong antimutagenic activity are represented by the order sinapaldehyde (**5**) > *trans*-coniferylaldehyde (**4**) > syringaldehyde (**3**) > coixol (**6**) (Table 2). Toxicity and mutagenic tests were also carried out on the samples on the same bacterial stain, and the result demonstrated that the sample concentrations employed in the antimutagenic test were found to be nontoxic and nonmutagenic (data not shown).

Of six purified compounds, the structures of **1**−**5** belonged to phenolic aldedhyes, including benzaldehye derivatives (compounds **1**−**3**) and cinnamaldehyde derivatives (compounds **4** and **5**). Compound **6** is a benzoxazinoid derivative. In general, the suppressive effect of cinnamaldehydes against mutagen is better than that of benzaldehyes. This result suggested that the α,β-unsaturated aldehyde moiety is more important in suppressing IQ-induced mutagenecity in *S. typhimurium* TA98 than the aldehyde moiety. In addition, phenolic aldehydes, either benzaldehyes or cinnamaldehydes, substituted in the C3- or C5-position with a methoxyl group significantly increased antimutagenic activity in a number of methoxyl group substitution-dependent manner (Table 2). Because vanillin (**2**) has been demonstrated to exhibit antimutagenic and anticarcinogenic activities,[29,30] we suggested that phenolic aldehydes isolated from adlay hull with an α,β-unsaturated aldehyde moiety and methoxyl group substitution at C3- and C5-position, including sinapaldehyde (**5**) and *trans*-coniferylaldehyde (**4**), exhibit more antimutagenic potency than known antimutagen vanillin (**2**). Furthermore, it is interesting to note that coixol (**6**), which had been demonstrated to stimulate reproductive responses and modulate hormone systems in animals,[31−33] also showed antimutagenic efficacy for the first time in this study.

In addition to purifying thoese antimutagenic constitutes from subfraction F3 of the acetone extract of adlay hull, it is interesting to note that subfractions F2 and F4 also exhibited potent activity against IQ-induced mutagenicity in *S. typhimurium* TA98. Therefore, we could not rule out the possibility that other bioactive compounds against mutagenicity might exist in adlay hull, which would merit further investigation.

Adlay Antimutagenic Constituents Exhibit Potent Activity in Scavenging DPPH Radical and Inhibiting TPA-Stimulated Superoxide Anion Generation in Neutrophil-like Leukocytes. Among the naturally occurring antimutagens, the antioxidants present in the human diet are of great interest as possible protective agents against oxidative damage.[34] DPPH is a free radical compound and has been used extensively to predict the antioxidant activities of various chemicals.[2,35] Many plant extracts and phytochemicals showed a significant correlation between DPPH scavenging activity and inhibition of carcinogen-induced oxidative damage during carcinogenesis.[2,9,36,37] Hence, we wondered whether those antimutagenic constituents possess potent activity in scavenging DPPH radicals. As shown in Table 3, sinapaldehyde (**5**), *trans*-coniferylaldehyde (**4**), syringaldehyde (**3**), and vanillin (**2**) exhibited DPPH radical scavenging effect with EC_{50} values of 118, 136, 146, and 180 μg/mL, respectively. However, *p*-hydroxybenzaldehyde (**1**) and coixol (**6**) were inactive in scavenging DPPH radicals.

Reactive oxygen species such as superoxide radical anions, hydrogen peroxide, and hydroxyl radicals are produced by several biochemical reactions during the metabolism of molecular oxygen. It has been suggested that free radicals and related active species may play a role in tumor promotion.[27,38] TPA-induced superoxide anion radical formation through the NADPH oxidase system in neutrophil-like leukocytes is a biochemical marker of carcinogenesis, which has been used for screening of potential

Table 3. Effect of Antimutagenic Constituents in Scavenging DPPH Radicals

test compound	scavenging effect	
	scavenging[a] (%)	EC_{50} value[b] (μg/mL)
p-hydroxybenzaldehyde (**1**)	5.2 ± 0.9	>200
vanillin (**2**)	52.9 ± 2.6	180.2 ± 9.8
syringaldehyde (**3**)	62.1 ± 3.7	146.5 ± 7.4
trans-coniferylaldehyde (**4**)	69.8 ± 5.6	136.4 ± 6.9
sinapaldehyde (**5**)	78.1 ± 3.2	118.4 ± 10.3
coixol (**6**)	11.7 ± 2.9	>200

[a] Data are expressed as the mean ± SD. This represents the results of three separate experiments. Each treatment in each experiment has at least three replicates. Test compound in concentrations of 200 μg/mL was mixed with 300 μM DPPH radicals and kept in the dark for 90 min. The decrease of absorbance of DPPH was recorded spectrophotometrically 90 min after mixing at 517 nm. [b] For the determination of EC_{50} (the efficient concentration of test compound decreasing initial DPPH concentration by 50%), each sample was measured at seven different concentrations in the DPPH test. The EC_{50} was obtained by interpolation from linear regression analysis.

