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Suppressive Effect of the Ethanolic Extract of Adlay Bran on Cytochrome P-450 Enzymes in Rat Liver and Lungs

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ABSTRACT: Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is a grass crop and is reported to protect against various diseases such as cancer. To investigate the effect of the ethanolic extract of adlay bran (ABE) on drug-metabolizing enzymes and glutathione-related antioxidant enzymes in rats, three groups of eight male Sprague−Dawley rats each were fed a control diet or a diet containing 5 or 10% ABE for 4 weeks. Significant decreases in microsomal cytochrome P-450 (CYP) 1A1-catalyzed ethoxyresorufin *O*-deethylation, CYP2C-catalyzed diclofenac 4-hydroxylation, CYP2D-catalyzed dextromethorphan *O*-demethylation, and CYP3A-catalyzed testosterone 6β-hydroxylation in the liver and CYP1A1-catalyzed ethoxyresorufin *O*-deethylation in the lungs of rats fed ABE were observed. Immunoblot analyses also showed decreases of CYP1A1, 1A2, 2C6, 2C11, 2D1, 2E1, 3A1, and 3A2 in the liver and CYP1A1 in the lungs. Furthermore, rats fed the 10% ABE diet had a higher glutathione content and glutathione peroxidase, glutathione reductase, and glutathione *S*-transferase activities in the lungs, but such an increase was not noted in the liver. Inhibition of various CYP-catalyzed enzyme reactions by ABE in rat and human liver microsomes had also been shown. The results of this study indicate that ABE feeding may suppress CYP enzyme activities and CYP protein expression in the liver and lungs of rats. Moreover, the increase of the antioxidant potential by ABE is tissue-specific.

KEYWORDS: adlay bran, cytochrome P-450, drug-metabolizing enzymes, glutathione-related antioxidant enzymes, rats

INTRODUCTION

Drug-metabolizing enzymes (DMEs) include phase I and II enzyme systems. Mammalian cytochrome P-450 (CYP) enzymes are phase I monooxygenases that catalyze the oxidative metabolism of various xenobiotics, including drugs and chemical carcinogens, and many endogenous substrates such as steroids, fatty acids, and eicosanoids.[1] In general, the phase I oxidative metabolic reactions of drugs and chemicals result in the formation of more-water-soluble and less-toxic metabolites. However, some CYP enzymes such as CYP1A1, 3A, and 2E1 are also involved in the metabolic activation of carcinogens such as benzo(*a*)pyrene, *N*-nitrosodimethylamine, and aflatoxin B_1, and their metabolites are more toxic than the parent compounds.[2] In addition to oxidative metabolism, conjugation reactions are also involved in the biotransformation of drugs and chemical carcinogens in mammalian systems. Microsomal UDP-glucuronosyltransferase (UGT) and cytosolic glutathione *S*-transferase (GST) are two important phase II enzymes that catalyze the conjugation reactions resulting in the formation of water-soluble glucuronate and glutathione conjugates to facilitate the excretion of xenobiotics. Several CYP isoforms and phase II enzymes have been shown to be inhibited or induced by functional foods[3,4] and herbal medicines.[5,6] Phytochemicals, especially phenolic acid and flavonoids, present in plants were shown to modulate DMEs with or without affecting protein and gene expressions.[7,8] Induction or inhibition of DMEs may change the pharmacological activities and toxicities of drugs and carcinogens and may also cause drug interactions.[9]

Adlay or Job's tears (*Coix lachrymal-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 including the hull, testa, bran, and endosperm. It has long been used in food supplements and as a nourishing food. Although the exact active components were not known, several pharmacological effects including antioxidant and anticancer activities derived from whole seeds or the bran part of adlay were demonstrated.[10−13] The bran part of adlay has attracted attention because it contains a considerable amount of neutral oil (approximately 25% of the dry weight) mainly present in the form of triglyceride (>90%).[14,15] Oleic acid and linoleic acid are two of the most abundant fatty acid components in adlay bran oil.[15] In addition, considerable amounts of phytosterols[16] and phenolic compounds[12,13] are also present in the adlay bran. Animal study revealed that adlay bran oil, partially or fully substituted for dietary fat in experimental diets, reduces plasma lipids and lipid peroxidation in rats without obvious adverse effects.[15] In addition, the ethyl acetate-soluble fraction of the methanol extract of adlay bran possesses anti-inflammatory activity and antiproliferative effects on colon and lung cancer cell lines.[12] Recently, the ethyl acetate fraction of the adlay bran ethanolic extract was demonstrated to reduce colon carcinogenesis in F344 rats induced by 1,2-dimethylhydrazine (DMH),[13] a carcinogen that requires activation by liver CYP enzymes to form

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DNA-reactive metabolites.[17,18] All of these results suggest that adlay bran may have the potential to be developed as a functional food with chemopreventive effects against carcinogenesis. However, it is not known whether adlay bran could modulate DME activity, resulting in changes of the pharmacological activities and toxicities of chemicals.

The present study was designed to investigate the effect of the ethanolic extract of adlay bran (ABE) on DMEs including CYP, GST, and UGT and glutathione-related antioxidant enzymes in the liver and lungs of rats following its administration in diet for 4 weeks. In addition, the inhibitory effects of ABE on CYP-catalyzed enzyme reactions in rat and human liver microsomes were also evaluated.

MATERIALS AND METHODS

Materials. Testosterone, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, resorufin, *p*-nitrophenol, diclofenac, dextromethorphan, 4-nitrocatechol, lauric acid, 12-hydroxylauric acid, NADPH, GSH, glutathione reductase (GSH-Rd), *p*-nitrophenol glucuronic acid, 1-chloro-2,4-dinitrobenzene, sodium dodecyl sulfate (SDS), cytochrome *c*, heparin, Ponceau S, 4-hydroxybenzoic acid, nobiletin, tangeritin, rutin, ferulic acid, quercetin, vanillic acid, *p*-coumaric acid, chrysoeriol, protocatechuic acid, naringenin, *p*-hydroxybenzaldehyde, isoliquiritigenin, formononetin, and caffeic acid were obtained from Sigma Chemical Co. (St. Louis, MO). 6β-Hydroxytestosterone, 4-hydroxydiclofenac, and dextrophen were purchased from Ultrafine Chemicals (Manchester, U.K.). All other chemicals and reagents were of analytical grade and were obtained commercially.

