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Phytochemical Profiles and Antioxidant Activity of Adlay Varieties

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ABSTRACT: Consumption of whole grains has been associated with reduced risk of developing major chronic diseases. These health benefits have been attributed in part to their unique phytochemicals. Little is known about the complete profiles of phytochemicals and antioxidant activities of different adlay varieties. The objectives of this study were to determine the phytochemicals profiles of the three adlay varieties, including both free and bound of total phenolics and total flavonoids, and to determine the total antioxidant activity of adlay. The free, bound, and total phenolic contents of adlay samples ranged from 31.23 to 45.19 mg of gallic acid equiv/100 g of sample, from 28.07 to 30.86 mg of gallic acid equiv/100 g of sample, and from 59.30 to 76.04 mg of gallic acid equiv/100 g of sample, respectively. On average, the bound phenolics contributed 45.3% of total phenolic content of the adlay varieties analyzed. The free, bound, and total flavonoid contents of adlay samples ranged from 6.21 to 18.24 mg of catechin equiv/100 g, from 18.68 to 35.27 mg of catechin equiv/100 g, and from 24.88 to 52.86 mg of catechin equiv/100 g, respectively. The average values of bound flavonoids contributed 71.1% of total flavonoids of the adlay varieties analyzed. The percentage contribution of flavonoid content to phenolic content of free, bound, and total ranged from 11.6 to 35.2%, from 50.5 to 66.8%, and from 24.6 to 50.5%. The free, bound, and total oxygen radical absorbance capacity (ORAC) values of adlay samples ranged from 231.9 to 316.6 mg of Trolox equiv/100 g, from 209.0 to 351.4 mg of Trolox equiv/100 g, and from 440.9 to 668.0 mg of Trolox equiv/100 g, respectively. The average ORAC values of bound phytochemicals contributed 48.1% of total antioxidant activity of the adlay varieties analyzed. The content of total polyphenol and the antioxidant capacity are obviously different among different species. Liaoning 5 adlay and Longyi 1 adlay are significantly better than Guizhou heigu adlay. The adlay extracts have obvious proliferate inhibition on human liver cancer cells, and substantially in the experimental concentration range, the adlay sample itself has no cytotoxicity. Knowing the phytochemical profiles and antioxidant activity of adlay gives insights to its potential application to promote health.

KEYWORDS: *phytochemicals, phenolics, flavonoids, adlay, antioxidant activity*

INTRODUCTION

Epidemiological studies have shown that increased consumption of whole grains and their products has been consistently associated with reduced risk of developing chronic diseases, such as cardiovascular disease,[1,2] type II diabetes,[3] obesity,[4] and some cancera.[5] Because of the increased evidence indicating the health benefits of whole grain, the recommendation for whole grain consumption has been changed from 1995 to 2010.[6] The 2010 Dietary Guidelines for Americans recommend that all Americans make half or more of their grains as whole grains in their plates. This means eating three to five servings or more of whole grains every day.[7] The recognition of the potential health benefits of whole grain consumption is an important step in the direction to improve the health of the American public. In the meantime, industry has responded to the increased recommendation for whole grain consumption, and more and more whole grain products have been introduced into the market in recent years.

A whole grain consists of the intact, ground, cracked, or flaked caryopsis, whose principal anatomical components—the starchy endosperm, germ, and bran—are present in the same relative proportions as they exist in the intact caryopsis.[8] A whole grain product can be defined as a product that has the original components of a whole grain recombined to the relative proportion naturally occurring in the grain kernel.[9] There are many whole grains such as wheat, corn, barley, rice, oats, adlay, millet, spelt, and rye. Those grains, both whole and refined, are commonly consumed on a daily basis in a number of products around the world.[10] Wheat has become the prominent grain based on consumption. Rice is rarely eaten as a whole grain because generally we eat the endosperm fraction, polished rice, without the bran and germ fractions. Corn is another commonly eaten grain that recently has gained more attention because of its antioxidant content. Oats are almost always eaten whole because their bran and germ fractions are rarely removed.[10]

Whole grain phytochemicals are proposed to be responsible for the health benefits of whole grains.[11] Phytochemicals are the bioactive non-nutrient plant compounds found in fruits, vegetables, whole grain, and other plant foods and are classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organic sulfur compounds.[12] Whole grain phytochemicals have potent antioxidant activity and are able to scavenge free radicals that may increase oxidative stress and potentially damage large biological molecules, such as lipids, protein, and DNA.[11,13] Previous studies reported the phytochemical content and antioxidant activity of whole

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grain.[14−16] However, these studies reported only free phytochemicals and did not include bound phytochemicals and the contributions of bound fractions to the total phenolics and total antioxidant activity as Adom and Liu pointed out previously.[11] Adom and Liu developed a methodology to determine the complete phenolic profiles of whole grains.[17] This method identifies and quantifies the free, soluble-conjugated, and insoluble-bound forms of phenolics, as well as their antioxidant activity in corn, wheat, oats, and rice. This research indicated that the major portion of the phytochemicals in the grains is present in the bound form (85% in corn, 76% in wheat, and 75% in oats). These results suggest that the total phytochemical contents of grains have been commonly underestimated because traditional methodologies do not include bound phenolics.[17]

Adlay (*Coix lacrryma-jobi* L. var. ma-yuan Stapf), or Job's tears, is native in India, Burma, China, and Malaysia and had been grown extensively in South Asia before corn became popular as a major agricultural crop.[18] Over the years, adlay has been used as a food source for humans and livestock. The adlay seed consists of four parts including the hull, testa, bran, and endosperm.[19] It has long been used in Traditional Chinese Medicine to treat warts, chapped skin, rheumatism, and neuralgia and as an anti-inflammatory or antihelmintic agent.

Few studies reported bound phytochemicals of adlay and their contribution to the total antioxidant activity previously. Obviously, their phenolic content and antioxidant activity were underestimated without inclusion of the bound phytochemicals as described previously.[11,17] Many recent papers have indicated that the consumption of adlay is beneficial. Kuo et al. reported that the methanol extract of adlay hull had antiproliferative activity against human histolytic lymphoma U937 monocytic cell,[20] Chang et al reported that adaly extract exhibited antiproliferative activity against A549 lung cancer cell by inducing cell cycle arrest and apoptosis.[21] Shih et al. showed that dehulled adlay suppressed early events in colon carcinogenesis and reduced COX-2 protein expression.[29]

Compared to other whole grains, previous studies on adlay mainly focus on adlay bran extracts and their anticancer and anti-inflammation activities.[21−25] Limited studies reported phytochemical profiles and antioxidant activity of adlay.[26,27] However, these studies did not report the bound phytochemicals and their antioxidant activity. Therefore, more complete analyses of phytochemical profiles including bound phytochemcals and their antioxidant activity of diverse varieties of adlay are needed.

Chemical methods for the assessment of antioxidant activity include the oxygen radical absorbance capacity (ORAC)[28] and the peroxyl radical scavenging capacity (PSC) assays.[30] Currently, the chemical methods for the in vitro assessment of antioxidant capacity in cereal crops include the DPPH radical scavenging method,[10] the total oxygen radical scavenging method (TOAC),[13] and the ferric reducing antioxidant power (FRAP) assay, among others. Liu[31−33] reported that the cellular antioxidant activity (CAA) assay reflects the absorption, metabolism, and distribution of antioxidants at the cellular level. Compared with the chemical methods, CAA can assess the cellular antioxidant activity with better accuracy.