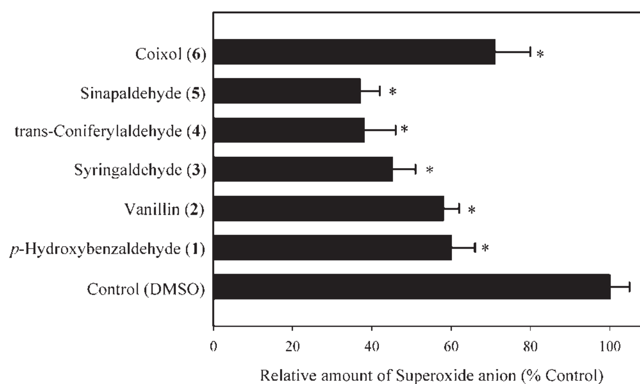

Figure 3. Effects of antimutagenic constituents on TPA-stimulated superoxide anion radical generation by neutrophil-like leukocytes. In HL-60 cells, differentiation was induced for 5 days by means of 1.3% DMSO treatment. These differentiated cells were treated with test compounds at a concentration of 100 μg/mL for 20 min and then stimulated with 8.1 μM TPA for another 60 min. Superoxide anion production was determined by cytochrome *c* reduction. Results are expressed as the mean ± standard deviation of three replicated experiments. A *p* value of <0.05 was considered to be statistically significant from control groups by Student's *t* test.

chemopreventive agents popularly.[27] To investigate whether these antimutagenic constituents are able to block tumor promoter induced oxidative stress in live cells, we investigated the inhibitory effect of these compounds on TPA-stimulated superoxide anion generation in a cellular model.

As a result, six purified compounds revealed inhibitory activity of superoxide anion radical generation in neutrophil-like leukocytes (differentiated HL-60 cells), and the order of potency for mitigation of TPA-mediated oxidative processes by those antimutagenic constituents was as follows: sinapaldehyde (**5**) = *trans*-coniferylaldehyde (**4**) > syringaldehyde (**3**) > vanillin (**2**) = *p*-hydroxybenzaldehyde (**1**) > coixol (**6**) (Figure 3). This result indicated that adlay antimutagenic constituents also function as superoxide formation inhibitors showing promise in preventing carcinogenesis. Similar to antimutagenic activity, phenolic aldehydes with an α,β-unsaturated aldehyde moiety and methoxyl group substitution at the C3- and C5-positions also have better antioxidant activity in scavenging DPPH radical and inhibiting tumor promoter TPA-induced oxidative stress in leukocytes.

Adlay Antimutagenic Constituents Induce Antioxidant-Responsive Element (ARE)-Driven Luciferase Activity in HSC-3 Cells. Current strategies adopted in the cancer chemoprevention field are aimed to interfere in all stages of the carcinogenesis process by various chemopreventive agents. To block the initiation of carcinogenesis, enhancement of the metabolizing/detoxifying and antioxidant enzymes by chemopreventive agents has been demonstrated to effectively lower the occurrence of cancer in populations exposed to environmental toxins through increasing carcinogen excretion and decreasing availability of carcinogen reactive metabolites capable of interacting with DNA.[39] The mechanisms to efficiently neutralize and eliminate both endogenous and exogenous carcinogenic species by chemopreventive agents are mainly mediated by the Nrf2/ARE pathway.[40]

Nuclear factor erythroid-2-related factor (Nrf2), a leucin-zipper transcription factor belonging to the Cap 'n' collar (CNC) family, transcriptionally activates several detoxifying and antioxidant genes through the direct binding of the ARE sequence located in their promoter regions. The Nrf2/ARE pathway is important in protection against carcinogenesis and oxidative stress.[41,42] Many natural chemopreventive agents, such as polyphenols (e.g., curcumin, caffeic acid phenethyl ester, 4′-bromoflavone, EGCG, resveratrol), sulfur-containing compounds (e.g., isothiocyanate sulforaphane, phenethyl isothiocyanate), indoles (e.g., indole-3-carbinol), terpenoids (e.g., cafestol, kahweol), carotenoids (e.g., β-carotene), tetrapyrroles (e.g., chlorophyll), and inorganic (e.g., selenium), are well-investigated in the activation of Nrf2/ARE-dependent genes to eliminate the cell damage caused by ROS or caricongens.[41−43]

In this study, we generated an in vitro ARE modulator-screening system, a nine copy of ARE-luciferase reporter plasmid prepared using the pGL3 vector and transiently transfected into HSC-3 cells. Luciferase activities were determined after cells had been treated with adlay antimutagenic constituents in nontoxic concentration (50 μM) for 24 h. *t*-BHQ, a well-known inducer in ARE-controlled gene transcription, showed a 3.3-fold induction of ARE-driven luciferase activity, indicating that the in vitro ARE modulator-screening system is well established. Sinapaldehyde (**5**) possesses comparable activity with *t*-BHQ treatment. Notably, *trans*-coniferylaldehyde (**4**) is more potent than *t*-BHQ, with a 6-fold increment in inducing luciferase activity. However, *p*-hydroxybenzaldehyde (**1**) and vanillin (**2**) did not induce luciferase activity in our study, and syringaldehyde (**3**) and coixol (**6**) showed a moderate effect in inducing reporter gene activity (Figure 4). This result suggested that the α,β-unsaturated aldehyde moiety in phenolic aldehydes is necessary for the activity of ARE-driven gene transactivation and that *trans*-coniferylaldehyde (**4**) may have potential for preventing carcinogenesis in an ARE-dependent manner.

