Antagonism of Free-Radical-Induced Damage of Adlay Seed and Its Antiproliferative Effect in Human Histolytic Lymphoma U937 Monocytic Cells

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The goal of our current research was to investigate the antioxidative effects of methanolic extracts from different parts of adlay seed and their antiproliferative activity in malignant human cells. The methanolic extracts from different parts of adlay seeds were from the hull (AHM), testa (ATM), bran (ABM), and polished adlay (PAM). AHM exhibited greater capacity to scavenge superoxide anion radicals in the PMS−NADH system than ATM, ABM, or PAM. The scavenging capacities of AHM and ATM on hydrogen peroxides were about 20% at a dose of 250 μg/mL. Using the method of deoxyribose degradation to assess damage caused by hydroxyl radicals, AHM was found to inhibit damage in deoxyribose at a higher concentration. However, ATM, ABM, and PAM exhibited prooxidative activity at the same concentration. The inhibitory effect on enzymatic oxidation of xanthine to uric acid was found to follow the order AHM > ATM ≒ ABM. However, PAM was inactive. All test samples were positive for inhibition of TPA-induced free radical formation on neutrophil-like leukocytes and were found to follow the order AHM > ATM > ABM > PAM. When human histolytic lymphoma U937 monocytic cells were exposed to *tert*-butyl hydroperoxide, AHM protected the cells against the cytotoxicity caused by *tert*-butyl hydroperoxide. In addition, AHM exhibited antiproliferative activity against human histolytic lymphoma U937 monocytic cells in a dose-dependent manner. The antiproliferative properties of AHM appear to be attributable to its induction of apoptotic cell death as determined by flow cytometry. These results show that AHM displays multiple antioxidant effects and induces apoptosis of malignant human cells.

Keywords: *Adlay seed; hull; antioxidant; free radical; cytotoxicity; apoptosis*

INTRODUCTION

Adlay ("soft-shelled job's tears", *Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is a grass crop that has long been used in traditional Chinese medicine and as a nourishing food. According to the ancient Chinese medical book *Pen-Tsao-Kang-Mu* (*1*), the seed of adlay was used in China for the treatment of warts, chapped skin, rheumatism, and neuralgia, and as an anti-inflammatory or antihelmintic agent. Adlay also has been said to have stomachic, diuretic, antipholgistic, anodynic, antispasmodic, and antitumor effects. It is widely planted in Taiwan, China, and Japan, where it is considered a healthy food supplement.

A number of recent studies have shown some physiological effects of adlay extracts. Ukita and Tanimura (*2*) reported that the growth of Ehrlich ascites sarcoma was inhibited by adlay and identified the active component as coixenolide (*3*). Nagao et al. (*4*) isolated from adlay seed a number of benzoxazinones that showed anti-inflammatory activity. Takahashi et al. (*5*) reported that coixans A, B, and C isolated from adlay seed had hypoglycemic activity in rats. Park et al. (*6*) found that lipid components in plasma and feces decreased in rats fed with adlay seed. Hidaka et al. (*7*) demonstrated that ingestion of adlay seed tablets could increase the activities of cytotoxic T-lymphocytes and natural killer cells. Check and K'Ombut (*8*) also found decreased fibrinolytic activities of blood plasma of Wistar rats fed an adlay mixed diet. Numerous other reports have indicated that the consumption of adlay seed is beneficial to humans (*9*−*11*). Results from our laboratory have shown that some extracts of adlay seed are anti-allergic (*12*), antimutagenic (*13*), and hypolipidemic (*14*), and they exhibit prebiotic activity (*15*).

Reactive oxygen species (ROS) and oxygen free radicals play important roles, both beneficial and detrimental, in aerobic life (*16*). Excess ROS has been implicated in a variety of pathophysiological phenomena, such as inflammation, aging, atherosclerosis, cancer, rheumatoid arthritis, hepatotoxicity, and reperfusion injury (*17*, *18*). ROS includes superoxide anion radical, hydrogen peroxide, and hydroxyl radical.

Methanolic extracts from adlay seed have been reported to have a moderate antioxidative effect on the peroxidation of linoleic acid (*19*, *20*). Liou et al. (*21*) also showed that methanolic extract of dehulled adlay acted as reductant, chelator of ferrous ions, and scavenger of

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DPPH radicals. However, the effects of extracts of different parts of adlay seed (including adlay hull, testa, bran, and polished adlay) on the antagonism of free-radical-induced damage and cytotoxic function in cancer cells has not been studied systematically.