Few studies have been conducted on the cytotoxicity and antiproliferative effects of adlay extracts in cells. On the other hand, studies have mainly focused on the physiological functions of adlay extracts.[24,26] Chang et al.[21] reported that adlay, which has antitumor properties, can inhibit sarcoma-180 tumors in mice. Methanol extracts of adlay husks have been shown to have antiproliferative effects in U937 leukemia cells[20] and in A549 lung cancer cells.[21] Even though the total polyphenol content of adlay bran has been assessed, there is no evidence of the content ratio between the free and bound polyphenols.[26,34]

The objectives of this study were (1) to determine the phytochemical profiles of the three adlay varieties, including both free and bound total phenolics and total flavonoids, (2) to determine the total antioxidant activity of adlay, and (3) to analyze the cytotoxicity and antiproliferative effect on HepG2 human hepatoma cells of adlay extracts and to evaluate and analyze the functions of adlay extracts on a cellular level. We report here that adlay grains are rich in phytochemicals and have high antioxidant activity. Bound phenolics contributed 45% of total phenolics, and bound flavonoids accounted for 71% of total flavonoids in adlay grains. Bound phytochemicals may provide additional health benefits in the colon after fermentation by colon bacteria. The total polyphenol content and the antioxidant capacity are different among different species. For example, Liaoning 5 adlay and Longyi 1 adlay have higher total polyphenol content and antioxidant activity than Guizhou heigu adlay. Adlay extracts have antiproliferative properties in human liver cancer cells. In the concentration range used in this study, adlay extract had no cytotoxic effects.

MATERIALS AND METHODS

Chemicals and Reagents. Methanol (MeOH), ethanol (EtOH), hexanes, ethyl acetate, hydrochloric acid (HCl), acetic acid (HAC), potassium chloride (KCl), sodium acetate (NaAC), sodium carbonate ($NaCO_3$), sodium hydroxide (NaOH), potassium phosphate monobasic (KH_2PO_4), and potassium phosphate dibasic (K_2HPO_4) were of analytical grade and were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). 2′,7′-Dichlorofluorescin diaacetate (DCFH-DA), fluorescein disodium salt, sodium borohydride ($NaBH_4$, reagent grade), chloranil (analytical grade), vanillin (analytical grade), catechin hydrate, Folin−Ciocalteu reagent, ascorbic acid, cholera toxin, hydrocortisone, penicillin, streptomycin, and gentamicin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Tetrahydrofuran (THF, analytical grade) and aluminum chloride ($AlCl_3{\cdot}6H_2O$, analytical grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Folin−Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), trifluoroacetic acid (TFA, chromatographic grade), and acetonitrile (chromatographic grade) were purchased from Sigma. Gallic acid was purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemicals USA, and HepG2 human hepatoma cells were purchased from American Type Culture Collection (ATCC). WME medium, Hank's balanced salt solution (HBSS), epidermal growth factor, heparin, insulin, and other cell culture reagents were purchased from Gibco U.S. Biotechnology Co. Fetal bovine serum (FBS) was purchased from Atlanta Biotech Co. Inc. (Richmond, VA, USA).

Adlay Samples and Samples Preparation. Descriptions of the three adlay varieties used in this study are given in Table 1. The samples of three adlay varieties were obtained from the collection of

Table 1. Descriptions of the Three Adlay Varieties Used in This Study

variety	description	moisture content (%)	ref
Guizhou Heigu	hard hull, black	9.68	Zhiming et al.[49]
Liaoning 5	soft hull, black	10.84	Hui et al.[50]
Longyi 1	hard hull, brown	11.65	Li et al.[51]

Jiangsu Key Laboratory of Quality Control and Processing for Cereals and Oils at Nanjing University of Finance and Economics, China. All three varieties of adlay used in this study were commercially available. All grain samples of the three adlay varieties were dehusked on a Satake Rice Machine (Satake Co., Japan) and milled into flour by passing through a 60-mesh sieve on a Cyclone sample mill (UDY Corp., Fort Collins, CO, USA). All adlay flour samples were stored at −20 °C until analysis within 1 month. The moisture content of all samples was determined using an oven-drying method at 105 °C for 16 h (Table 1).

Extraction of Free Phenolic Compounds. Free phenolic compounds of adlay samples were extracted using the modified method reported previously from our laboratory.[17,35,36] Briefly, 4 g of adlay flour was blended with 30 mL of 80% chilled acetone. The mixture was then centrifuged at 2500*g* for 10 min. The supernatant was removed, and the remaining pellet was extracted again with 30 mL of 80% chilled acetone and repeated twice. The supernatants were pooled and evaporated using a rotary evaporator at 45 °C to dryness. The solution was then reconstituted in 10 mL of 70% methanol. The extracts were stored at −40 °C until analysis. All extractions were performed at least five times for each sample.

Extraction of Bound Phenolic Compounds. Bound phenolics of adlay samples were extracted using a modification of the method previously reported by our laboratory.[11,17,28,36] Briefly, bound phenolics were extracted from the residue from the free phenolic extraction. The residue was first digested with 20 mL of 2 M sodium hydroxide at room temperature for 1 h while shaking under nitrogen. The mixture was then neutralized with concentrated hydrochloric acid. Hexanes were used to extract lipids in the mixture. The remaining mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated using a rotary evaporator at 45 °C to dryness. The bound phenolics were then reconstituted to 10 mL of 70% methanol. The extracts were stored at −40 °C until analysis. Each sample was extracted at least five times.

Determination of Total Phenolic Content. The total phenolic content of adlay samples was determined using the Folin−Ciocalteu colorimetric method described by Singleton et al.[37] and modified in our laboratory.[35,38] Briefly, all extracts were diluted 1:20 with distilled water to obtain readings within the standard curve ranges of 0.0−600.0 μg of gallic acid/mL. The extracts were reacted with Folin−Ciocalteu reagent and then neutralized with sodium carbonate. After 90 min, the absorbance of the resulting solution was measured at 760 nm at room temperature by an MRX II Dynex plate reader (Dynex Technologies Inc., Chantilly, VA, USA). Gallic acid was used as the standard, and total phenolic content was expressed as milligrams of gallic acid equivalents per 100 g of dry weight (DW) of sample.

Determination of Total Flavonoid Content. The total flavonoid content of each adlay sample was determined using the sodium borohydride/chloranil-based assay developed by our laboratory.[39] Briefly, 1 mL extracts of tested samples were added into test tubes (15 × 150 mm), dried to dryness under nitrogen gas, and reconstituted in 0.2 mL of terahydrofuran/ethanol (THF/EtOH, 1:1, v/v). Catechin standards (0.1−10.0 mM) were prepared fresh before use in 0.2 mL of THF/EtOH (1:1, v/v). Then 0.1 mL of 50 mM $NaBH_4$ solution and 0.1 mL of 74.6 mM $AlCl_3$ solution were added into each test tube with 1 mL of sample solution or 1 mL of catechin standard solution. Then the test tubes were shaken in an orbital shaker at room temperature for 30 min. An additional 0.1 mL of 50.0 mM $NaBH_4$ solution was added into each test tube with shaking continued for another 30 min at room temperature. Then, 0.4 mL of cold 0.8 M acetic acid solution was added into each test tube, and the solutions were kept in the dark for 15 min after a thorough mix. Then 0.2 mL of 20.0 mM chloranil was added into each tube, which was heated at 95 °C with shaking for 60 min. The reaction solutions were cooled using tap water, and the final volume was brought to 1 mL using methanol. Then, 0.2 mL of 1052 mM vanillin was added into each tube and mixed. Then 0.4 mL of 12 M HCl was added to each tube, and the reaction solutions were kept in the dark for 15 min after a thorough mix. Aliquots of final reaction solutions (200 μL) were added into each well of a 96-well plate, and the absorbances were measured at 490 nm using an MRX microplate reader with Revelation workstation (Dynex Technologies, Inc.). Total flavonoid content was expressed as milligrams of catechin equivalents per 100 g of DW of sample. Data were reported as the mean ± standard deviation (SD) of at least five times for each sample.

Determination of Total Antioxidant Activity by ORAC Assay. The total antioxidant activity was determined using the ORAC assay described by Huang et al.[40] and modified in our laboratory.[13,32] Briefly, adlay flour extract dilutions were prepared with 75 mM phosphate buffer (pH 7.4). The assay was performed in black-walled 96-well plates (Corning Scientific, Corning, NY, USA). The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Each well contained 20 μL of extracts or 20 μL of Trolox standard (range 6.25−50 μM), and 200 μL of fluoroscein (final concentration of 0.96 μM), which were incubated at 37 °C for 20 min. After incubation, 20 μL of 119 mM ABAP was added to each well. Fluorescence intensity was measured using a Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA, USA) at excitation of 485 nm and emission of 520 nm for 35 cycles every 5 min. ORAC values were expressed as milligrams of Trolox equivalents per 100 g of DW of sample. Data were reported as the mean ± SD at least five times for each sample.