Potential Antimutagenic Constituent, *trans*-Coniferylaldehyde, Possesses Cytoprotective Efficacy against *tert*-Butyl Hydroperoxide (*t*-BOOH)-Induced DNA Double-Strand Breaks (DSBs) in HSC-3 Cells. Because *trans*-coniferylaldehyde exhibits potent activity in inhibiting mutagenicity of IQ to *S. typhimurium* TA98, scavenging DPPH radicals, inhibiting TPA stimulated superoxide anion generation in neutrophil-like leukocytes, and inducing Nrf2/ARE-driven luciferase activity in HSC-3 cells, we wondered whether *trans*-coniferylaldehyde is

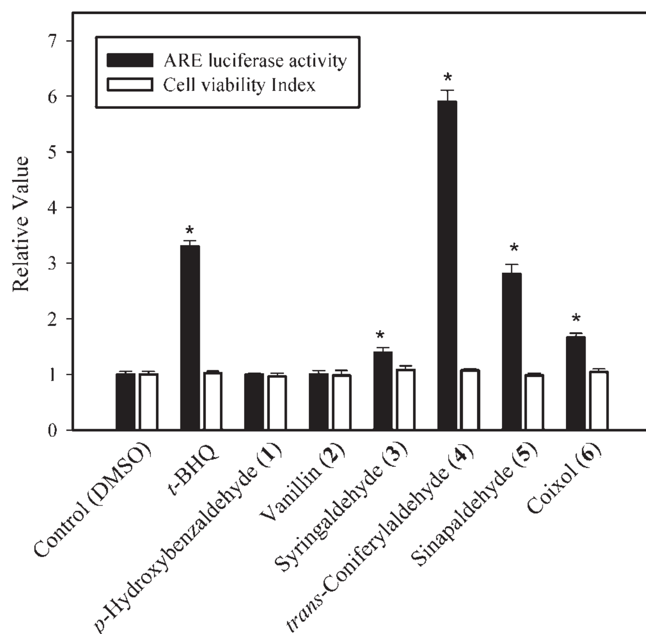

Figure 4. Effect of antimutagenic constituents on ARE-dependent transcriptional activity and cell viability of HSC-3 cells. ARE luciferase activity: HSC-3 cells were seeded at 2×10^5 per well in a 24-well plate. The 9×ARE pGL3 plasmid was cotransfected with pRL-TK plasmid for 24 h. The cells were then treated with 50 μM levels of compounds **1**−**6** or *t*-BHQ (positive control) for another 24 h, and luciferase reporter activity was determined as detailed under Materials and Methods. Cell viability: Cells were exposed to 50 μM levels of compounds **1**−**6** or *t*-BHQ for 24 h, and cell viability was determined by MTT assay. For control experiments, the same volume of DMSO was added to the medium. The value of relative luciferase activity and cell viability index of HSC-3 cells treated with DMSO was set at 1. Each treatment in each experiment has at least three replicates. Data are expressed as the mean ± SD. This represents the results of three separate experiments. A *p* value of <0.05 was considered to be statistically significant from control groups by Student's *t* test.

able to prevent carcinogen-induced DNA damage in cultured cells. DSBs, the DNA lesions potentially caused by carcinogens, rapidly respond to an extensive phosphorylation on the histone H2AX at the position of Ser139 in the carboxy-terminal SQE motif to create γH2AX and therefore are a hallmark of DSBs in vertebrates.[44] In this study, we explored the protective effect of *trans*-coniferylaldehyde against DNA DSBs induced by *t*-BOOH, a short-chain analogue of lipid hydroperoxide mentioned to act as a mutagenic and carcinogenic agent, in HSC-3 cells.[45]

As shown in Figure 5, cells treated with *t*-BOOH significantly increased the percentage of γH2AX-positive populations. Inhibition of *t*-BOOH-induced DNA damage was observed in cells pretreated with *trans*-coniferylaldehyde for 1, 4, 8, and 24 h. Notably, cells pretreated with *trans*-coniferylaldehyde for 8 and 24 h possess more potent cytoprotective activity against *t*-BOOH-induced DBSs as compared to pretreatment for 1 and 4 h. This result leads to the conclusion that the protective effect of *trans*-coniferylaldehyde is mediated not only by direct interaction with free radicals but also by induction of cellular defense mechanism against oxidative and mutagenic stress through the Nrf2/ARE pathway.