The objectives of this work were to investigate the antioxidant properties of methanolic extracts from different parts of adlay seed (including the elimination of ROS), to assay the inhibitory effect on free-radical-related enzymes, and to determine both the cytoprotective effect on cultured cells exposed to *tert*-butyl hydroperoxide and the level of inhibition of the growth of cancer cells.

MATERIALS AND METHODS

Materials. Allopurinol, L-ascorbic acid, *tert*-butyl hydroperoxide, 2-deoxy-D-ribose, cytochrome C, *N*,*N*-dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), ferric chloride hexahydrate, D-(+)-glucose, horseradish peroxidase, D-mannitol, α-nicotinamide-adenine-dinucleotide (reducing form) (α-NADH), nitro blue tetrazolium (NBT), penicillin G, phenazine methosulfate (PMS), phenol red, propidium iodide (PI), RNase A, sodium bicarbonate, sodium dodecyl sulfate (SDS), streptomycin sulfate, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), 2-thiobarbituric acid (TBA), tris(hydroxylmethyl)aminomethane, trypan blue, xanthine, and xanthine oxidase were purchased from the Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS), L-glutamine, and RPMI medium 1640 were purchased from the Gibco BRL Co. (Gaithersburg, MD). Hydrogen peroxide was purchased from the Showa Chemical Co. (Tokyo, Japan). Methanol and quercetin were purchased from the Riedel-deHaen Chemical Co. (Germany). Trichloroacetic acid (TCA) was purchased from the E. Merck Co. (Darmstadt, Germany).

Cell Culture. The human histolytic lymphoma U937 monocytic cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The human promyelocytic leukemia HL-60 cell line was obtained from the Sloan-Kettering Cancer Center (New York, NY). U937 and HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μL/mL streptomycin in a humidified atmosphere of 5% carbon dioxide. The cells were passaged every third day, and the cell density in cultures did not exceed 5×10^5 cells/mL.

Plant Material. Adlay was purchased from a local farmer who planted Taichung Shuenyu No.4 (TCS4) of *Coix lachryma-jobi* L. var. *Ma-yuen* Stapf in Taichung, Taiwan in March 1997 and harvested it in July of the same year. The air-dried adlay seed was separated into four different parts, including adlay hull, adlay testa, adlay bran, and polished adlay. All the materials were blended in powder form, and screened through a 20-mesh sieve (aperture 0.94 mm).

Sample Preparation. Each sample powder (100 g) was extracted with 1 L of methanol stirred on a Thermolyn Nuova II stirring/heating plate (Dubuque, IA) at room temperture for 24 h. Contents were filtered through #1 filter paper (Whatman Inc., Hillsboro, OR). The filtrate was concentrated to dryness in vacuo to obtain methanolic extract and stored at −20 °C.

Superoxide Anion Radical-Scavenging Property. Superoxide anion radicals were generated by the reaction of NADH with PMS, and detected through the reduction of NBT as described by Robak and Gryglewsk (*22*). The reaction mixture contained 1 mL of sample, 1 mL of 936 μM NADH, and 1 mL of 300 μM NBT. After incubation at room temperature for 10 min, the reaction was started by adding 1 mL of 120 μM PMS. Then the reaction mixture was incubated at ambient temperature for 5 min. The results was determined by reading the absorbance at 560 nm against blank samples. Quercetin was used as a positive scavenger, and its effect on scavenging of superoxide anion radicals was also determined.

Hydrogen Peroxides-Scavenging Property. Hydrogen peroxides were measured using the horseradish peroxidase assay (*23*). Briefly, 1 mL of sample was first mixed with 400 μL of 4 mM H_2O_2 solution and allowed to incubate for 20 min at room temperature. Then 600 μL of phenol red solution (7.5 mM phenol red and 500 μg/mL horseradish peroxidase in 100 mM phosphate buffer) was added to the reaction mixture. After 10 min, the sample absorbance was determined using a spectrophotometer set at 610 nm. Sample absorbances were converted to mM H_2O_2 by interpolating from a standard curve. Ascorbic acid was used as a positive scavenger, and its effect on scavenging of hydrogen peroxide was also determined.

Hydroxyl Radical-Scavenging Property. The method of deoxyribose degradation was used to assess damage caused by hydroxyl radicals as described by Halliwell and associates (*24*). The reaction mixture contained 100 μL of 28 mM deoxyribose, 400 μL of 40 mM phosphate buffer (pH 7.4), 100 μL of fresh ferric chloride, 100 μL of 1.04 mM EDTA, 100 μL of 10 mM hydrogen peroxide, 100 μL of 1 mM fresh ascorbic acid, and 100 μL of sample. The mixture was incubated for 1 h at 37 °C. Then 500 μL of 1% TBA in 50 mM sodium hydroxide and 500 μL of 2.8% trichloroacetic acid were added, and the mixture was left to react at 100 °C for 10 min. Absorbance of the chromogen produced was measured spectrophotometrically at 532 nm. Mannitol was used as a positive scavenger, and its effect on inhibiting deoxyribose damage was also determined.