Quantification of Total Antioxidant Activity by PSC Assay. The total antioxidant activity was determined using the PSC assay.[30] Thermal degradation of ABAP (2,2′-azobisamidinopropane) produced peroxyl radicals ($ROO^{\bullet}$), which oxidized nonfluorescent dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF). The degree of inhibition of DCFH oxidation by antioxidants that scavenge peroxyl radicals was used as the basis for calculating antioxidant activity. Fluorescence was monitored at 485 nm excitation and 538 nm emission with a fluorescent spectrophotometer (Fluoroskan Ascent FL, Thermo Electron Corp., Asheville, NC, USA). The results were calculated as milligrams of vitamin C equivalents per 100 g of DW of sample. Data were reported as the mean ± SD of at least triplicates for each sample.

Cell Culture. HepG2 cells, grown in growth medium (i.e., WME supplemented with 5% FBS, 10 mM Hepes, 2 mM L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamycin), were maintained at 37 °C and 5% CO_2.[41,42] For the experiments, cells were used between passages 18 and 28.

CAA of Adlay Extracts. The CAA assay has been previously described.[31−33] Briefly, HepG2 cells were seeded at a density of 6×10^4 cells/well on a 96-well microplate in 100 μL of growth medium/well. Twenty-four hours postseeding, the growth medium was removed and the wells were washed with PBS. Triplicate wells were treated for 1 h with 100 μL of medium containing adlay extracts plus 25 μM DCFH-DA. Certain wells were washed with 100 μL of PBS (i.e., PBS wash protocol) and certain wells were not washed (i.e., no PBS wash protocol). PBS wash protocol means that cells are pretreated with adlay extract before the APAP is added; on the other hand, no PBS wash means that cells are cotreated with adlay extracts and APAP. ABAP (600 μM) in 100 μL of HBSS was added to the cells. The 96-well microplate was placed in a Fluoroskan Ascent FL plate reader (Thermo Labsystems) at 37 °C. The emission wavelength at 538 nm was measured after an excitation at 485 nm every 5 min for 1 h.

Quantification of CAA. After the subtraction of the blank and the initial fluorescence values, the area under the fluorescence versus time curve was calculated to determine the CAA value at each adlay extract concentration. The following equation was used:

$$\text{CAA (units)} = 1 - \left(\int \text{SA} / \int \text{CA}\right)$$

$\int$SA is the integrated area under the sample in the fluorescence versus time curve, and $\int$CA is the integrated area under the control in the fluorescence versus time curve. The median effective dose (EC_{50}) of the adlay extracts was calculated from the median effect plot of $\log(f_a/f_u)$ versus log(dose), where f_a is the fraction affected by the treatment (CAA unit) and f_u is the fraction unaffected (1 − CAA unist) by the treatment. The EC_{50} values were expressed as the mean ± SD using

triplicate data sets obtained from the same experiment. EC_{50} values were converted to CAA values, which were expressed as micromoles of quercetin equivalents (QE) per 100 g of grain, using the mean EC_{50} value for quercetin from three separate experiments.

Cytotoxicity Test. A cytotoxicity test was performed using the modified methylene blue assay.[43,44] Briefly, HepG2 cells were seeded at a density of 4×10^4 cells/well on a 96-well microplate in 100 μL of growth medium/well. The cells were incubated for 24 h at 37 °C. After the cells had attached to the wells, the growth medium was removed and the cells were washed with PBS. Then 100 μL of medium with different concentrations of adlay extract was added to each well; wells that received medium without adlay extract served as the control. After 24 h of incubation at 37 °C, the medium was removed and the wells were washed with PBS. The cells were then incubated for 1 h at 37 °C after the addition of 50 μL of methylene blue (98% HBSS, 0.67% glutaraldehyde, and 0.6% methylene blue) to each well. After the incubation, the staining solution was removed and the cells were washed six times in deionized water until the water was clear. Subsequently, 100 μL of elution buffer (49% PBS, 50% ethanol, and 1% acetic acid) was added to each well. The plates were placed on a table oscillator for 20 min, and absorbance was measured at 570 nm in a microplate reader. The different concentrations of adlay extract were compared to the control; if a certain concentration of adlay extract reduced cell viability by >10% compared to the control, then that concentration was considered to be cytotoxic.[44]

Cell Proliferation Inhibiting Test. The antiproliferative effects of adlay extracts were assessed in a modified HepG2 cell methylene blue colorimetric method.[43−45] Briefly, HepG2 cells were seeded at a density of 2.5×10^4 cells/well on a 96-well microplate. Only 100 μL of cell-free medium was added to the peripheral wells of the 96-well microplate. In the central wells of the 96-well microplate, 100 μL of the cell suspension was added. The 96-well microplate was incubated for 4 h at 37 °C. The medium was then removed, and 100 μL of fresh medium containing different concentrations of adlay extracts (10, 20, 40, 60, 80, and 100 mg/mL) was added. The wells receiving cell suspension without adlay extract served as the control. The plates were incubated for 96 h at 37 °C. Following the incubation, the staining solution was removed, and the 96-well microplates were washed six times in deionized water until the water was clear. Then 100 μL of elution buffer (49% PBS, 50% ethanol, and 1% acetic acid) was added to each well. The 96-well microplates were transferred to a table oscillator for 20 min. Absorbance was measured at 570 nm using a microplate reader. Each sample was measured at least three times. The antiproliferative effects were assessed by the EC_{50} values, which were expressed as milligrams of adlay extracts per milliliter.

Statistical Analyses. Data, which were expressed as the mean ± SD, included at least three replicates per sample. ANOVA and Tukey's test were performed using SPSS (Statistics for Social Science) version 17.0. All graphical representations were performed using Sigmaplot version 11.0 (SPSS, USA). Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Total Phenolic Content. The free, bound, and total phenolic contents of three adlay varieties are presented in Figure 1, expressed as milligrams of gallic acid equivalents per 100 g of sample on a DW basis. The free phenolic content was highest ($p < 0.05$) in Longyi 1 (45.19 ± 0.91 mg of gallic acid equiv/100 g of sample, DW), followed by those in Liaoning 5 (32.87 ± 0.71 mg of gallic acid equiv/100 g of grains, DW) and Guizhou Heigu (31.23 ± 2.13 mg of gallic acid equiv/100 g of sample, DW). The bound phenolic content was highest ($p < 0.05$) in Longyi 1 (30.86 ± 1.18 mg of gallic acid equiv/100 g of sample, DW), followed by those in Liaoning 5 (28.50 ± 0.90 mg of gallic acid equiv/100 g of sample, DW) and Guizhou Heigu (28.07 ± 0.44 mg of gallic acid equiv/100 g of sample, DW). The total phenolic content was highest ($p < 0.05$) in Longyi 1 (76.04 ± 1.99 mg of gallic acid equiv/100 g of sample, DW), followed by those in Liaoning 5 (61.38 ± 0.90 mg of gallic acid equiv/100 g of sample, DW) and Guizhou Heigu (59.30 ± 2.06 mg of gallic acid equiv/100 g of sample, DW). There were significant differences in total phenolic contents among the three varieties ($p < 0.05$).

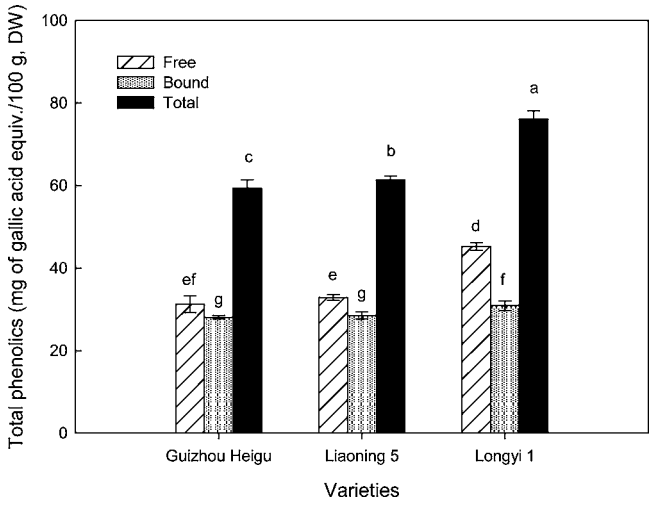

Figure 1. Free, bound, and total phenolic content of the three adlay varieties (mean ± SD, $n = 5$). Bars with no letters in common are significantly different ($p < 0.05$).