Preparation of ABE. ABE, prepared according to the method of Chung et al.,[13] was provided by Dr. W. Chiang, National Taiwan University. In brief, adlay seeds were purchased from local farmers who planted Taichung Shuenyu no. 4 (TCS4) of *C. lachrymal-jobi* L. var. *ma-yuen* Stapf in Taichung, Taiwan. Adlay bran was separated from dehulled adlay, blended into a powder, and screened through a 20-mesh sieve. Adlay bran powder was protected from light and extracted with ethanol (1:6; w/v) 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. In general, each gram of ABE residue was derived from 10 g of adlay bran powder.

Determination of Phytosterols in ABE. ABE was dissolved in ethanol (1:9, w/v). The contents of phytosterols, including β-sitosterol, campesterol, and stigmasterol, were determined by high-performance liquid chromatography (HPLC) with a UV detector.[19]

Determination of Total Phenols and Phenolic Compounds in ABE. To avoid the interference of neutral oil on measuring total phenolic contents, the ABE phenolic extract was prepared by phase partitioning according to the method of Christophoridou et al.[20] In brief, 3 g of ABE was dissolved in hexane (1:1, w/v), and the solution was extracted with 5 × 3 mL of methanol/water (80:20, v/v). The mixture was stirred for 2 min in a vortex apparatus and centrifuged at 3000*g* for 15 min. The methanolic layers of the extracts were combined and washed with hexane. The hexane wash was discarded, and the methanolic solution was evaporated under vacuum to dryness. The residue was then dissolved in methanol (w/v, 1:10) and filtered through a 0.45-μm filter. The filtrate was used to determine total phenols and phenolic compounds by a Folin−Ciocalteu method[21] and an Agilent 1100 series HPLC−mass spectrometer (MS) (Palo Alto, CA), respectively.

The phenolic compounds were identified by their retention times, compared to those of the reference standards, in two different HPLC systems and by the mass of the selected ions. The phenolic compounds in our in-house library were used as the reference standards. In the first HPLC system, an Alltech Alltima C18 column (5 μm, 250 × 4.6 mm i.d.) was used. The mobile phase consisted of solvents A (10 mM ammonia acetate containing 0.5% formic acid) and B (methanol containing 0.5% formic acid). The flow rate was 0.5 mL/min. The column temperature was 25 °C. The gradient system was 5−10% B (0−20 min), 10−30% B (20−50 min), 30−60% B (50−100 min), 60−90% B (100−120 min), 90% B (120−140 min), 90−5% B (140−145 min), and 5% B (145−160 min). Data acquisition was via selected ion monitoring (SIM). Ions representing positive (only for tangeritin and nobiletin) or negative species of the compounds were selected, and peak areas were measured. The tentatively identified phenolic compounds were further confirmed and quantified by a second HPLC-MS system. Again, reference standards were used. In the second HPLC-MS system, an Agilent Zorbax Eclipse XDB-C8 column (5 μm, 150 × 3.0 mm i.d.) was used. The mobile phase consisted of solvents A (10 mM ammonia acetate containing 0.5% formic acid) and B (acetonitrile containing 0.5% formic acid). The gradient system was 10−90% B (0−45 min), 90−10% B (45−50 min), and 10% B (50−60 min). All other conditions were the same as those of the first HPLC-MS system. In the present study, 15 phenolic compounds, including phenolic acids (protocatechuic acid, 7.7 min; 4-hydroxybenzoic acid, 10.6 min; caffeic acid, 11.0 min; vanillic acid, 11.2 min; *p*-coumaric acid, 13.9 min; and ferulic acid, 14.5 min), flavonoids (rutin, 11.7 min; quercetin, 19.3 min; chrysoeriol, 21.8 min; naringenin, 22.2 min; isoliquiritigenin, 23.9 min; formononetin, 24.3 min; nobiletin, 26.2 min; and tangeritin, 28.8 min), and a phenolic aldehyde (*p*-hydroxybenzaldehyde, 13.5 min), were identified and quantified by the second HPLC-MS system (Table 1). The calibration curves of authentic standards were linear over the concentration range of 0.02−40 μg/mL with correlation coefficients of ≥0.99.

Table 1. Phytochemical Contents in the Ethanolic Extract of Adlay Bran (ABE)

component	amount (μg/g)
phytosterols	
β-sitosterol	1700
campesterol	970
stigmasterol	443
phenolic compounds	
total phenols[a]	3583
4-hydroxybenzoic acid	167
nobiletin	54.6
tangeritin	44.5
rutin	41.2
ferulic acid	27.5
quercetin	26.4
vanillic acid	22.2
p-coumaric acid	9.0
chrysoeriol	5.0
protocatechuic acid	4.9
naringenin	2.7
p-hydroxybenzaldehyde	2.4
isoliquiritigenin	2.1
formononetin	1.9
caffeic acid	1.6

[a] The total phenols content of the ABE is expressed in μg of gallic acid equiv/g of ABE. Values are the mean of three determinations.