***trans*-Coniferylaldehyde Exhibits Chemopreventive Potency through Modulating Kinase Signaling in HSC-3 Cells.**

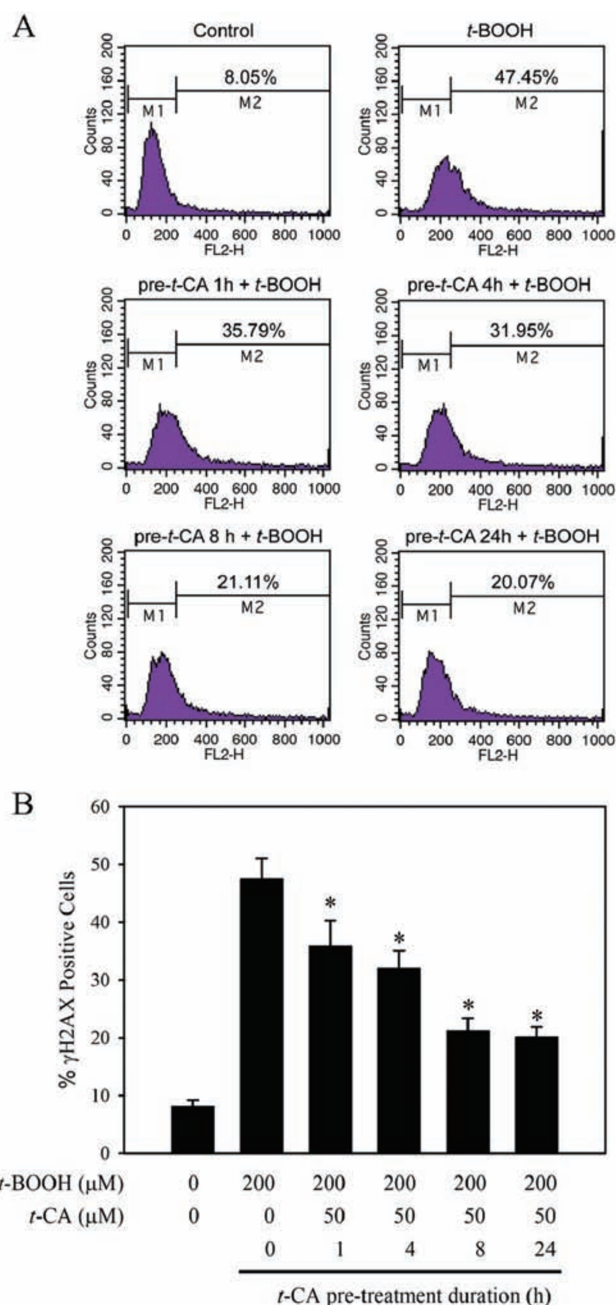

Figure 5. *trans*-Coniferylaldehyde prevents *t*-BOOH-induced DSBs in HSC-3 cells. (A) Detection of DSBs using histone H2AX phosphorylation assay: cells were treated with 50 μM *trans*-coniferylaldehyde (*t*-CA) for 1, 4, 8, or 24 h and then exposed to 200 μM *t*-BOOH for an additional 4 h. The cells were fixed and labeled with an antiphospho-histone H_2AX monoclonal antibody before flow cytometric analysis. Percentage of γH_2AX positive cells (M2) population was quantified and expressed as a function of drug treatment. (B) The graph of quantification of γH_2AX positive cells. The values represent the mean ± SD from three independent experiments. A *p* value of <0.05 was considered to be statistically significant from *t*-BOOH alone groups by Student's *t* test.

Protein phosphorylation is a major posttranslational modification in signaling processes. Several classes of kinase have been shown to activate Nrf2/ARE signaling, including mitogen-activated protein kinases (MAPK), protein kinase C (PKC), or phosphatidylinositol 3-kinase (PI3K).[46] To elucidate the signaling