Inhibitory Test of Xanthine Oxidase Activity. Xanthine oxidase (EC 1.1.3.22) activity was evaluated through spectrophotometric measurement of the formation of uric acid from xanthine as described by Terada and co-workers (*25*). The reaction was carried out in a mixture of 100 mM phosphate buffer (pH 7.5), 0.04 units/mL of xanthine oxidase, 100 μM of xanthine, and various concentrations of test samples. Each test sample was first mixed with xanthine oxidase and allowed to incubate for 10 min at 37 °C. Xanthine was then added, and the absorbance of uric acid at 290 nm was measured after 1 h. Allopurinol was used as a positive inhibitor, and its effect on XOD activity was also determined.

Inhibitory Test of TPA-Induced Superoxide Generation on Neutrophil-Like Leukocytes. An inhibitory test of TPA-induced superoxide generation was conducted as previously reported (*26*). Briefly, human promyelocytic leukemia HL-60 cells were preincubated in complete culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) with 1.3% (v/v) dimethyl sulfoxide (DMSO) at 37 °C in a 5% CO_2 incubator for 5 days to differentiate cells into neutrophil-like leukocytes. Morphological evaluation of the differentiation cells revealed mostly band and segmented cells. The nitro blue tetrazolium (NBT) test (*27*) was used to screen cells for their capacity to undergo oxidative metabolism. More than 65% of the differentiation HL-60 cells were routinely found to reduce NBT after exposure to TPA. Differentiated cells (5×10^5/mL) were plated in a 96-well microplate suspended in Hank's balanced salt solution. Free radical formation was induced by the addition of TPA (8.1 μM), followed by the addition of samples and cytochrome *c* (60 μM), and incubated at 37 °C for 60 min. Cytochrome *c* reduction was measured at 550 nm. Using induction by TPA alone as a measure of maximum free radical formation, the percentage of radical formation in TPA plus agent-treated samples was determined.

Cytoprotective Assay on Cultured Human Histolytic Lymphoma U937 Monocytic Cells Exposed to *tert*-Butyl Hydroperoxide. A cytoprotective test on human histolytic lymphoma U937 monocytic cells was conducted as previously reported (*28*). Human histolytic lymphoma U937 monocytic cells (1×10^6/mL) were maintained in continuous cell suspension at 37 °C under 5% CO_2 and 95% air in complete medium (RPMI 1640 medium supplemented 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) with test samples at various concentrations.

After 30 min of incubation, cells were treated with 500 μM *tert*-butyl hydroperoxide for another 3 h. Cell viability was assessed by trypan blue exclusion.

MTT-Microculture Tetrazolium Assay. The colorimetric assay for cellular growth and survival was described by Hansen et al. (*29*). Suspensions of human histolytic lymphoma U937 monocytic cells (2.8×10^4 cells/mL) were cultured with or without test samples (at various concentrations in 20 μL of suspension) in a 96-well microplate (180 μL suspension/well). After 72 h, 25 μL of MTT solution (5 mg/mL) was added to each well, and the cells were incubated at 37 °C for 4 h. Then, 100 μL of lysis buffer was added to each well, and the cells were again incubated at 37 °C for overnight (about 16 h) to dissolve the dark blue crystals. Each well was completely pipetted, and then the absorption of formazan solution at 570 nm was measured using a microplate reader.

Cell Cycle Analysis and Apoptosis Measured by Flow Cytometry. For cell cycle analysis, the cells were plated at an initial density of 1×10^5/mL in complete medium with or without test samples. After 24 or 48 h of treatment, the cells were removed from the culture plates and centrifuged at 300*g* for 10 min. The cell pellets were then suspended in PBS, fixed in 70% ethanol for 1 h, and, after washing in PBS twice, stained with a PBS solution containing 100 μg/mL of RNase A and 40 μg/mL of propidium iodide at 37 °C in the dark for 30 min. DNA content was analyzed using a flow cytometer (FAScan, Becton Dickinson, Franklin Lakes, NJ) with a CELL FIT profile analyzer (*30*).

Statistical Analysis. The results were statistically evaluated by the Student *t* test. Comparison was made only between the control (in the absence of test samples) and experimental values (in the presence of test samples).