The total phenolic contents of the three adlay varieties in this study ranged from 59.30 (Guizhou Heigu) to 76.04 (Longyi 1) mg of gallic acid equiv/100 g of grains, DW (Figure 1). Choi et al. previously reported the total phenolic content of adlay was 43 mg of gallic acid equiv/100 g of sample,[46] which was significantly lower than those of adlay reported in the present study. The difference between the two studies is mainly due to the methodology used for grain phytochemical analyses as Adom and Liu pointed out previously.[17] The previous reports for whole grain phytochemicals did not include the determination of bound phytochemicals. Our group developed a methodology to determine the complete phenolic profiles of whole grains,[17] which included both free and bound phytochemicals. Whole grain phytochemicals will be significantly underestimated without determination of bound phytochemicals. Here we reported adlay grains contained 54.7% of free phenolics and 45.3% of bound phenolics (Table 2). Therefore, without accounting for bound phytochemicals, total phenolic content of adlay grains will be underestimated by 45%. In addition, bound phytochemicals are resistant to stomach and small intestine digestion and may reach the colon to release phytochemicals after the fermentation by colon bacteria.[11] Consumption of whole grains and their products has been associated with reduced risk of developing chronic diseases, such as cardiovascular disease, diabetics, obesity, and some cancers.[10] These health benefits have been attributed in part to the unique phytochemicals of whole grains.[11,17] Adlay and their products are commonly consumed in Asia. This study may partially be responsible for the health benefits of whole grain consumption lowering the risk of colon cancer.

Total Flavonoid Content. This study was the first to report total flavonoid content in adlays. Determination of total flavonoids in foods is challenging. Previously, the most common methods for total flavonoid analyses included aluminum chloride ($AlCl_3$) colorimetric assay,[47] as well as high-performance liquid chromatography (HPLC). However, all of these assays have limitations. The $AlCl_3$ colorimetric assay

Table 2. Average Phytochemical Content, ORAC Values, and PSC Values of Adlay (Mean ± SD)[a]

	free	bound	total
phenolics (mg of gallic acid equiv/100 g, DW)	36.43 ± 6.57b (54.72)	29.14 ± 1.51c (45.28)	66.57 ± 7.88a
flavonoids (mg of catechin equiv/100 g, DW)	12.01 ± 5.18b (28.92)	29.53 ± 8.30a (71.08)	41.53 ± 12.76a
ORAC values (mg of Trolox equiv/100 g, DW)	279.9 ± 41.2b (51.92)	259.2 ± 71.2c (48.08)	539.1 ± 101.3a
PSC values (mg of vitamin C equiv/100 g, DW)	58.51 ± 18.60b (56.36)	45.30 ± 3.29b (43.64)	103.8 ± 20.9a
CAA values (μmol QE/100 g, PBS)	2.62 ± 0.35b (45.00)	3.21 ± 0.75b (55.00)	5.83 ± 0.96a
CAA values (μmol QE/100 g, no PBS)	10.86 ± 0.50c (38.00)	18.16 ± 1.86b (62.00)	29.02 ± 2.21a

[a]Percent contribution to total is in parentheses. Values with no letters in common in each row are significantly different ($p < 0.05$).

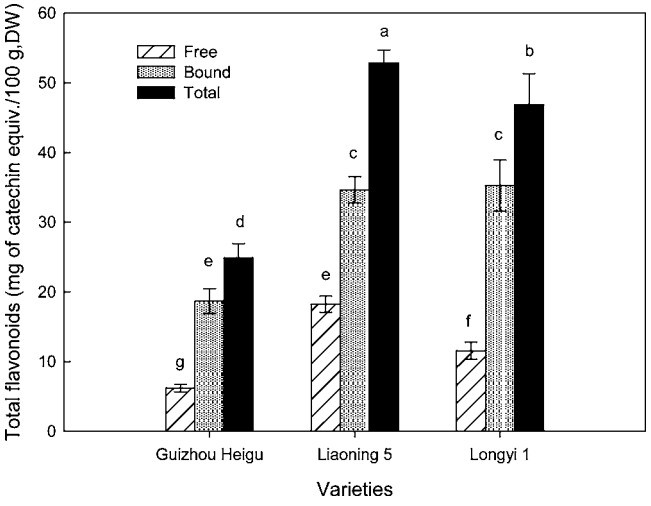

Figure 2. Free, bound, and total flavonoid content of the three adlay varieties (mean ± SD, $n = 5$). Bars with no letters in common are significantly different ($p < 0.05$).

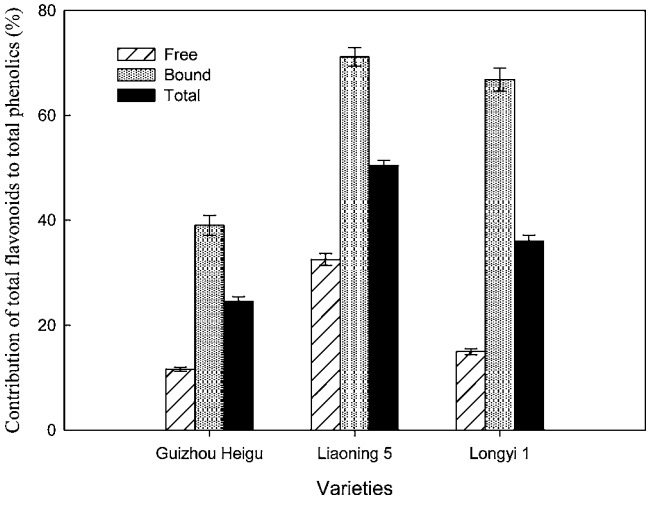

Figure 3. Percent contribution of total flavonoids to total phenolics (mean ± SD, $n = 5$).

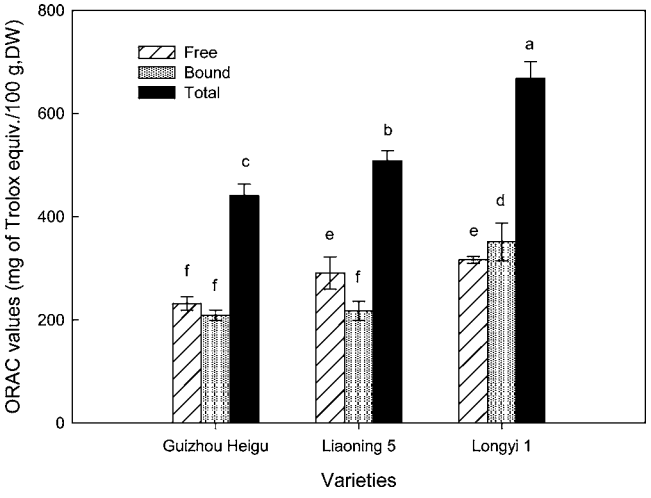

Figure 4. ORAC values of the three adlay varieties (mean ± SD, $n = 5$). Bars with no letters in common are significantly different ($p < 0.05$).