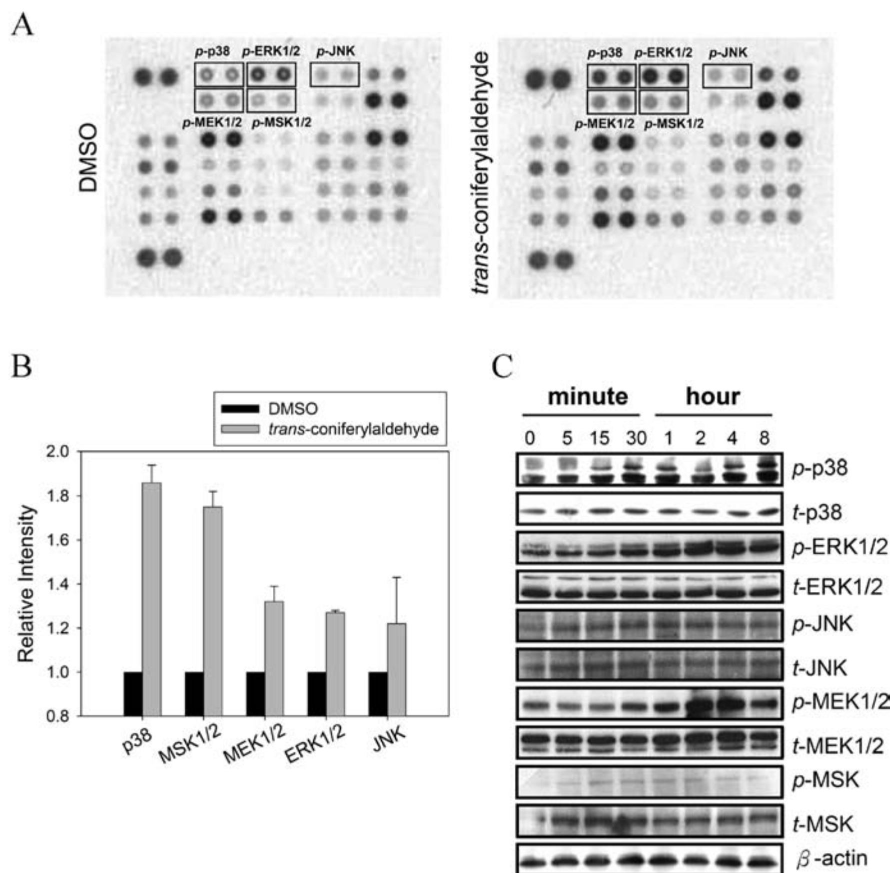

Figure 6. Expression and activation of signaling pathways by *trans*-coniferylaldehyde in HSC-3 cells. (A) Human phosphokinase array analysis in response to *trans*-coniferylaldehyde treatment. Briefly, cells were treated with 50 μM *trans*-coniferylaldehyde for 3 h. Total cell lysates were prepared and hybridized with the antibody-coated membranes according to the manufacturer's instructions. Increased signals of *p*-p38, *p*-ERK1/2, *p*-JNK, *p*-MEK1/2, and *p*-MSK1/2 in response to *trans*-coniferylaldehyde are indicated by boxes. (B) Relative spot intensities of phosphoproteins quantified using Image J software and normalized by those of DMSO control on the membrane. (C) Verification of *p*-p38, *p*-ERK1/2, *p*-JNK, *p*-MEK1/2, and *p*-MSK1/2 in response to *trans*-coniferylaldehyde by Western blot analysis. Cells were treated with 50 μM *trans*-coniferylaldehyde for 5, 15, and 30 min or 1, 2, 4, and 8 h. Forty micrograms of protein lysates was analyzed for phosphorylated and total form of kinases including p38, ERK1/2, JNK, MEK, and MSK by Western blot analysis. β-Actin has been used as internal loading control.

pathway involving in the chemopreventive potency activated by *trans*-coniferylaldehyde, the phosphokinase array analysis was performed. As shown in Figure 6A,B, the most evident effects of *trans*-coniferylaldehyde were indicated by a significant up-regulation of the phosphorylated proteins of p38, ERK1/2, JNK, MEK1/2, and MSK1/2 when HSC-3 cells were treated with this compound for 3 h.

To determine the activation time point of these kinase proteins, we next studied the kinetics of p38, ERK1/2, JNK, MEK1/2, and MSK1/2 activation by *trans*-coniferylaldehyde treatment. Western blot analysis revealed a good agreement with the results obtained with the phosphokinase array in HSC-3 cells (Figure 6C). In HSC-3 cells, 50 μM *trans*-coniferylaldehyde induced phsophorylation of JNK (*p*-JNK) and MSK (*p*-MSK) as early as 5 min and decreased the level around 4−8 h; however, the increased level of *p*-MSK is lower than that of *p*-JNK. *p*-p38 was increased from 15 min and reached a peak at 8 h. ERK1/2 and its upstream kinase, MEK1/2, were activated from 30 min and decreased at 8 h (Figure 6C). Different kinetics of p38, ERK1/2, JNK, MEK1/2, and MSK1/2 activation suggest that these kinases are differentially regulated in response to *trans*-coniferylaldehyde. Because p38, ERK1/2, and JNK are the major components of the MAPK cascades, this result seems to indicate that MAPK may play an important role in mediating the *trans*-coniferylaldehyde-induced ARE-driven gene transactivation.

Several lines of evidence have indicated that MAPK cascades affect the Nrf2/ARE pathway by certain chemopreventive agents. For example, ERK and JNK signaling have been showed to play important and positive roles in BHA and phenethyl isothiocyanate-induced and Nrf2-dependent regulation of ARE-mediated gene expression in cultured cells.[47,48] Induction of ARE-driven gene product, γ-glutamate cysteine ligases, by pyrrolidine dithiocarbamate was mediated by ERK2 and p38 pathways.[49] However, p38 was also shown to negatively regulate the induction of phase II enzymes that detoxify carcinogens.[50] Taken together, these results suggested that the signaling pathway of Nrf2/ARE induced by various compounds may be compound-specific and cell type-specific.

Concluding Remarks. The discovery and exploration of chemical compounds that contain antimutagenic potency is at present of great importance because of the undesirable consequences of an increasing rate of mutations and their relative possible risks of cancer in human. In this study, we have characterized for the first time six potent antimutagenic

phytochemicals, *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, *trans*-coniferylaldehyde, sinapaldehyde, and coixol, from adlay hull. All compounds were classified as phenolic aldehydes except coixol. According to our results, we suggest that phenolic aldehydes with an α,*β*-unsaturated aldehyde moiety and methoxyl group substitution at the C3- or C5-position (i.e., sinapaldehyde and *trans*-coniferylaldehyde) exhibit the most antimutagenic potency against IQ-induced mutagenicity in *S. typhimurium* TA98.