RESULTS AND DISCUSSION

Scavenging Effect of Reactive Oxygen Species (ROS). The scavenging effects of superoxide anion radicals by methanolic extracts from different parts of adlay seed were found to follow the order quercetin (positive control) > AHM > ATM. The scavenging effects of AHM and ATM increased with increasing concentration. Superoxide anion radicals were scavenged 50 and 25% by AHM and ATM at a dose of 250 μg/mL, respectively. PAM was inactive on scavenging superoxide anion radicals. However, ABM promoted the formation of superoxide anion radicals in the PMS/NADH system (Figure 1). Robak and Gryglewski (*22*) reported that the antioxidant properties of several flavonoids, such as quercetin, myricetin, and rutin, were affected mainly via scavenging of superoxide anion radicals. On the basis of the ^{1}H NMR spectra, we believe that the active fractions from AHM are rich in flavonoids. Chemical structure analyses of these active ingredients are currently underway.

The scavenging effects of hydrogen peroxides by methanolic extracts from different parts of adlay seed were found to follow the order ascorbic acid (positive control) (73%) > ATM (21%) ≒ AHM (19%) at a dose of 250 μg/mL. PAM and ABM were not capable of reacting directly with hydrogen peroxide in our assay (Figure 2). These data indicate that the methanolic extracts from different parts of adlay seed are not effective hydrogen peroxide scavengers.

The effects of methanolic extracts from different parts of adlay seed on oxidative damage in deoxyribose induced by the ascorbic acid/Fe^{3+}-EDTA/H_2O_2 system are shown in Figure 3. No prooxidant activity was found in mannitol (the positive control). AHM accelerated oxidative damage in deoxyribose at a concentration of 250 μg/mL, but its prooxidant activity was weaker than that of ATM, ABM, and PAM. AHM induced deoxyribose damage at lower concentrations, but inhibited damage at higher concentrations. The decrease in prooxidant activity at higher concentrations might have resulted from competition between antioxidative and prooxidative action. Many lipid-soluble, chain-breaking antioxidants can have prooxidant properties under certain circumstances in vitro, often because they can reduce Fe^{3+} or Cu^{2+} ions to Fe^{2+} or Cu^{+} (*31*, *32*). Yen and associates (*33*) found that tea extracts seemed to act as antioxidants or prooxidants, depending on their ability to reduce iron and scavenge hydroxyl radicals. Yen et al. (*34*) also suggested that the prooxidant activity of some antioxidants was related to their reducing power. AHM potentially possesses great reducing power (unpublished data). Comparing the reducing power of ascorbic acid with that of AHM, 500 μg/mL of AHM is found to be equal to 135 μM of ascorbic acid. Thus, AHM

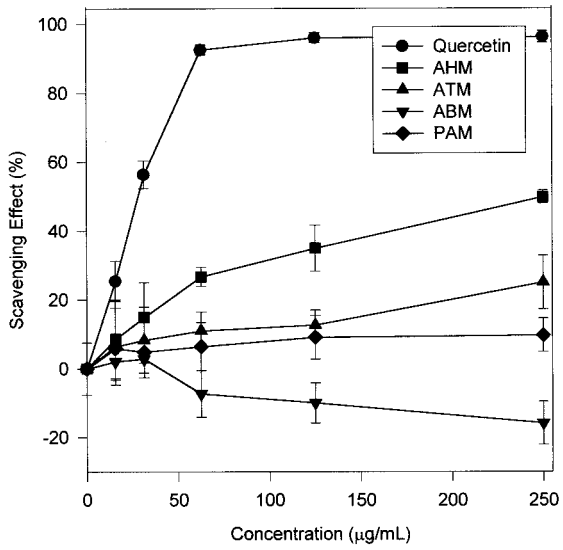

Figure 1. Scavenging effects of methanolic extracts from different parts of adlay seed on superoxide anion radicals (PMS−NADH system). Negative values show that the samples did not have any scavenging effects. A high negative value indicates a large facilitating effect on superoxide anion. Each value was quadruplicated in the test (mean ± standard deviation).

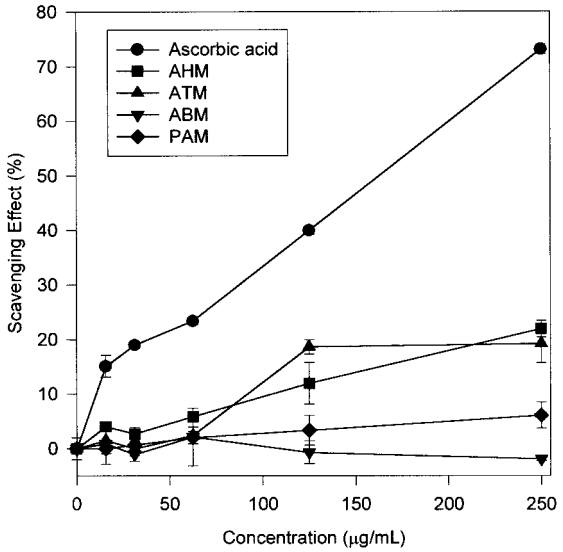

Figure 2. Scavenging effects of methanolic extracts from different parts of adlay seed on hydrogen peroxide. Each value was quadruplicated in the test (mean ± standard deviation).