Determination of the Fatty Acid Composition in ABE. The fatty acid composition of the lipid fraction of ABE was determined by gas chromatography (GC) according to the method previously reported.[4] Fatty acids in ABE were converted to their corresponding methyl esters

Table 2. Composition of the Experimental Diets

	control	5% ABE[a]	10% ABE
ingredient (%, w/w)			
casein	20	20	20
soybean oil	3	3	3
olive oil	10	5	
adlay bran ethanolic extract		5	10
vitamin mixture[b]	1	1	1
mineral mixture[b]	4	4	4
choline chloride	0.2	0.2	0.2
cellulose	5	5	5
corn starch	56.8	56.8	56.8
total	100	100	100
fatty acid composition (%)			
palmitic acid ($C_{16:0}$)	12.2	12.3	14.3
palmitoleic acid ($C_{16:1}$)	0.4	0.2	0.2
stearic acid ($C_{18:0}$)	4.5	3.2	2.7
oleic acid ($C_{18:1}$)	58.5	51.8	44.9
linoleic acid ($C_{18:2}$)	23	31.3	36.6
linolenic acid ($C_{18:3}$)	1.4	1.2	1.3

[a] ABE, adlay bran ethanolic extract. Compositions of the fatty acids in ABE were 18.0% palmitic acid ($C_{16:0}$), 0.2% palmitoleic acid ($C_{16:1}$), 1.0% stearic acid ($C_{18:0}$), 48.1% oleic acid (C18:1), 32.4% linoleic acid ($C_{18:2}$), and 0.3% linolenic acid ($C_{18:3}$). [b] AIN 93 vitamin and mineral mixtures were procured from ICN Biochemicals (Costa Mesa, CA).

before analysis by GC. Fatty acid profiles were identified according to the retention times of appropriate standard fatty acid methyl esters.

Animals and Treatments. Male Sprague−Dawley rats weighing approximately 250 g each (6 weeks old) were obtained from BioLASCO, Taiwan (Ilan, Taiwan). Rats were fed a chow diet for 1 week, and then the animals were randomly divided into three groups with eight rats per group: (1) control group, (2) 5% ABE diet group, and (3) 10% ABE diet group. The compositions of the experimental diets given to the test animals are shown in Table 2. Rats were housed in individual plastic cages in a room kept at 23 ± 1 °C and 60% ± 5% relative humidity with a 12-h light/dark cycle. Food and drinking water were available ad libitum for 4 weeks. This study was approved by the Animal Center Management Committee of China Medical University. Animals were maintained in accordance with guidelines for the care and use of laboratory animals as issued by the Animal Center of the National Science Council, Taiwan.

Collection of Blood and Tissue Samples. At the end of the experiment, animals were fasted for 12 h prior to being sacrificed. Animals were killed by exsanguination via the abdominal aorta while under carbon dioxide (70%/30%, CO_2/O_2) anesthesia. Heparin was used as the anticoagulant. Plasma was separated from the blood by centrifugation (1750*g*) at 4 °C for 20 min. The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were immediately measured with commercial kits (Randox Laboratories, Antrum, U.K.). The liver and lungs from each animal were immediately removed, weighed, and stored at −80 °C. Microsomal preparation and enzyme activity assays were performed within 2 weeks.

Microsome Preparation. Liver and lung samples were homogenized (1:4, w/v) in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA. The homogenate was first centrifuged at 10000*g* for 15 min at 4 °C. The supernatant was then centrifuged at 105000*g* for 60 min. The resulting microsomal pellets were suspended in a 0.25 M sucrose solution containing 1 mM EDTA and stored at −80 °C until use. The protein concentration of the microsomal preparations was determined with a BCA protein assay kit (Pierce, Rockford, IL).

DME Assays. Total CYP and cytochrome *b*5 contents were quantified according to the method of Omura and Sato.[22] NADPH-CYP reductase activity was measured according to the procedure described by Phillips and Langdon[23] using cytochrome *c* as the substrate. CYP enzyme activity was determined as reported previously.[4,24] Ethoxyresorufin (2 μM), methoxyresorufin (5 μM), and penthoxyresorufin (5 μM) were respectively used as the probe substrates for ethoxyresorufin *O*-deethylation (CYP1A1), methoxyresorufin *O*-demethylation (CYP1A2), and pentoxyresorufin *O*-depentylation (CYP2B), whereas *p*-nitrophenol (50 μM) and testosterone (60 μM) were respectively used as the probe substrates for *p*-nitrophenol 6-hydroxylation (CYP2E1) and testosterone 6*β*-hydroxylation (CYP3A). The microsomal protein concentration of all incubations was 0.2 mg/mL, and the incubation time was 15 min. The metabolites of various CYP enzyme reactions were determined by HPLC-MS methods as reported previously.[4,24] Enzyme activities are expressed as picomoles of metabolite formation per minute per milligram of protein.

The microsomal UGT activity was determined using *p*-nitrophenol as the substrate. The rate of formation of *p*-nitrophenol glucuronate was measured by HPLC.[25]

Determination of Lipid Peroxides and GSH Contents and GSH-Related Antioxidant Enzyme Activities. Liver and lung homogenates were prepared by homogenizing tissues in ice-cold 1.15% KCl to obtain a 10% solution and centrifuged at 10000*g* for 15 min at 4 °C. The resulting supernatant was used to determine the lipid peroxides and GSH contents and GSH peroxidase (GSH-Px), GSH reductase (GSH-Rd), and GSH *S*-transferase (GST) activities. The lipid peroxide level in tissue homogenates was assessed by measuring thiobarbituric acid-reactive substance (TBARS) values according to the method of Uehiyama and Mihara.[26] The calibration curve of a 1,1,3,3-tetraethoxypropane standard solution was used to determine the concentrations of TBARS in each sample. Fluorescence was measured at respective excitation and emission wavelengths of 515 and 553 nm. The contents of reduced (GSH) and oxidized glutathione (GSSG) in liver and lung homogenates were determined by HPLC-MS.[27] GST activity was determined spectrophotometrically according to the method of Habig and Jakoby.[28] Enzyme activity was expressed as nanomoles of CDNB-GSH formation per minute per milligram of protein. GSH-Px and GSH-Rd activities were determined spectrophotometrically according to the method of Mohandas et al.[29] GSH-Px and GSH-Rd activities were expressed as the decrease in nanomoles of NADPH per minute per milligram of protein.

Determination of the Concentrations of Phenolic Compounds in Plasma and Tissues. Three male Sprague−Dawley rats (250 g) were fasted overnight before being administrated the ABE (2 g/kg body weight by intragastric gavage). At 1 h after dosing, rats were sacrificed, and plasma, liver, and lungs were collected according to the same method described above. For determining phenolic compounds concentration in liver and lungs, 1 g of tissue was homogenized with deionized water (1:4, w/v), and the resulting homogenates were mixed with 3 volumes of ethyl acetate. The mixture was vortexed and centrifuged at 10000*g* for 10 min at 4 °C. The resulting supernatant was collected and evaporated to dryness under nitrogen. The residual was dissolved in methanol and used for determination of phenolic compounds. For determining phenolic compounds concentration in plasma, plasma (100 μL) was mixed with 200 μL of methanol (1:2, v/v), vortexed, and then centrifuged at 10000*g* for 20 min. The supernatant was used to determine the phenolic compounds. Calibration standards of mixed phenolic compounds were prepared by serial dilution of a mixed phenolic compound stock solution with blank plasma or tissue homogenates. The concentrations of phenolic compounds in the plasma and tissues were determined by HPLC-MS as described above.