In addition, sinapaldehyde and *trans*-coniferylaldehyde possess desirable chemopreventive potential in scavenging DPPH radicals, inhibiting TPA-stimulated superoxide anion generation in neutrophil-like leukocytes, and inducing Nrf2/ARE-driven luciferase activity in HSC-3 cells. Notably, *trans*-coniferylaldehyde is more potent than the standard ARE activator *t*-BHQ in inducing ARE-driven luciferase activity. Moreover, *trans*-coniferylaldehyde possesses potent cytoprotective efficacy against *tert*-butyl hydroperoxide-induced DNA double-strand breaks in cultured cells through direct interaction with free radicals and induction of ARE-mediated cellular defense system. Furthermore, kinase signals, including p38, ERK1/2, JNK, MEK1/2, and MSK1/2, may play an important role in mediating *trans*-coniferylaldehyde-induced ARE-driven gene transactivation. It will be especially valuable to investigate *trans*-coniferylaldehyde as a promising chemopreventive agent against carcinogenesis, and elucidation of regulation details in upstream kinase signaling network and downstream ARE gene products of this compound merits further investigation.

AUTHOR INFORMATION

Corresponding Author

*Postal address: National Institute of Cancer Research, National Health Research Institutes, No. 367 Shengli Road, Tainan 70456, Taiwan, ROC. Phone: +886-6-7000123, ext. 65115. Fax: +886-6-2083427. E-mail: cckuo@nhri.org.tw.

Author Contributions

‖Equal contribution.

Funding Sources

The study was supported by grants from the National Health Research Institutes (CA-100-PP-05), the National Science Council (NSC98-2320-B-400-003-MY3, NSC99-2323-B-400-006), and the Department of Health (DOH100-TD-C-111-004, DOH100-TD-B-111-005), Taiwan, ROC.

ABBREVIATIONS USED

Ames test, Ames *Salmonella typhimurium* mutagenicity assay; B[a]P, benzo(*a*)pyrene; *t*BHQ, *tert*-butylhydroquinone; *t*-BOOH, *tert*-butyl hydroperoxide; DPPH, 2,2′-diphenyl-1-picrylhydrazyl radicals; DSBs, DNA double-strand breaks; EtOAc, ethyl acetate; Hex, *n*-hexane; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MAPK, mitogen-activated protein kinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium; NQNO, 4-nitroquinoline-*N*-oxide; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TPA, 12-*O*- tetradecanoylphorbol 13-acetate.

REFERENCES

(1) Li, S. C. *Pen-t′sao kang mu (Systematic Pharmacopoeia)*; China, 1596.

(2) Kuo, C. C.; Chiang, W.; Liu, G. P.; Chien, Y. L.; Chang, J. Y.; Lee, C. K.; Lo, J. M.; Huang, S. L.; Shih, M. C.; Kuo, Y. H. 2,2′-Diphenyl-1-picrylhydrazyl radical-scavenging active components from adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) hulls. *J. Agric. Food Chem.* **2002**, *50* (21), 5850–5855.

(3) Huang, D. W.; Kuo, Y. H.; Lin, F. Y.; Lin, Y. L.; Chiang, W. Effect of adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) testa and its phenolic components on Cu^{2+}-treated low-density lipoprotein (LDL) oxidation and lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 macrophages. *J. Agric. Food Chem.* **2009**, *57* (6), 2259–2266.

(4) Otsuka, H.; Hirai, Y.; Nagao, T.; Yamasaki, K. Anti-inflammatory activity of benzoxazinoids from roots of *Coix lachryma-jobi* var. *ma-yuen*. *J. Nat. Prod.* **1988**, *51* (1), 74–79.

(5) Huang, D. W.; Chung, C. P.; Kuo, Y. H.; Lin, Y. L.; Chiang, W. Identification of compounds in adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) seed hull extracts that inhibit lipopolysaccharide-induced inflammation in RAW 264.7 macrophages. *J. Agric. Food Chem.* **2009**, *57* (22), 10651–10657.

(6) Numata, M.; Yamamoto, A.; Moribayashi, A.; Yamada, H. Antitumor components isolated from the Chinese herbal medicine *Coix lachryma*-jobi. *Planta Med.* **1994**, *60* (4), 356–359.

(7) Tanimura, A. Studies on the antitumor components in the seeds of *Coix lachryma-jobi* L. var. *ma-yuen* (Roman.) Stapf. II. The structure of coixenolide. *Chem. Pharm. Bull. (Tokyo)* **1961**, *9*, 47–53.

(8) Lee, M. Y.; Lin, H. Y.; Cheng, F.; Chiang, W.; Kuo, Y. H. Isolation and characterization of new lactam compounds that inhibit lung and colon cancer cells from adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) bran. *Food Chem. Toxicol.* **2008**, *46* (6), 1933–1939.