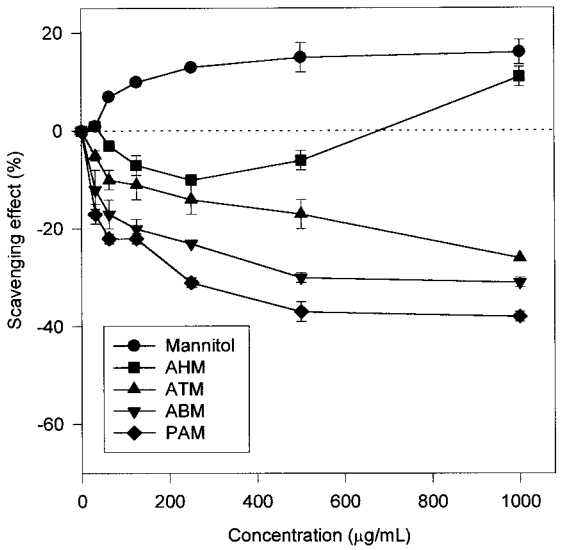

Figure 3. Scavenging effects of methanolic extracts from different parts of adlay seed on hydroxyl radicals. The hydroxyl radicals were generated by the ascorbic acid/Fe^{3+}-EDTA/H_2O_2 system. Test samples inhibited the generation of hydroxyl radicals as measured by deoxyribose degradation. Each value was quadruplicated in the test (mean ± standard deviation).

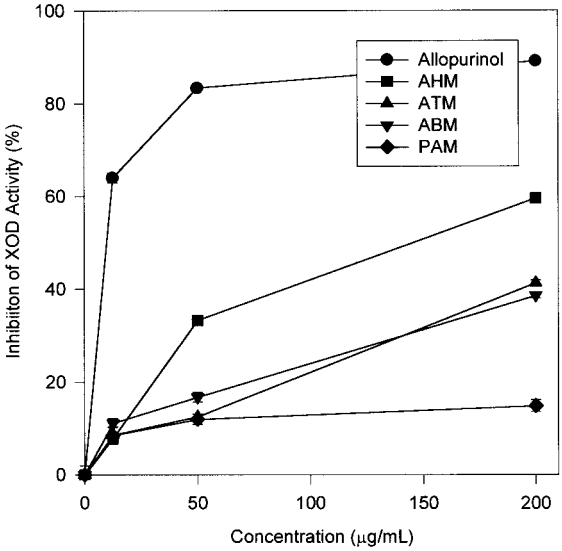

Figure 4. Inhibition of xanthine oxidase (XOD) activity by methanolic extracts from different parts of adlay seed. Inhibition was determined by measuring the amount of uric acid generated from xanthine. Results are expressed as mean ± standard deviation of quadruplicated experiments.

may exhibit moderate scavenging activity on the hydroxyl radicals at higher concentrations, thus reducing its prooxidant activity.

Inhibitory Effect of Xanthine Oxidase activity. Xanthine oxidase (XOD) produces uric acid and reactive oxygen species (ROS) during catabolism of purines. Excess amounts of the former can lead to gout, and excess amounts of the latter can increase oxidative stress, mutagenesis, and possibly cancer rick (*35*). Figure 4 shows the effect of methanolic extracts from different parts of adlay seed on the activity of XOD. The inhibitory effect of enzymatic oxidation of xanthine to uric acid was found to follow the order allopurinol (89%) > AHM (60%) > ATM (41%) ≒ ABM (39%). However, PAM did not inhibit XOD activity. The intestinal mucosa and liver are the richest sources of XOD. XOD

Table 1. Effects of Methanolic Extracts from Different Parts of Adlay Seed on TPA-Stimulated Superoxide Anion Radicals Generation by Neutrophil-like Leukocytes[a]

test sample	$O_2^{\bullet-}$ scavenging ratio (%) at concentration of 50 μg/mL	100 μg/mL	200 μg/mL	EC_{50}[b] (μg/mL)
AHM	35.20 ± 5.33	56.80 ± 3.60	81.86 ± 1.41	80
ATM	21.81 ± 1.11	31.97 ± 1.48	54.86 ± 3.02	180
ABM	7.99 ± 3.49	12.74 ± 4.68	28.94 ± 2.85	>200
PAM	6.86 ± 3.18	7.34 ± 6.40	19.65 ± 1.87	>200