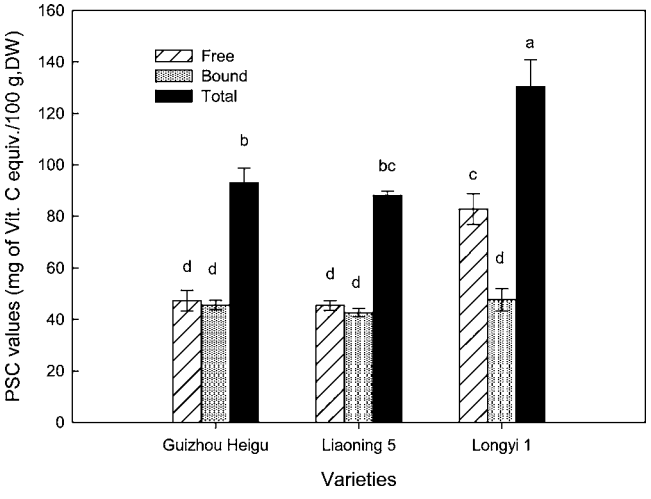

Figure 5. Total antioxidant activity of the three adlay varieties expressed as PSC values (mean ± SD, $n = 3$). Bars with no letters in common are significantly different ($p < 0.05$).

measures only partial flavonoids and cannot be used to determine total flavonoids.[26,48] The HPLC method is excellent to determine individual flavonoids, but cannot be used to determine the total flavonoids because it is limited by the flavonoid standards available and the many unidentified flavonoids present in foods.[48] The SBC assay developed recently can detect all subgroups of flavonoids, including flavones, flavonols, flavonones, flavononols, isoflavonoids, and anthocyanidines.[39]

The free, bound, and total flavonoid contents of three adlay varieties are presented in Figure 2, expressed as milligrams of catechin equivalents per 100 g of sample on a DW basis. The free flavonoid content was highest in Liaoning 5 (18.24 ± 0.55 mg of catechin equiv/100 g of sample, DW), followed by those in Longyi 1 (11.57 ± 1.21 mg of catechin equiv/100 g of sample, DW) and Guizhou Heigu (6.21 ± 0.55 mg of catechin equiv/100 g of sample, DW). The bound flavonoid content was highest in Longyi 1 (35.27 ± 3.66 mg of catechin equiv/100 g of sample, DW), followed by those in Liaoning 5 (34.63 ± 1.89

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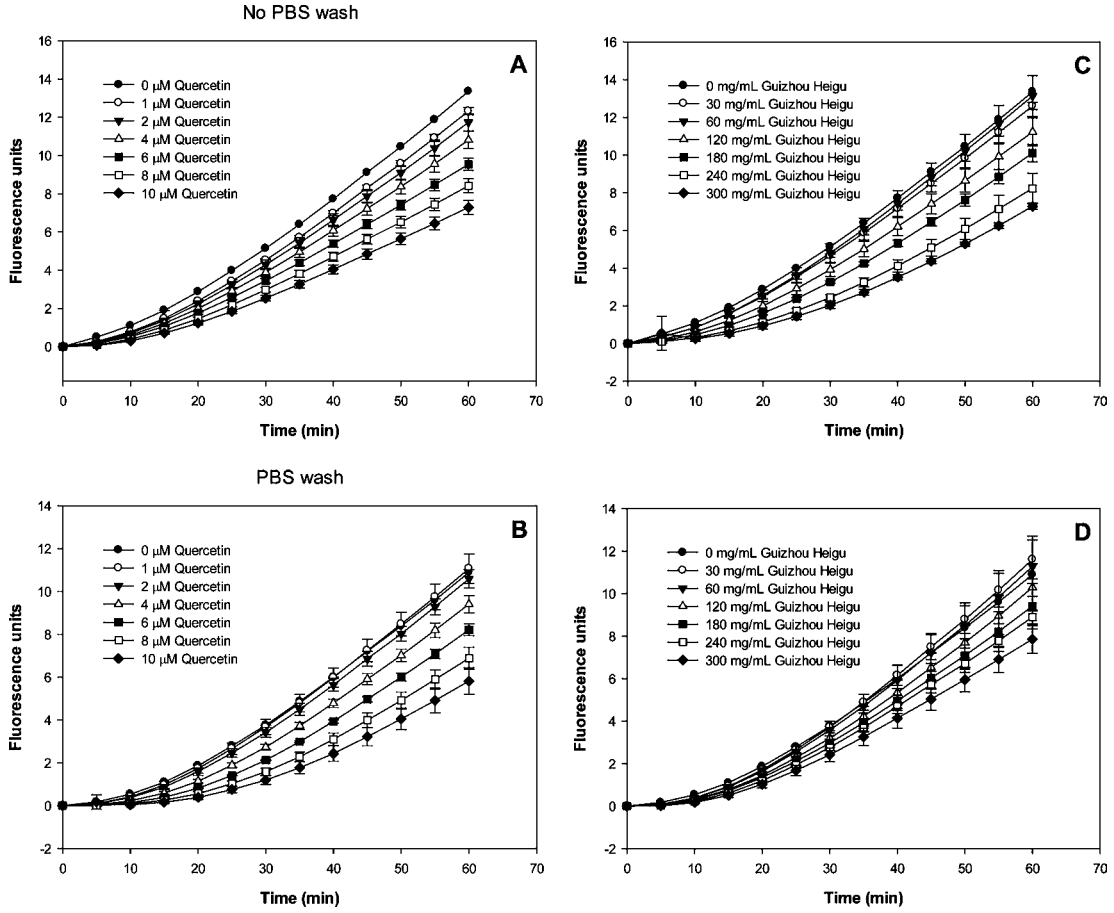

Figure 6. Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by quercetin (A, B) and adlay cultivar (C, D) over time. The curves shown in each graph are from a single experiment (mean ± SD, n = 3).

Table 3. Variation of EC_{50} Values of Three Adlay Cultivars in This Study

		PBS wash, EC_{50}(mg/mL)			no PBS wash, EC_{50}(mg/mL)		
variety		mean	SD	CV (%)	mean	SD	CV (%)
Guizhou Heigu	free	1328.79	51.97	3.91	357.39	9.86	2.76
	bound	990.46	43.24	4.37	150.06	3.52	2.35
Liaoning 5	free	554.35	24.89	4.49	243.53	13.14	5.40
	bound	646.26	37.87	5.86	425.45	23.56	5.54
Longyi 1	free	844.92	40.57	4.80	318.34	15.43	4.85
	bound	532.14	28.96	5.44	144.20	7.31	5.70

mg of catechin equiv/100 g of sample, DW) and Guizhou Heigu (18.68 ± 1.77 mg of catechin equiv/100 g of sample, DW). The total flavonoid content was highest in Liaoning 5 (52.86 ± 1.85 mg of catechin equiv/100 g of sample, DW), followed by those in Longyi 1 (46.85 ± 4.48 mg of catechin equiv/100 g of sample, DW) and Guizhou Heigu (24.88 ± 2.06 mg of catechin equiv/100 g of sample, DW). There were significant differences in total flavonoids among the three varieties ($p < 0.05$). The free flavonoid content of Liaoning 5 was significantly higher than those Guizhou Heigu and Longyi 1 ($p < 0.05$). In addition, the free flavonoid content of Longyi 1 was significantly higher than those of Guizhou Heigu ($p < 0.05$). There was no significant difference in bound flavonoid content between Liaoning 5 and Longyi 1, but their bound flavonoid contents were significantly higher than those in Guizhou Heigu ($p < 0.05$). These results are higher than these reported in previous studies using the $AlCl_3$ method,[26] which measured only partial flavonoids, not total. The $AlCl_3$ assay should not be used for total flavonoid analysis in the future.[39]

Contribution of Total Flavonoids to Total Phenolics. The contribution of flavonoids to phenolics was calculated on a micromole basis. The contributions of free flavonoids to free phenolics and of bound flavonoids to bound phenolics ranged from 11.6 to 32.5% and from 39.0 to 71.2%, respectively. The contribution of total flavonoids to total phenolics ranged from 24.6 to 50.5%, indicating flavonoids are one of the major phytochemicals in adlay (Table 2). The contributions of total flavonoid content to the total phenolic content are presented in Figure 3. The contribution of free flavonoids to free phenolics was highest in Liaoning 5 (32.5%), followed by Longyi 1 (15.0%) and Guizhou Heigu (11.6%). The contribution of bound flavonoids to bound phenolics was highest in Liaoning 5 (71.2%), followed by Longyi 1 (66.8%) and Guizhou Heigu (39.0%). The contribution of total flavonoids to total phenolics