Western Blot Analysis. Liver microsomes were analyzed for expression of CYP1A1, 1A2, 2C6, 2C11, 2D1, 2E1, 3A1, and 3A2, and lung microsomes were analyzed for the expression of CYP1A1 by immunoblot analyses. Equal amounts of microsomal proteins from

Table 3. Activities of Drug-Metabolizing Enzymes in the Liver of Rats[a]

	control	5% ABE	10% ABE
cytochrome P-450 (pmol/mg protein)	738.3 ± 87.4a	649.2 ± 83.9ab	617.8 ± 111.3b
cytochrome b5 (pmol/mg protein)	235.3 ± 32.6	224.7 ± 27.3	207.1 ± 20.8
NADPH-cytochrome P-450 reductase (μmol/min/mg protein)	42.9 ± 9.0	51.4 ± 13.3	42.9 ± 6.7
ethoxyresorufin *O*-deethylase (CYP1A1) (pmol/min/mg protein)	52.8 ± 13.6a	28.9 ± 4.4b	23.3 ± 3.0b
methoxyresorufin *O*-demethylase (CYP1A2) (pmol/min/mg protein)	34.2 ± 4.9a	30.0 ± 7.3a	20.8 ± 4.5b
pentoxyresorufin *O*-depentylase (CYP2B) (pmol/min/mg protein)	12.3 ± 3.4	10.4 ± 3.4	9.9 ± 2.9
diclofenac 4-hydroxylase (CYP2C) (pmol/min/mg protein)	114.0 ± 33.0a	74.5 ± 19.1b	64.6 ± 11.6b
dextromethorphan *O*-demethylase (CYP2D) (pmol/min/mg protein)	235.2 ± 45.7a	175.4 ± 37.5b	167.6 ± 30.2b
p-nitrophenol 6-hydroxylase (CYP2E1) (pmol/min/mg protein)	156.6 ± 28.5a	139.2 ± 20.1a	120.4 ± 34.7b
testosterone 6β-hydroxylase (CYP3A) (pmol/min/mg protein)	1849.6 ± 433.9a	1405.0 ± 337.7b	1299.4 ± 205.6b
lauric acid 12-hydroxylase (CYP4A) (pmol/min/mg protein)	608.2 ± 79.0	571.4 ± 157.3	592.9 ± 92.9
glutathione *S*-transferase (nmol/min/mg protein)	377.1 ± 36.6	401.4 ± 48.3	418.5 ± 89.2
UDP-glucurosyltransferase (nmol/min/mg protein)	43.0 ± 4.9	44.1 ± 6.5	42.3 ± 6.9

[a] Values are the mean ± SD, $n = 8$. Rats were fed one of following diets for 4 weeks: control diet, 5% ethanolic adlay extract (5% ABE) diet, or 10% ABE diet. Values in the same row with different letters significantly differ ($p < 0.05$).

pooled microsomes of each group (CYP1A1 and 1A2, 12.5 μg; CYP2C6, 2C11, 3A1, and 3A2, 4 μg; CYP2D1 and 2E1, 10 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Subsequently, the membranes were blocked with 5% skim milk in PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.1% Tween-20) for 1 h at room temperature and then hybridized with primary antibodies against CYP1A1, 2C6, 2D1, 3A1, or 3A2 (Chemicon International, Temecula, CA), CYP1A2 (Millipore, Billerica, MA), or CYP2C11 or 2E1 (Abcam, Cambridge, U.K.) with gentle agitation overnight at 4 °C. After two washings with PBST, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody from Abcam for 1 h at room temperature. The immunoreactive bands were visualized using Western Lightning Western Blot Chemiluminescence Reagents (Blossom Biotechnologies, Blossom, TX). Densitometric measurements of the bands were made using an image analysis system with Image pro plus 5.1 software (Media Cybernetics, Silver Spring, MD). Equal loading across the lanes was confirmed by staining the blot with Ponceau S solution.

In Vitro Inhibitory Effect of ABE on CYP Enzymes in Rat and Human Liver Microsomes. To examine the selective inhibition of ABE on rat and human CYP enzymes, several CYP probe substrates in the absence or presence of ABE (0−1000 μg/mL) were separately incubated with rat and human liver microsomes. Pooled rat liver microsomes from eight control rats were prepared in our laboratory. Human liver microsomes were purchased from BD Gentest (Woburn, MA). The probe substrates and their concentrations, microsomal protein concentrations, and incubation times for all reactions were same as those described above. Incubations without ABE (ethanol only) were used as the controls. The effects of phytosterols (β-sitosterol, campesterol, and stigmasterol; 1−100 μM) on CYP enzyme activities in rat and human liver microsomes were also evaluated. To examine whether ABE inhibited CYP enzymes in a mechanism-based manner, an index of irreversible inhibition, ABE (10 μg/mL) was preincubated with rat or human liver microsomes at 37 °C for 15 min in the reaction mixture with the presence of NADPH according to the method described above. All CYP-catalyzed enzymatic reactions were conducted in duplicate. To avoid misinterpretation, the results with >10% deviations between replicates were repeated to confirm the results.

Statistical Analysis. For the animal study, statistical differences among groups were analyzed by using one-way analysis of variance (ANOVA; SPSS 10.0.7; Chicago, IL) and were considered to be significant at $p < 0.05$ as determined by Duncan's new multiple-range test. For the in vitro CYP enzyme inhibition study, the relative CYP isozyme activity was calculated by dividing the metabolite formed in the incubation containing ABE by that of the negative control (incubation without ABE). IC_{50} values were determined graphically.