[a] In HL-60 cells, differentiation was induced for 5 days by means of 1.3% DMSO treatment. These differentiated cells were treated with various concentrations of test samples for 20 min and then stimulated with 8.1 μM TPA for 60 min. Superoxide anion production was determined by cytochrome C reduction. Results are expressed as mean ± standard deviation of quadruplicated experiments. [b] The efficient concentration required to inhibit superoxide anion radical generation by 50%.

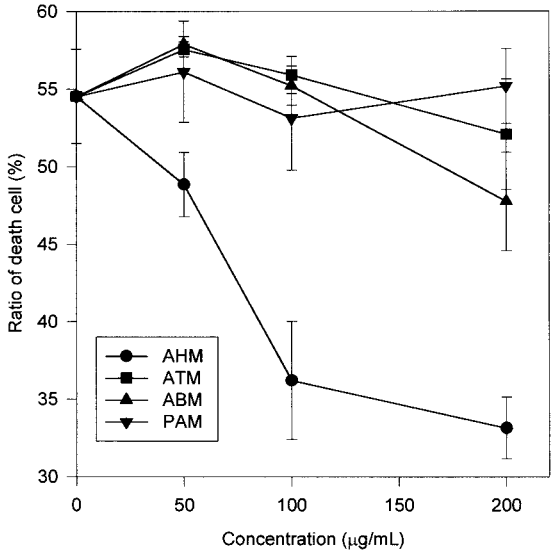

Figure 5. Cytoprotective influence of methanolic extracts from different parts of adlay seed on cultured U937 cells exposed to *tert*-butyl hydroperoxide assessed using the Trypan blue exclusion assay. Values were determined after a 180 min incubation period with *tert*-butyl hydroperoxide and represent mean ± SD derived from quadruplicate determinations.

is the major source of ROS in the ischemic small intestine. Allopurinol, an XOD inhibitor, is effective in preventing any increase in vascular permeability or mucosal lesions produced by ischemia (*36*). Recent reports have shown that crude extracts of anthocyanins and other phenolic compounds from plants inhibit XOD by competing with the substrate, xanthine (*35*, *37*, *38*).

Inhibitory Effect of TPA-Induced Superoxide Generation on Neutrophil-Like Leukocytes. Neutrophil-like leukocytes (differentiated HL-60 cells) are able to generate superoxide anion radicals along with stimulation of TPA through the NADPH oxidase system (*39*). The mitigation of TPA-mediated oxidative processes by methanolic extracts from different parts of adlay seed was determined by measuring superoxide anion radical production of TPA-treated neutrophil-like leukocytes as shown in Table 1. Methanolic extracts from different parts of adlay seed inhibited free radical formation in a dose-dependent manner. AHM and ATM at a concentration of 200 μg/mL significantly inhibited superoxide anion radical generation by 82 and 55%, respectively. ABM and PAM showed little potential for inhibition (inhibition effects of 29 and 20%, respectively). Phagocyte-derived free radicals are involved in

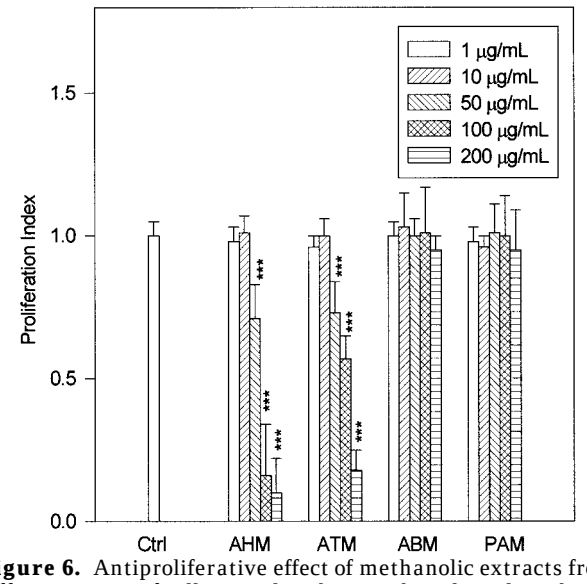

Figure 6. Antiproliferative effect of methanolic extracts from different parts of adlay seed in human histolytic lymphoma U937 monocytic cells. U937 cells (2.8×10^4/mL) were cultured with or without test samples for 72 h and detected using the MTT assay. The results are expressed as the percentage of living cells cultured in the presence of test samples relative to a parallel culture that did not receive any treatment. Each bar represents mean ± S.D. quadruplicated in the test. *** $p < 0.001$.