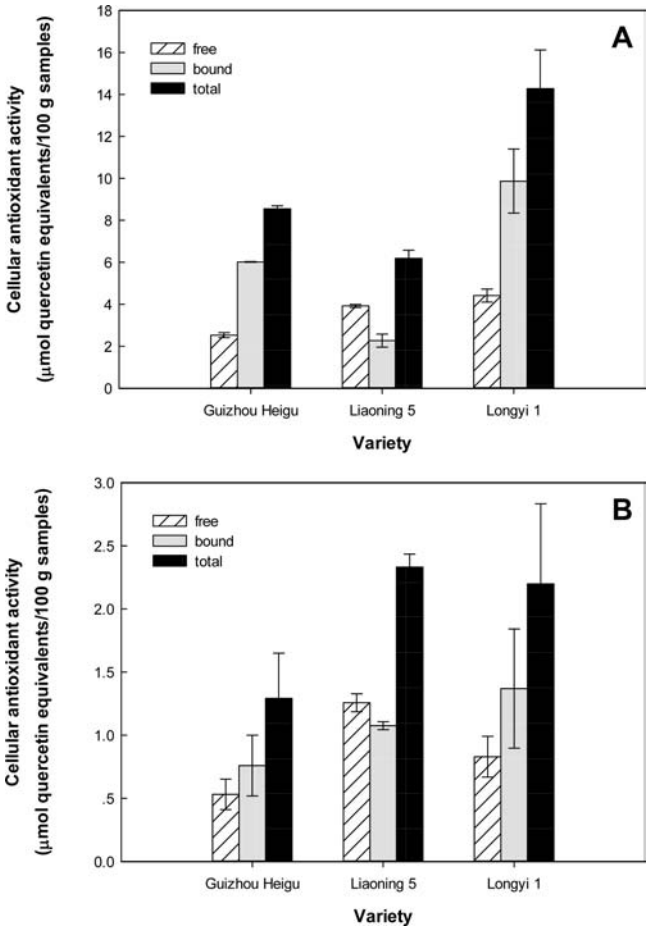

Figure 7. CAA quality of three adlay cultivars in the (A) no PBS wash protocol and (B) PBS wash protocol (mean ± SD, n = 3).

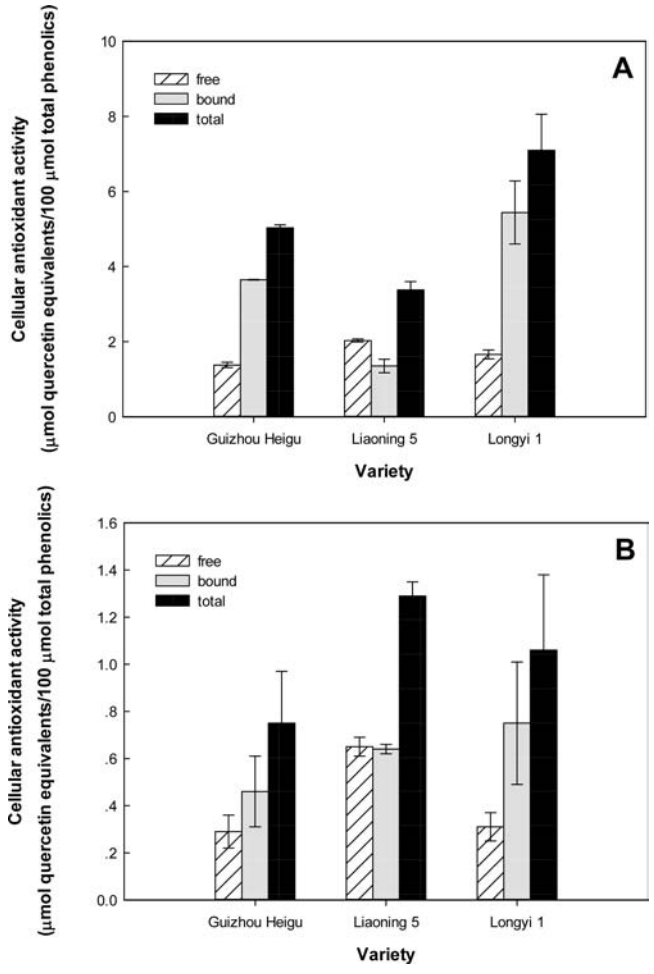

Figure 8. CAA quanlity of three adlay cultivars of total phenolics in the (A) no PBS wash protocol and (B) PBS wash protocol (mean ± SD, n = 3).

was highest in Liaoning 5 (50.5%), followed by Longyi 1 (36.0%) and Guizhou Heigu (24.6%).

Total Antioxidant Activity Measured by ORAC. The means of free, bound, and total ORAC values of the 3three adlay varieties were 279.9, 259.2, and 539.1 mg of Trolox equiv/100 g of sample, DW. On average, the contribution of bound ORAC values to the total ORAC values was 48.1% (Table 2).

Therefore, determination of total antioxidant activity of adlay should include both free and bound. Otherwise, the total antioxidant activity of adlay will be underestimated as suggested previously.[11] The free, bound, and total antioxidant activities of three adlay varieties are presented in Figure 4, expressed as ORAC values (mg of Trolox equiv/100 g of sample, DW). The

Table 4. Comparison of the Average EC_{50} Values and CAA Values among the Different Adlays

variety		CAA (μmol QE[a]/100 g DW)	CAA (μmol QE/100 μmol total flavonoids)	CAA (μmol QE/100 μmol total phenolics)
		PBS Wash		
Guizhou Heigu	free	0.53 ± 0.12b	2.48 ± 0.56a	0.29 ± 0.07c
	bound	0.76 ± 0.24ab	1.18 ± 0.37bcd	0.46 ± 0.15abc
Liaoning 5	free	1.26 ± 0.07a	2.01 ± 0.11abc	0.65 ± 0.04ab
	bound	1.08 ± 0.03ab	0.91 ± 0.03d	0.64 ± 0.02ab
Longyi 1	free	0.83 ± 0.16ab	2.08 ± 0.40ab	0.31 ± 0.06bc
	bound	1.37 ± 0.48a	1.13 ± 0.39cd	0.75 ± 0.26a
		No PBS Wash		
Guizhou Heigu	free	2.53 ± 0.12d	11.83 ± 0.56a	1.38 ± 0.07c
	bound	6.02 ± 0.02b	9.36 ± 0.03ab	3.65 ± 0.01b
Liaoning 5	free	3.92 ± 0.07cd	6.24 ± 0.11c	2.03 ± 0.04c
	bound	2.27 ± 0.31[d]	1.90 ± 0.26[d]	1.35 ± 0.18[c]
Longyi 1	free	4.41 ± 0.31bc	11.06 ± 0.78a	1.66 ± 0.12c
	bound	9.87 ± 1.53a	8.12 ± 1.26bc	5.44 ± 0.84a

[a]QE, quercetin equivalents.