(9) Kuo, C. C.; Shih, M. C.; Kuo, Y. H.; Chiang, W. Antagonism of free-radical-induced damage of adlay seed and its antiproliferative effect in human histolytic lymphoma U937 monocytic cells. *J. Agric. Food Chem.* **2001**, *49* (3), 1564–1570.

(10) Chen, H. J.; Shih, C. K.; Hsu, H. Y.; Chiang, W. Mast cell-dependent allergic responses are inhibited by ethanolic extract of adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) testa. *J. Agric. Food Chem.* **2010**, *58* (4), 2596–2601.

(11) Takahashi, M.; Konno, C.; Hikino, H. Isolation and hypoglycemic activity of coixans A, B and C, glycans of *Coix lachryma-jobi* var. *ma-yuen* seeds. *Planta Med.* **1986**, *52* (1), 64–65.

(12) Ishiguro, Y.; Okamoto, K.; Sakamoto, H.; Sonoda, Y. Antimicrobial substances coixindens A and B in etiolated seedlings of adlay. *Nippon Nogei Kagaku Kaishi* **1993**, *67*, 1405–1410.

(13) Kondo, Y.; Nakajima, K.; Nozoe, S.; Suzuki, S. Isolation of ovulatory-active substances from crops of Job's tears (*Coix lacryma-jobi* L. var. *ma-yuen* STAPF.). *Chem. Pharm. Bull. (Tokyo)* **1988**, *36*, 3147–3152.

(14) Khan, M.; Pelengaris, S. *The Molecular Biology of Cancer*, 1st ed.; Blackwell Publishing: Malden, MA, 2006.

(15) Huang, M.-T.; Osawa, T.; Ho, C.-T.; Rosen, R. T. *Food Phytochemicals for Cancer Prevention I. Fruits and Vegetables*; ACS Symposium Series 546; American Chemical Society: Washington, DC, 1994.

(16) Brenner, D. E.; Gescher, A. J. Cancer chemoprevention: lessons learned and future directions. *Br. J. Cancer* **2005**, *93* (7), 735–739.

(17) Ohta, T. Modification of genotoxicity by naturally occurring flavorings and their derivatives. *Crit. Rev. Toxicol.* **1993**, *23* (2), 127–146.

(18) Mortelmans, K.; Zeiger, E. The Ames *Salmonella*/microsome mutagenicity assay. *Mutat. Res.* **2000**, *455* (1−2), 29–60.

(19) Weisburger, J. H. Antimutagenesis and anticarcinogenesis, from the past to the future. *Mutat. Res.* **2001**, *480−481*, 23–35.

(20) Huang, S.-L.; Chiang, W. Composition of the different fractions of adlay seed and the demutagenic effect of their acetone extract. *Food Sci.* **1999**, *26*, 121–130.

(21) Lee, C. K.; Lu, C. K.; Kuo, Y. H.; Chen, J. Z.; Sun, G. Z. New prenylated flavones from the roots of *Ficus beecheyana*. *J. Chin. Chem. Soc.* **2004**, *51* (2), 437–441.

(22) Lee, T. H.; Chiou, J. L.; Lee, C. K.; Kuo, Y. H. Separation and determination of chemical constituents in the roots of *Rhus javanica* L. var. *roxburghiana*. *J. Chin. Chem. Soc.* **2005**, *52* (4), 833–841.

(23) Miyazawa, M.; Hisama, M. Antimutagenic activity of phenylpropanoids from clove (*Syzygium aromaticum*). *J. Agric. Food Chem.* **2003**, *51* (22), 6413–6422.

(24) Yahara, S.; Nishiyori, T.; Kohda, A.; Nohara, T.; Nishioka, I. Isolation and characterization of phenolic compounds from magnoliae cortex produced in China. *Chem. Pharm. Bull.* **1991**, *39* (8), 2024–2036.

(25) Yue, Q.; Bacon, C. W.; Richardson, M. D. Biotransformation of 2-benzoxazolinone and 6-methoxy-benzoxazolinone by *Fusarium moniliforme*. *Phytochemistry* **1998**, *48* (3), 451–454.

(26) Maron, D. M.; Ames, B. N. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* **1983**, *113* (3−4), 173–215.

(27) Sharma, S.; Stutzman, J. D.; Kelloff, G. J.; Steele, V. E. Screening of potential chemopreventive agents using biochemical markers of carcinogenesis. *Cancer Res.* **1994**, *54* (22), 5848–5855.

(28) Leonce, S.; Kraus-Berthier, L.; Golsteyn, R. M.; vid-Cordonnier, M. H.; Tardy, C.; Lansiaux, A.; Poindessous, V.; Larsen, A. K.; Pierre, A. Generation of replication-dependent double-strand breaks by the novel N2-G-alkylator S23906-1. *Cancer Res.* **2006**, *66* (14), 7203–7210.

(29) Ferguson, L. R. Antimutagens as cancer chemopreventive agents in the diet. *Mutat. Res.* **1994**, *307* (1), 395–410.