tissue damage, and antioxidant protection can be achieved not only by scavenging these species, but also by preventing their formation. Sharma et al. (*26*) found that many phenolic and sulfur compounds were strong superoxide anion radical scavengers, and some antiinflammatory compounds have been identified as free radical inhibitors. Tumor-promoter-induced ROS generation has been suggested to play an important role in tumor promotion; thus, superoxide formation inhibitors are effective and show promise for preventing of tumorigenesis (*26, 40*).

Cytoprotective Effect on Cultured Human Histolytic Lymphoma U937 Monocytic Cells Exposed to *tert*-Butyl Hydroperoxide. We examined the protective effects of methanolic extracts from different parts of adlay seeds against oxidative damage induced by *tert*-butyl hydroperoxide in a culture of human histolytic lymphoma U937 monocytic cells. *tert*-Butyl hydroperoxide has been widely used as a model compound for inducing oxidative stress (*41, 42*). Under our experimental conditions, methanolic extracts from different parts of adlay seed did not exhibit any cytotoxic effect (data not shown). AHM-treated cells showed increased resistance to oxidative challenge, as revealed by a higher percent of survival compared to that of control cells during *tert*-butyl hydroperoxide treatment, and a dose-dependent effect was also evident (Figure 5). However, ATM, ABM, and PAM did not exhibit any cytoprotective effect in this model system. Our results indicate that AHM is able to modulate cellular response to oxidative challenge. Devaraj et al. (*43*) reported that supplemen-