RESULTS

Phytochemical Constituents of ABE. As shown in Table 1, a total of 0.31% (w/w) phytosterols was found in ABE. The relative abundance among phytosterols was in the order of β-sitosterol (1700 μg/g) > campesterol (970 μg/g) > stigmasterol (443 μg/g). In addition to phytosterols, a considerable amount of total phenols (3583 μg/g) was also found in ABE. Fifteen phenolic compounds were identified and quantified by HPLC-MS comparison with the reference standards. Among those, 4-hydroxybenzoic acid (167.3 μg/g) had the highest concentration, which was followed by nobiletin (54.6 μg/g), tangeritin (44.5 μg/g), rutin (41.2 μg/g), ferulic acid (27.5 μg/g), quercetin (26.4 μg/g), and vanillic acid (22.2 μg/g). Relatively smaller amounts (<10 μg/g) of other phenolic compounds including *p*-coumaric acid, chrysoeriol, protocatechuic acid, naringenin, *p*-hydroxybenzaldehyde, isoliquiritigenin, formononetin, and caffeic acid were also found in ABE.

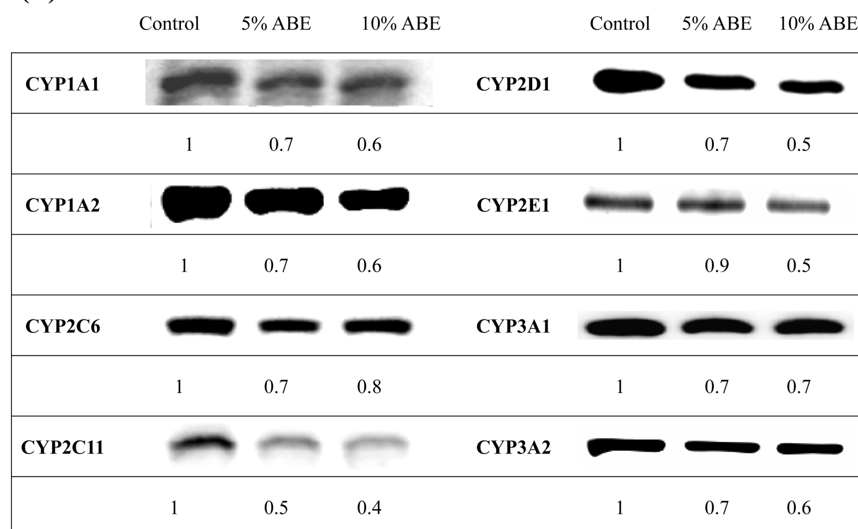
Effect of ABE Feeding on the Body Weight and Liver Weight of Rats. After 4 weeks of feeding of ABE, no changes of body weight and liver weight were observed in the treated rats. No differences in plasma aminotransaminases (AST and ALT) were noted (data not shown), indicating that ABE caused no hepatotoxicity.

DME Activities. Table 3 shows the DME activities in rat liver. Decrease of total CYP contents was observed in rats fed ABE, but the change was significant only in rats fed the 10% ABE diet. ABE treatment had no effect on the cytochrome *b*5 content and NADPH-cytochrome P-450 reductase activity in rats. The activities of hepatic testosterone 6β-hydroxylase (CYP3A), diclofenac 4-hydroxylase (CYP2C), dextromethorphan *O*-demethylase (CYP2D), and ethoxyresorufin *O*-deethylase (CYP1A1) decreased dose-dependently in rats fed diet containing ABE ($p < 0.05$). Decreases of *p*-nitrophenol 6-hydroxylase (CYP2E1) and methoxyresorufin *O*-demethylase (CYP1A2) activities were also observed in the ABE-treated rats, but the change was significant only in rats fed the 10% ABE diet ($p < 0.05$). ABE had no effect on the activities of lauric acid 12-hydroxylase (CYP4A) or

Table 4. Activities of Drug-Metabolizing Enzymes in the Lungs of Rats[a]

	control	5% ABE	10% ABE
ethoxyresorufin *O*-deethylase (CYP1A1) (pmol/min/mg protein)	2.4 ± 1.3a	1.3 ± 0.8ab	0.4 ± 0.2b
methoxyresorufin *O*-demethylase (CYP1A2) (pmol/min/mg protein)	0.8 ± 0.3	0.6 ± 0.1	0.6 ± 0.1
pentoxyresorufin *O*-depentylase (CYP2B) (pmol/min/mg protein)	2.8 ± 1.7	2.9 ± 1.7	3.0 ± 1.1
glutathione *S*-transferase (nmol/min/mg protein)	52.3 ± 11.4b	55.5 ± 9.3ab	69.3 ± 15.5a
UDP-glucurosyltransferase (nmol/min/mg protein)	35.3 ± 10.5	33.8 ± 5.8	35.2 ± 10.4

[a] Values are the mean ± SD, $n = 8$. Rats were fed one of following diets for 4 weeks: control diet, 5% ethanolic adlay extract (5% ABE) diet, or 10% ABE diet. Values in the same row with different letters differ significantly ($p < 0.05$).



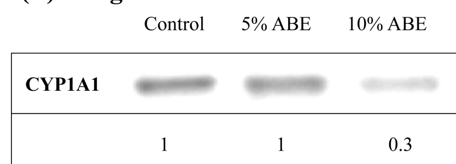


Figure 1. Effect of the ethanolic extract of adlay bran (ABE) on CYP expression in the liver and lungs of rats. Protein contents in the pooled liver (A) and lung (B) microsomes from individual rats ($n = 8$) in each group were measured by immunoblotting assay. The protein band was quantified by densitometry, and the level of control was set at 1.

pentoxyresorufin *O*-depentylase (CYP2B). These results indicate that ABE treatment may reduce the metabolism of xenobiotics catalyzed by CYP1A1, 1A2, 2C, 2D, 2E1, and 3A in the liver. No differences in the hepatic activities of GST or UGT were observed after ABE treatment.