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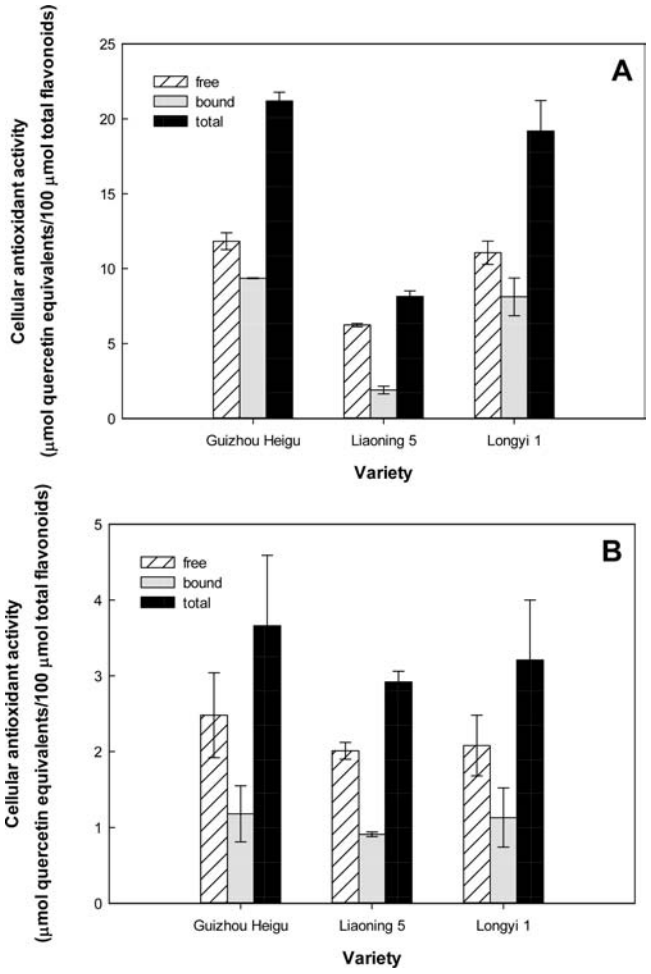

Figure 9. CAA quality of three adlay cultivars total flavonoids in the (A) no PBS wash protocol and (B) PBS wash protocol (mean ± SD, n = 3).

free ORAC values of Longyi 1 (316.6 ± 6.4 mg of Trolox equiv/100 g of sample, DW) and Liaoning 5 (291.1 ± 31.5 mg of Trolox equiv/100 g of sample, DW) were higher than those of Guizhou Heigu (231.9 ± 13.3 mg of Trolox equiv/100 g of sample, DW). The bound ORAC values were highest (p < 0.05) in Longyi 1 (351.4 ± 36.3 mg of Trolox equiv/100 g of sample, DW), followed by Liaoning 5 (217.3 ± 18.6 mg of Trolox equiv/100 g of sample, DW) and Guizhou Heigu (209.0 ± 10.1 mg of Trolox equiv/100 g of sample, DW). The total ORAC values were highest (p < 0.05) in Longyi 1 (668.0 ± 32.7 mg of Trolox equiv/100 g of sample, DW), followed by Liaoning 5 (508.4 ± 19.3 mg of Trolox equiv/100 g of sample, DW). Guizhou Heigu had the lowest ORAC values (440.9 ± 22.2 mg of Trolox equiv/100 g of sample, DW) among the three varieties tested. This order was consistent with those of total phenolics. There were significant differences in total ORAC values among all three varieties (p < 0.05). The free ORAC values of Longyi 1 and Liaoning 5 were significantly higher than that of Guizhou Heigu (p < 0.05), and the bound ORAC values of Longyi 1 were significantly higher than those of Liaoning 5 and Guizhou Heigu (p < 0.05).

Total Antioxidant Activity Measured by PSC Assay. The free, bound, and total antioxidant activities of three adlay varieties measured by PSC assay are presented in Figure 5, expressed as PSC values (mg of vitamin C equiv/100 g of sample, DW). The free PSC values were highest in Longyi 1 (82.77 ± 6.05 mg of vitamin C equiv/100 g of sample, DW), followed by Guizhou Heigu (47.35 ± 4.01 mg of vitamin C equiv/100 g of sample, DW) and Liaoning 5 (45.42 ± 1.93 mg of vitamin C equiv/100 g of sample, DW). The bound PSC values were highest in Longyi 1 (47.64 ± 4.32 mg of vitamin C equiv/100 g of sample, DW), followed by Guizhou Heigu (45.60 ± 1.86 mg of vitamin C equiv/100 g of sample, DW) and Liaoning 5 (42.67 ± 1.55 mg of vitamin C equiv/100 g of sample, DW). The total PSC values were highest in Longyi 1 (130.4 ± 10.4 mg of vitamin C equiv/100 g of sample, DW), followed by Guizhou Heigu (92.95 ± 5.86 mg of vitamin C equiv/100 g of sample, DW) and Liaoning 5 (88.09 ± 1.65 mg of vitamin C equiv/100 g of sample, DW). In the total PSC values, Longyi 1 had the highest peroxyl radical scavenging capacity (p < 0.05), whereas Guizhou Heigu and Liaoning 5 had lower peroxyl radical scavenging capacities among the three adlay varieties tested. This order was consistent with those of total phenolics and total ORAC values. The free PSC values of Longyi 1 were significantly higher than those of Guizhou Heigu and Liaoning 5 (p < 0.05), and the bound PSC values of the three adlay varieties were not significantly different.

The mean of free, bound, and total PSC values of the three adlay varieties were 58.51, 45.30, and 103.8 mg of vitamin C equiv/100 g of sample, DW, respectively. The contribution of bound PSC values to the total PSC values was 43.64%, indicating the antioxidant activity of bound fraction should be included for the total antioxidant activity as described above.

EC_{50} Values of Adlay Extracts by CAA. The kinetics of DCFH oxidation by peroxyl radicals generated from ABAP are shown in Figure 6. The increase in fluorescence from the formation of DCF was inhibited by both quercetin and adlay extracts in a dose-dependent manner (Figure 6A,B for quercetin and Figure 6C,D for adlay extracts). DCFH oxidation was inhibited regardless of whether the cells had been washed with PBS (Figure 6B,D) or not (Figure 6A,C) between the antioxidant and the ABAP treatments.

Compared to the PBS wash protocol, the no PBS wash protocol had significantly lower EC_{50} values and higher antioxidant capacity. This result is attributed to the PBS, which can affect the extracellular antioxidant capacity, thereby reducing the intracellular antioxidant capacity.

Table 3 shows the EC_{50} values of the three adlay extracts. The EC_{50} values ranged from 532.14 to 1328.79 mg/mL in the PBS wash protocol and from 144.20 to 425.45 mg/mL in the no PBS wash protocol. In both methods, the bound Longyi 1 adlay had the lowest EC_{50} values (532.14 ± 28.96 in the PBS wash protocol and 144.20 ± 7.31 mg/mL in the no PBS wash protocol). In addition, among the three adlay extracts, EC_{50} values of bound Liaoning 5 extracts were higher than those of free Liaoning 5 extracts regardless of the protocol. However, the opposite was observed in the case of the Guizhou Heigu and Longyi 1 adlay extracts.

Cellular Antioxidant Activities of Adlay Extracts. The CAA values for adlay extracts in the no PBS wash protocol are shown in Figure 7A. CAA values ranged from 2.27 to 9.87 μmol of QE/100 g of adlay. The average CAA values were 4.84 μmol of QE/100 g of adlay. Bound Longyi 1 adlay had the highest CAA value (9.87 ± 1.53 μmol of QE/100 g of adlay), whereas Liaoning 5 adlay had the lowest CAA value (2.27 ± 0.31 μmol of QE/100 g of adlay). In the PBS wash protocol, CAA values ranged from 0.53 to 1.37 μmol of QE/100 g of adlay. The average CAA value was 0.97 μmol of QE/100 g of adlay (Figure