(30) Shaughnessy, D. T.; Schaaper, R. M.; Umbach, D. M.; DeMarini, D. M. Inhibition of spontaneous mutagenesis by vanillin and cinnamaldehyde in *Escherichia coli*: dependence on recombinational repair. *Mutat. Res.* **2006**, *602* (1−2), 54–64.

(31) Butterstein, G. M.; Schadler, M. H.; Lysogorski, E.; Robin, L.; Sipperly, S. A naturally occurring plant compound, 6-methoxybenzoxazolinone, stimulates reproductive responses in rats. *Biol. Reprod.* **1985**, *32* (5), 1018–1023.

(32) Sanders, E. H.; Gardner, P. D.; Berger, P. J.; Negus, N. C. 6-Methoxybenzoxazolinone: a plant derivative that stimulates reproduction in *Microtus montanus*. *Science* **1981**, *214* (4516), 67–69.

(33) Yuwiler, A.; Winters, W. D. Effects of 6-methoxy-2-benzoxazolinone on the pineal melatonin generating system. *J. Pharmacol. Exp. Ther.* **1985**, *233* (1), 45–50.

(34) Hartman, P. E.; Shankel, D. M. Antimutagens and anticarcinogens: a survey of putative interceptor molecules. *Environ. Mol. Mutagen.* **1990**, *15* (3), 145–182.

(35) Yokozawa, T.; Chen, C. P.; Dong, E.; Tanaka, T.; Nonaka, G. I.; Nishioka, I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol.* **1998**, *56* (2), 213–222.

(36) Zessner, H.; Pan, L.; Will, F.; Klimo, K.; Knauft, J.; Niewohner, R.; Hummer, W.; Owen, R.; Richling, E.; Frank, N.; Schreier, P.; Becker, H.; Gerhauser, C. Fractionation of polyphenol-enriched apple juice extracts to identify constituents with cancer chemopreventive potential. *Mol. Nutr. Food Res.* **2008**, *52* (Suppl. 1), S28–S44.

(37) Gerhauser, C.; Klimo, K.; Heiss, E.; Neumann, I.; Gamal-Eldeen, A.; Knauft, J.; Liu, G. Y.; Sitthimonchai, S.; Frank, N. Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutat. Res.* **2003**, *523−524*, 163–172.

(38) Cerutti, P. A. Prooxidant states and tumor promotion. *Science* **1985**, *227* (4685), 375–381.

(39) Guengerich, F. P. Metabolism of chemical carcinogens. *Carcinogenesis* **2000**, *21* (3), 345–351.

(40) Eggler, A. L.; Gay, K. A.; Mesecar, A. D. Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2. *Mol. Nutr. Food Res.* **2008**, *52* (Suppl. 1), S84–S94.

(41) Giudice, A.; Montella, M. Activation of the Nrf2-ARE signaling pathway: a promising strategy in cancer prevention. *Bioessays* **2006**, *28* (2), 169–181.

(42) Zhang, Y.; Gordon, G. B. A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway. *Mol. Cancer Ther.* **2004**, *3* (7), 885–893.

(43) Sporn, M. B.; Liby, K. T. Cancer chemoprevention: scientific promise, clinical uncertainty. *Nat. Clin. Pract. Oncol.* **2005**, *2* (10), 518–525.

(44) Rogakou, E. P.; Pilch, D. R.; Orr, A. H.; Ivanova, V. S.; Bonner, W. M. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* **1998**, *273* (10), 5858–5868.

(45) Savio, M.; Stivala, L. A.; Bianchi, L.; Vannini, V.; Prosperi, E. Involvement of the proliferating cell nuclear antigen (PCNA) in DNA repair induced by alkylating agents and oxidative damage in human fibroblasts. *Carcinogenesis* **1998**, *19* (4), 591–596.

(46) Jaiswal, A. K. Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radical Biol. Med.* **2004**, *36* (10), 1199–1207.

(47) Yuan, X.; Xu, C.; Pan, Z.; Keum, Y. S.; Kim, J. H.; Shen, G.; Yu, S.; Oo, K. T.; Ma, J.; Kong, A. N. Butylated hydroxyanisole regulates ARE-mediated gene expression via Nrf2 coupled with ERK and JNK signaling pathway in HepG2 cells. *Mol. Carcinog.* **2006**, *45* (11), 841–850.

(48) Xu, C.; Yuan, X.; Pan, Z.; Shen, G.; Kim, J. H.; Yu, S.; Khor, T. O.; Li, W.; Ma, J.; Kong, A. N. Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. *Mol. Cancer Ther.* **2006**, *5* (8), 1918–1926.

(49) Zipper, L. M.; Mulcahy, R. T. Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. *Biochem. Biophys. Res. Commun.* **2000**, *278* (2), 484–492.

(50) Yu, R.; Mandlekar, S.; Lei, W.; Fahl, W. E.; Tan, T. H.; Kong, A. N. p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. *J. Biol. Chem.* **2000**, *275* (4), 2322–2327.