In the lungs, rats fed the ABE diet showed a dose-dependent decrease in ethoxyresorufin *O*-deethylase (CYP1A1) activity ($p < 0.05$) but had no effect on methoxyresorufin *O*-demethylase (CYP1A2) or pentoxyresorufin *O*-depentylase (CYP2B) activities (Table 4). An increase of GST activity was noted in rats fed the 10% ABE diet ($p < 0.05$). ABE had no effect on UGT activity in the lungs.

Figure 1 shows immunoblots of liver and lung microsomal CYP proteins. Similar to the changes of CYP enzyme activities, ABE decreased CYP1A1, 1A2, 2C6, 2C11, 2D1, 2E1, 3A1, and 3A2 protein expressions in liver microsomes and CYP1A1 protein expression in lung microsomes.

GSH Contents, GSH-Related Enzyme Activities, and Lipid Peroxidation. Rats fed the ABE diet for 4 weeks did not change GSH, GSSG, and TBARS contents, the GSH/GSSG ratio, or GSH-Px and GSH-Rd activities in the liver (Table 5). These results indicated that ABE had no effect on the oxidative stress status in the liver after feeding a diet containing up to 10% ABE for 4 weeks. In contrast, in the lungs, higher reduced GSH content, GSH/GSSG ratio, and GSH-Px and GSH-Rd activities were noted in rats fed the 10% ABE diet ($p < 0.05$). These findings indicate that ABE displays potent antioxidant activity in a tissue-specific manner.

Concentrations of Phenolic Compounds in Plasma and Tissues. At 1 h after the oral administration of a single dose (2 g/kg bw) of ABE to three rats, 4-hydroxyphenolic acid could be detected in the plasma, but the concentration was below the limit of quantitation (<0.5 μM). Other selected phenolic compounds in ABE were not detectable in the plasma.

Table 5. Effect of the Ethanolic Extract of Adlay Bran (ABE) on Reduced Glutathione (GSH), Oxidized Glutathione (GSSG), and GSH-Related Enzyme Activities and Lipid Peroxidation in the Liver and Lungs[a]

	liver			lung		
	control	5% ABE	10% ABE	control	5% ABE	10% ABE
GSH (nmol/mg protein)	22.6 ± 7.2	26.8 ± 5.1	24.9 ± 7.2	6.1 ± 3.3b	8.7 ± 4.1b	10.9 ± 4.1a
GSSG (nmol/mg protein)	1.2 ± 0.3	1.5 ± 0.4	1.1 ± 0.4	0.48 ± 0.14	0.42 ± 0.11	0.56 ± 0.12
GSH/GSSG	18.3 ± 7.9	18.6 ± 4.2	26.2 ± 10.8	12.2 ± 5.0b	20.6 ± 7.0a	19.5 ± 7.7a
glutathione peroxidase (nmol/min/mg protein)	234.6 ± 27.8	217.2 ± 46.1	223.6 ± 35.8	95.5 ± 10.0b	87.8 ± 10.2b	121.9 ± 18.0a
glutathione reductase (nmol/min/mg protein)	84.0 ± 13.9	79.5 ± 15.9	72.8 ± 16.8	47.5 ± 15.8b	36.6 ± 5.9b	65.6 ± 13.7a
TBARS (nmol/g protein)	155.9 ± 35.8	147.3 ± 37.6	180.3 ± 45.6	104.2 ± 9.5	111.6 ± 15.1	109.4 ± 17.0

[a] Values are the mean ± SD, $n = 8$. Rats were fed one of following diets for 4 weeks: control diet, 5% ethanolic adlay extract (5% ABE) diet, or 10% ABE diet. Values in the same row with different letters differ significantly ($p < 0.05$).

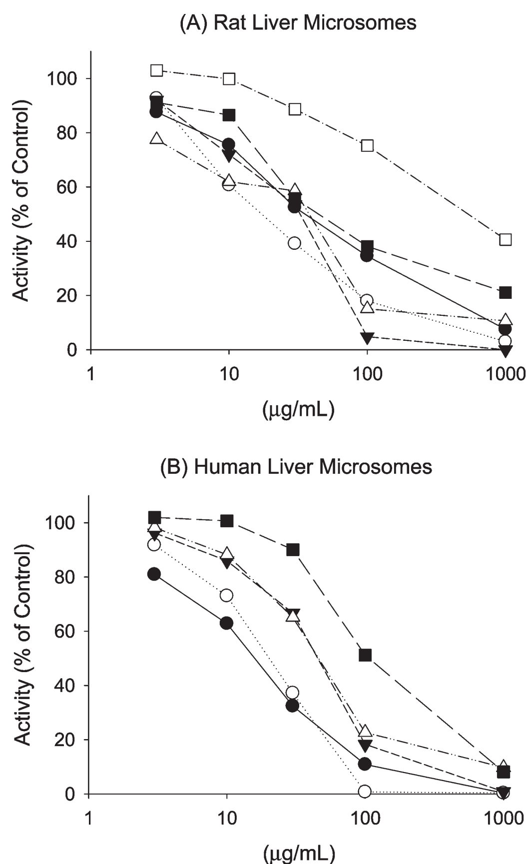


Figure 2. Effect of the ethanolic extract of adlay bran (ABE) on CYP-catalyzed reactions in rat and human liver microsomes. The enzyme reactions evaluated in rat liver microsomes (A) were diclofenac 4-hydroxylation (●), testosterone 6β-hydroxylation (○), dextromethorphan *O*-demethylation (▼), *p*-nitrophenol hydroxylation (□), ethoxyresorufin *O*-deethylation (■), and methoxyresorufin *O*-demethylation (□). The enzyme reactions evaluated in human liver microsomes (B) were diclofenac 4-hydroxylation (●), testosterone 6β-hydroxylation (○), dextromethorphan *O*-demethylation (▼), *p*-nitrophenol hydroxylation (□), and methoxyresorufin *O*-demethylation (■). Concentrations of the ABE in the incubations were 0−1000 μg/mL. The ABE was incubated under the conditions described under Materials and Methods. Each datum point represents the mean of duplicate determinations.

No phenolic compounds in ABE were observed in the liver or lungs.