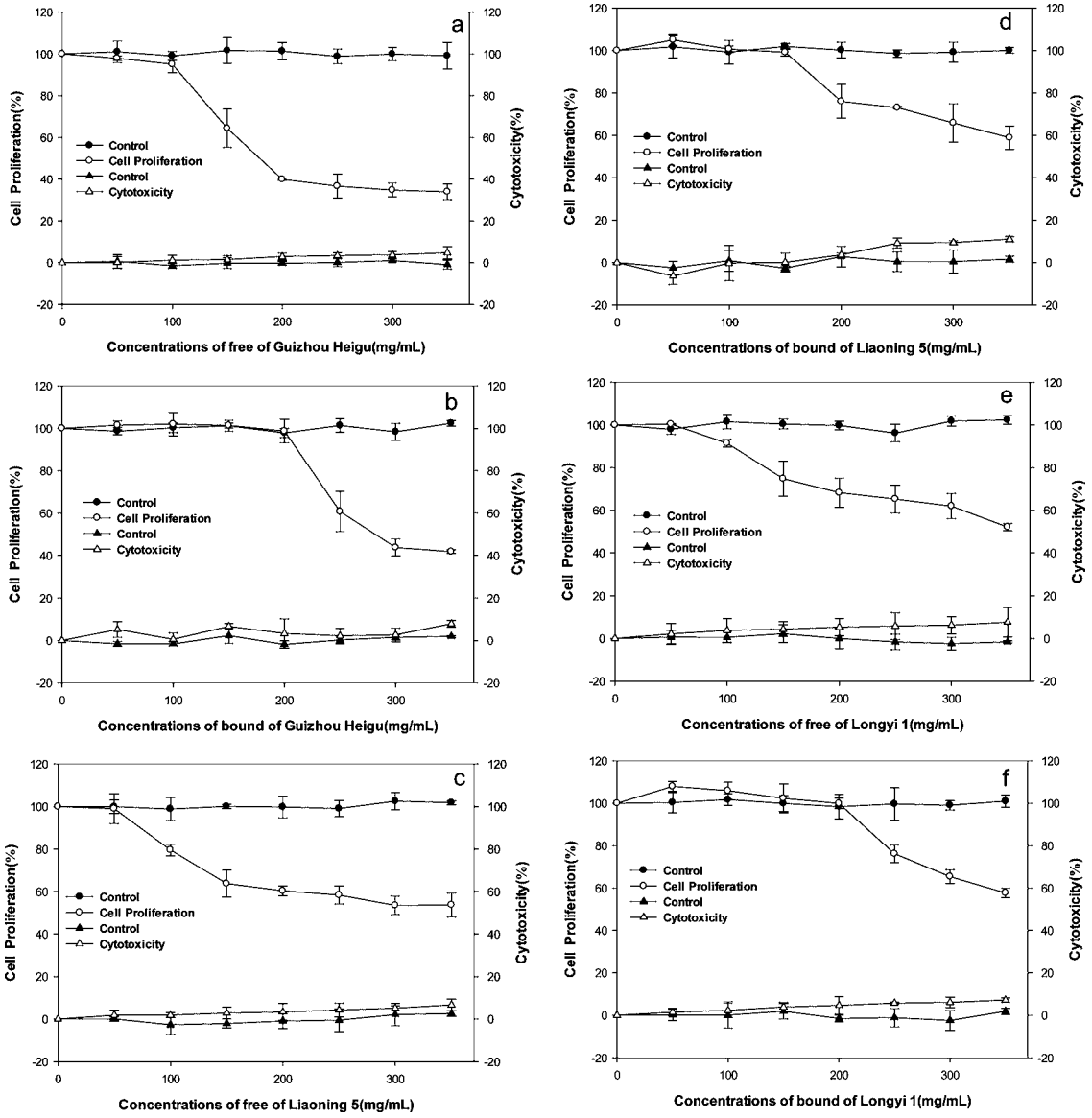

Figure 10. Percent inhibition of human HepG2 liver cancer cells proliferation and cytotoxicity by three adlay extracts.

Table 5. Antiproliferative Activities (EC_{25} and EC_{50}) and Cytotoxicities (CC_{50}) of Adlay Extracts toward Human HepG2 Liver Cancer Cells

		antiproliferative capacity of cells		
variety		EC_{25}(mg/mL)	EC_{50}(mg/mL)	cytotoxic capacity (CC_{50}, mg/mL)
Guizhou Heigu	free	147.57 ± 6.54	223.33 ± 8.02	>350
	bound	252.49 ± 8.37	296.62 ± 9.86	>350
Liaoning 5	free	167.59 ± 4.57	280.99 ± 3.29	>350
	bound	258.77 ± 7.68	332.63 ± 11.16	>350
Longyi 1	free	183.53 ± 5.34	324.14 ± 12.64	>350
	bound	251.09 ± 9.67	325.59 ± 10.87	>350

7B). Bound Longyi 1 adlay had the highest CAA value (1.37 ± 0.48 μmol of QE/100 g of adlay), whereas free Guizhou Heigu adlay had the lowest CAA value (0.53 ± 0.12 μmol QE/100 g of adlay).

The CAA and EC_{50} values of the different adlay extracts are shown in Tables 3 and 4. The EC_{50} values and CAA values were negatively correlated; the lower the EC_{50} value, the higher the CAA value. Regardless of the protocol, bound Longyi 1 adlay had the lowest EC_{50} value.

Cellular Antioxidant Quality of Adlay Extracts. The cellular antioxidant quality of the adlay extracts was determined from their CAA values, total flavonoids, and total phenolic contents (Table 4; Figures 8 and 9). CAA can be expressed as the number of quercetin equivalents per 100 μmol of total flavonoids or phenolics. In the no PBS wash protocol, free Guizhou Heigu adlay had the highest CAA value relative to the total flavonoids (11.83 ± 0.56 μmol QE/100 μmol total flavonoids), whereas bound Liaoning 5 adlay had the lowest CAA value (1.90 ± 0.26 μmol QE/100 μmol total flavonoids). In the Liaoning 5 and Longyi 1 adlay extracts there were significant differences between the bound and free sections ($p < 0.05$). Bound Longyi 1 adlay had the highest CAA value relative to the total phenolics (5.44 ± 0.84 μmol QE/100 μmol total phenolics), whereas bound Liaoning 5 adlay had the lowest CAA values (1.35 ± 0.18 μmol QE/100 μmol total phenolics).

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There were significant differences between the bound and free sections ($p < 0.05$) of Guizhou Heigu and Longyi 1 adlay extracts. However, there were no significant differences in CAA values between these extracts and Liaoning 5 adlay extract ($p > 0.05$). In the PBS wash protocol, free Guizhou Heigu adlay had the highest CAA value relative to the total flavonoids (2.48 ± 0.56 μmol QE/100 μmol total flavonoids), whereas Liaoning 5 adlay had the lowest CAA value (0.91 ± 0.03 μmol QE/100 μmol total flavonoids). There were significant differences ($p < 0.05$) among the free and bound adlay extracts. Bound Longyi 1 adlay had the highest CAA value relative to the total phenolics (0.75 ± 0.26 μmol QE/100 μmol total phenolics), whereas free Guizhou Heigu adlay had the lowest CAA value (0.29 ± 0.07 μmol QE/100 μmol total phenolics). There were significant differences between the bound and free sections ($p < 0.05$) of Longyi 1 adlay extract. However, there were no significant differences in CAA values between Longyi 1 adlay extract and Guizhou Heigu and Liaoning 5 adlay extracts ($p > 0.05$).

Antiproliferative Effect on HepG2 Human Liver Cancer Cells. The inhibition of HepG2 cell proliferation by the adlay extracts and the cytotoxic effects are shown in Figure 10. Adlay extracts inhibited cell proliferation, and there were few differences among the three adlay extracts. The free adlay polyphenol extracts had stronger inhibitory effects than the bound ones. The polyphenol extracts of Guizhou Heigu adlay (free and bound) had the highest inhibitory effects on cell proliferation; the inhibitory effect of Guizhou Heigu was higher than those of Longyi 1 and Liaoning 5 adlay extracts. The EC_{25} and EC_{50} values of the inhibitory effects by the different adlay extracts are shown in Figure 10.

At an adlay extract concentration of 350 mg/mL, the inhibitory effect ranged from 42% (for bound Liaoning 5 adlay; Figure 10D) to 66% (for free Guizhou Heigu adlay; Figure 10A). The corresponding EC_{50} values ranged from 332.63 ± 11.16 to 223.33 ± 8.02 mg/mL (Table 5). The three adlay extracts had relatively constant inhibitory effects on cell proliferation. However, there were no obvious differences among the three adlay extracts (Figure 10 and Table 5). The 350 mg/mL adlay concentration had no cytotoxic effects (Table 5), which indicated that the inhibitory effect was not attributed to a cytotoxic effect but to the antitumor effects of the extracts. In addition to being an edible whole grain food, adlay has good antitumor effects.

In summary, adlay grains are rich in phytochemicals and have high antioxidant activity. It is possible that bound phytochemicals may provide additional health benefits to the colon after being fermented by colonic bacteria. However, further research is needed to assess the effects of adlay in the prevention of colon cancer.

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Notes

The authors declare no competing financial interest.

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