Inhibitory Effects of ABE on CYP Enzyme Activity in Rat and Human Liver Microsomes. In an in vitro experiment, ABE inhibition on the activities of CYP enzymes in rat (Figure 2A) and human (Figure 2B) liver microsomes was determined. ABE inhibited ethoxyresorufin *O*-deethyllation (CYP1A1), diclofenac 4-hydroxylation (CYP2C), dextromethorphan *O*-demethylation (CYP2D), *p*-nitrophenol hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A) activities in rat liver microsomes. The IC_{50} values for each CYP enzyme ranged from 18.0 to 57.4 μg/mL (Table 6). In human liver microsomes, ABE was also found to inhibit CYP2C, 2D, 2E1, and 3A activities with IC_{50} values ranging from 15.4 to 44.8 μg/mL. The IC_{50} values for the CYP1A2 catalyzed MROD activity in rat and human liver microsomes were much higher than those of other CYP-isozyme catalyzed metabolic reactions, being 494 and 104 μg/mL, respectively.

To demonstrate whether the ABE inhibition on CYP enzyme activity was involved in a mechanism-based manner, 10 μg/mL of ABE was preincubated with rat and human liver microsomes in the absence and presence of NADPH at 37 °C for 15 min before the addition of the probe substrate for each CYP isozyme to initiate the metabolic reactions. The inhibition potency of ABE was not altered by the preincubation. The results indicated that ABE contained little or no mechanism-based inhibitors toward CYP enzymes. Moreover, the lack of effect of ABE (up to 1 mg/mL) on the microsomal CO binding spectra indicated that the ABE inhibition on CYP-catalyzed oxidation might not result from destruction of the heme moiety of CYP enzymes (data not shown).

CYP-catalyzed oxidation reactions require the transfer of electrons from NADPH to CYP by NADPH-CYP reductase. To determine whether inhibition of CYP enzyme activities by ABE was attributed to inhibition of NADPH-CYP reductase, ABE (0−1000 μg/mL) was incubated with rat and human liver microsomes using cytochrome *c* as the electron receptor. The results showed that ABE did not inhibit the NADPH-CYP reductase activity (data not shown).

DISCUSSION

In this study, we demonstrated that administration of a diet containing 5 or 10% of ABE (w/w) to rats for 4 weeks reduced CYP1A1, 1A2, 2C, 2D, and 3A activities in the liver and CYP1A1 activity in the lungs. Moreover, higher GSH content and GSH-related enzyme activities were noted in the lungs, but not in the liver, of rats fed the 10% ABE diet. These results suggest that, in rats, ABE may suppress CYP-mediated metabolism of drugs and

Table 6. Effects of the Ethanolic Extract of Adlay Bran (ABE) on CYP-Catalyzed Reactions in Rat and Human Liver Microsomes[a]

metabolic reaction (CYP enzyme)	IC_{50} (μg/mL)
rat liver microsomes	
ethoxyresorufin *O*-deethyllation (CYP1A1)	57.4
methoxyresorufin *O*-demethylation (CYP1A2)	494
diclofenac 4-hydroxylation (CYP2C)	38.9
dextromethorphan *O*-demethylation (CYP2D)	27.7
p-nitrophenol hydroxylation (CYP2E1)	24.9
testosterone 6β-hydroxylation (CYP3A)	18.0
human liver microsomes[b]	
methoxyresorufin *O*-demethylation (CYP1A2)	104
diclofenac 4-hydroxylation (CYP2C)	15.4
dextromethorphan *O*-demethylation (CYP2D)	44.5
p-nitrophenol hydroxylation (CYP2E1)	44.8
testosterone 6β-hydroxylation (CYP3A)	20.4

[a] Activities were determined in the absence or presence of the ABE (0−1000 μg/mL). The ABE was incubated under conditions described under Materials and Methods. [b] The effect on CYP1A1 activity in human liver microsomes was not determined due to low expression in that tissue.

chemical carcinogens in the liver and lungs and that the increased antioxidant potential after ABE treatment was tissue-specific.

The CYP1A family comprises the most active enzymes in the bioactivation of chemical carcinogens. In humans, CYP1A1 is poorly expressed in the liver, but it can be markedly induced by some chemicals in many extrahepatic tissues such as the lungs.[30] CYP1A1 is involved in the biotransformation of polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene, to carcinogenic metabolites.[31] Increased lung CYP1A1 gene expression and activity are associated with a high risk of lung cancer,[32] suggesting that CYP1A1-mediated chemical activation may result in pulmonary carcinogenesis. In contrast to CYP1A1, CYP1A2 is principally expressed in the liver.[30] CYP1A2 mainly metabolizes certain drugs and also activates a tobacco procarcinogen to a carcinogen (i.e., 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; NNK)[33] and foodborne toxicants aflatoxin B_1[34] and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline.[35] In this study, significantly lower CYP1A1 (−55.9%) and CYP1A2 (−39.1%) activities in the liver and CYP1A1 (−83.3%) activity in the lungs were noted in rats fed the 10% ABE diet. Because of their critical roles in activating chemical carcinogens, it is likely that ABE may be potent on anticarcinogenesis by inhibiting CYP1A1 and CYP1A2 activities. CYP3A, CYP2D, and CYP2C are three most important phase I DMEs responsible for the respective metabolism of 50, 25, and 20% of clinical drugs.[1] In this study, in addition to CYP1A1 and 1A2, ABE also effectively depressed hepatic activities of CYP3A, CYP2D, and CYP2C. This is likely to result in decreasing the metabolism of most therapeutic drugs catalyzed in the liver and, thus, enhancing the therapeutic efficacy in clinics. Consistent with the changes in CYP activity, immunoblots revealed that rats fed the ABE diets had lower protein expressions of CYP1A1, 1A2, 2C6, 2C11, 2D1, 2E1, 3A1, and 3A2 in the liver and CYP1A1 in the lungs. Taken together, our results suggest that ABE treatment may down-regulate CYP protein expression in the liver and lungs, which may, in turn, reduce the enzyme activity, thus resulting in a lower metabolism of drugs or chemical carcinogens catalyzed by CYP enzymes.

Phytochemicals are derived from plants and are present in dietary supplements and herbal remedies, which may have antioxidant, anti-inflammatory, and cancer-preventive activities. The phenolic compounds were suggested to partially contribute to the antioxidant and anticancer activities of adlay.[10,13,36] Moreover, several flavonoids were shown to reduce the biotransformation of drugs or chemicals by direct inhibition of CYP enzyme activity.[7] In our in vitro study, results demonstrated that ABE was able to inhibit CYP1A1, 2C, 2E1, 2D, and 3A enzyme activities in both rat and human liver microsomes with IC_{50} values ranging 15.4 to 57.4 μg/mL (Figure 2; Table 6). Fifteen phenolic compounds were identified in ABE, and it is interesting to find out which compound(s) contributed to this inhibition. In the case of incubation with 1 mg/mL of ABE, except for 4-hydroxybezonic acid, which had a concentration of 1.2 μM, the final concentrations of other phenolic compounds in the incubation mixture were all <1 μM. Although quercetin, tangeritin, rutin, narigenin, and furic acid have been shown to act as inhibitors of CYP enzymes,[8,37−39] they showed little or no inhibition on CYP enzyme activities at the estimated concentrations in ABE (data not shown). With regard to the richest 4-hydroxybezonic acid, only a mild inhibition on CYP1A activity (−18%) in rat liver microsomes was noted at a concentration of 10 μM, and it had no effect on the activity of other CYP. Similarly, three phytosterols at estimated concentrations in ABE did not change CYP enzyme activities (data not shown). Because multiple phenolic compounds coexisted in plant extracts, the combined action of all phenolics is responsible for their various biological activities such as anticancer.[40] Taken together, these findings indicated that the direct inhibition of ABE on CYP activity is more likely attributed to the additive or synergistic effect of all phenolic compounds rather than to an individual compound.

In the present study, the selected phenolic compounds in ABE were low or not detectable in the plasma and tissues after a single dose of ABE administration. The lower plasma concentrations of phenolic compounds may have been due to their poor bioavailability, which in turn is either due to poor absorption or due to extensive first-pass metabolism.[7] Therefore, direct inhibition of CYP in the liver or lungs by the individual phenolic compounds after ABE feeding may be insignificant. However, the modulation of CYP enzymes, either by direct inhibition in short-term exposure or by suppression of their protein expressions after long-term administration, by the phenolic metabolites of ABE cannot be excluded.[41,42] Nevertheless, due to local high concentrations of CYP inhibitors in the intestines after ABE administration, the CYP-mediated activation of chemical carcinogens in the colon might be suppressed. A recent study reported that rats fed the ethyl acetate fraction of ABE showed reduced DMH-induced colon carcinogenesis.[13] DMH can be activated by CYP enzymes to produce *O*(6)-methylguanine in the colorectal mucosa and liver, and reduced CYP activities, especially of CYP2E1 and CYP1A, which are known to be responsible for activation of DMH in the livers, may have chemopreventive effects against colon carcinogenesis in the initiation stage.[17,18] Therefore, the ability of ABE to suppress CYP activities observed in our in vitro and in vivo results may support the hypothesis that ABE may act as a chemopreventive agent to block CYP-mediated DMH activation, thus reducing colon carcinogenesis.

In addition to phenolic compounds, the possible physiological role of three phytosterols rich in ABE, that is, β-sitosterol, campesterol, and stigmasterol (Table 1), cannot be ruled out. Although absorption of plant sterols into the circulation is low,[43]

our recent study indicated that rats fed a diet containing phytosterols (25 mg/day, 75% β-sitosterol, and 10% campsterol) for 4 weeks lowered the hepatic CYP3A, 2C, and 2D activities (unpublished data). In this study, the amount of the daily intake of phytosterols in the 10% ABE diet group was about 7.5 mg/day, which was approximately 30% of the dose tested recently. To date, the exact mechanism of phytosterols on modulating CYP enzyme activities after long-term feeding is currently unknown; however, our results raise the possibility that the lower CYP enzyme activities in the liver after ABE treatment can be partly attributed to the phytosterols.

GSH, a vital protective antioxidant present in the lungs, is involved in protecting lungs against oxidant stress, inflammation, and injury.[44,45] In the present study, rats fed a diet containing 10% ABE for 4 weeks showed significantly increased GSH levels, GSH/GSSG ratio, and GSH-related enzyme activities including GST in the lungs. In contrast, such an increase of enzymatic and nonenzymatic antioxidant systems by ABE treatment was not noted in the liver, suggesting that ABE targeting appears to be tissue-specific. The increased GSH level and GSH/GSSG ratio in the lungs after ABE treatment display a higher antioxidant potential and protect the lungs against oxidative stress-induced injury.[45] Moreover, GST is a phase II detoxifying enzyme that can eliminate toxic metabolites by conjugation with GSH. Induction of GST-mediated detoxification is an important mechanism in the prevention of lung carcinogenesis, especially in subjects who smoke >20 cigarettes per day.[46] Therefore, in addition to the inhibition of CYP1A1 activity and its resultant carcinogen bioactivation as stated above, ABE enhancement on the antioxidant defense system and phase II detoxification apparently gives the lung tissues better capability against chemical insult.

In summary, the present study demonstrates that ABE suppresses CYP1A1, 1A2, 2C, 2D, and 3A activities in the liver and CYP1A1 activity in the lungs of rats after 4 weeks of feeding. This change may have resulted in a decrease in the metabolism of therapeutic drugs and chemical carcinogens catalyzed by CYP enzymes in these tissues. Moreover, ABE increases the antioxidant potential in the lungs in a tissue-specific manner.

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ABBREVIATIONS USED

ABE, adlay bran ethanolic extract; CYP, cytochrome P-450; DMEs, drug-metabolizing enzymes; DMH, 1,2-dimethylhydrazine; GC, gas chromatography; GSH, reduced glutathione; GST, glutathione *S*-transferase; GSH-Px, glutathione peroxidase; GSH-Rd, glutathione reductase; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBARSs, thiobarbituric acid reactive substances; NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; PAHs, polycyclic aromatic hydrocarbons; UGT, UDP-glucuronosyltransferase.

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