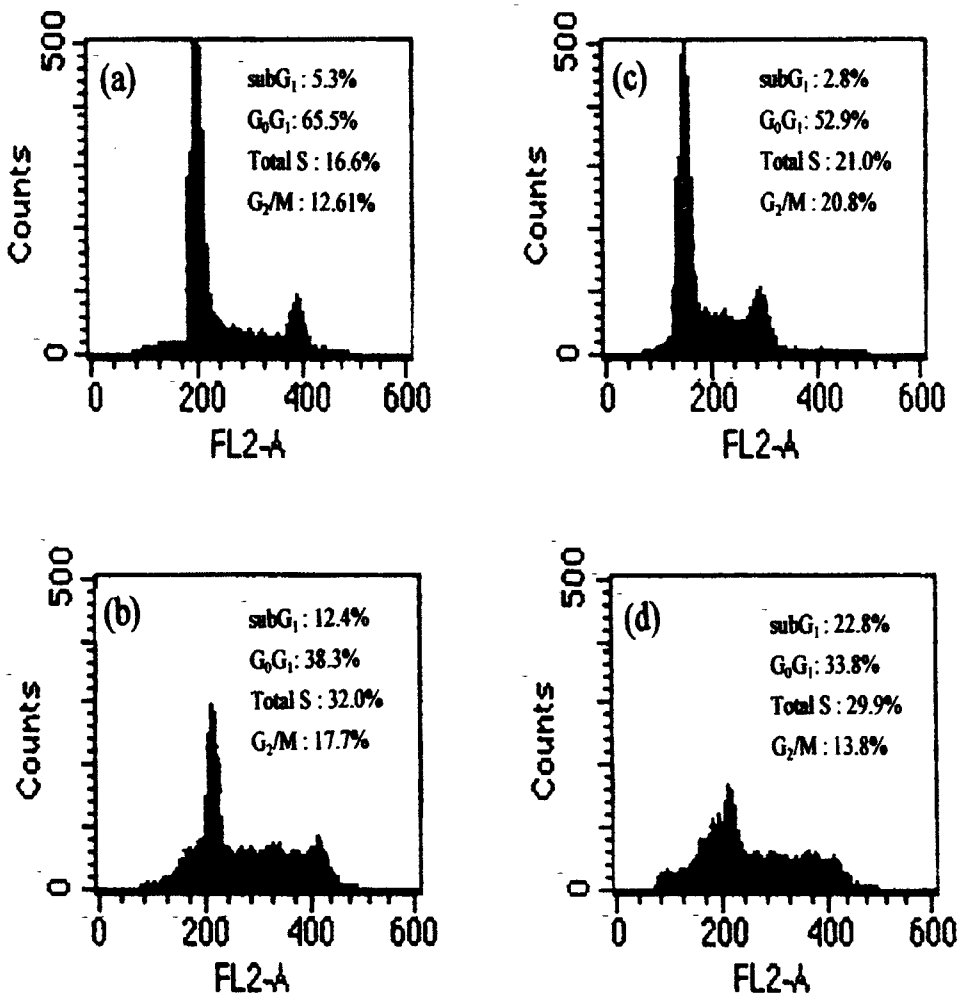

Figure 7. Effect of AHM on cell cycle distribution of U937 cells. Cells were cultured with or without test samples for 24 or 48 h, harvested, and then subjected to cytofluorimetric analysis to evaluate cell cycle distribution and apoptosis. (a) Control group, cultured for 24 h; (b) AHM 200 μg/mL, cultured for 24 h; (c) control group, cultured for 48 h; (d) AHM 200 μg/mL, cultured for 48 h.

tation of α-tocopherol in humans affected cellular function in monocytes. Nardini et al. (*28*) also reported that caffeic acid affected cellular response in human histolytic lymphoma U937 monocytic cells under *tert*-butyl hydroperoxide-induced oxidative stress.

Antiprolifertative Effect on Cultured Human Histolytic Lymphoma U937 Monocytic Cells. Are methanolic extracts from different parts of adlay seed toxic to tumor cells? We evaluated this possibility. U937 cells of a human histolytic lymphoma cell line were cultured in the presence and absence of test samples (data shown in Figure 6). AHM and ATM inhibited the proliferation of human histolytic lymphoma U937 monocytic cells in a dose-dependent manner. AHM is more effective than ATM in terms of cytotoxic activity. However, ABM and PAM did not demonstrate any cytotoxic effect at concentrations up to 200 μg/mL after 72 h of incubation. Among the four test samples tested, AHM was the most potent, inhibiting proliferation of cells by 50% at 70 μg/mL. We have not examined the antiproliferative effect of AHM on normal human cells yet, and this work will be done soon. However, the cytotoxicity is much weaker in RAW 264.7 macrophage cell line (unpublished data). AHM displays selective cytotoxicity on different types of cell lines, perhaps because of its composition. We believe that identification of the active components of AHM and evaluation of the antiproliferative effect of malignant human cells can make this phenomenon more clear. Chemical structure analyses of these active ingredients are currently underway.

To obtain information on whether the cell death was apoptotic or necrotic, the cell cycle of U937 cells at 24 and 48 h after treatment with AHM was examined. In the control cultures, the percentage of cells exhibiting $subG_1$ peaks was about 3−5%, indicating some degree of spontaneous apoptosis. However, exposure to 200 μg/mL of AHM for 48 h caused a dramatic decrease of the G_0/G_1 phase in the cells and a concomitant increase in the number of apoptotic cells ($subG_1$ peak at about 23%). The data demonstrate that AHM causes cell cycle arrest at the S Phase checkpoint (Figure 7). Many antioxidants or free radical scavengers have been reported to be potent antitumor agents, and such activity may occur through the induction of apoptosis (*44*−*46*). The apoptosis-inducing agent AHM shows promise as a source of chemopreventive agents.

Conclusions. Our study showed that methanolic extracts of adlay hull (AHM) displayed multiple antioxidant effects and induced apoptosis in malignant human cells. However, other parts of adlay seed, including testa, bran, and polished adlay, were not found to be effective free radical scavengers. Although this experiment showed that the antioxidative and antiproliferative effects of dehulled adlay seed are worse than those of adlay hull, the physiological properties found in some research done in our laboratory showed positive potential. According to our research studies, dehulled adlay seed has anti-allergic (*12*), probiotic (*14*), and hypolipidemic (*15*) properties. Because the hull of adlay is very hard, people usually eat dehulled adlay seed in China. However, there are some commercial products in Taiwan and Japan in which some adlay hull is added as an ingredient for some unknown reason. This is why we wanted to investigate the antioxidative potential and other physiological function (for the current research) of adlay hull. Recently, investigations of plant hulls from several plants and spices have been shown to possess antioxidative activities (*47*−*51*). Our results indicate that AHM is a strong scavenger of reactive oxygen species, and that it inhibits free-radical-generating enzymes, blocks tumor-promoter-generated oxidative processes in neutrophil-like leukocytes, exhibits a cytoprotective effect on cultured cells exposed to *tert*-butyl hydroperoxide, and obstructs the growth and viability of cancer cells, i.e., U937 leukemia cells, through apoptosis.

ABBREVIATIONS USED

ABM, the methanolic extract of adlay bran; AHM, the methanolic extract of adlay hull; ATM, the methanolic extract of adlay testa; PAM, the methanolic extract of polished adlay; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanolyphorbol-13-acetate; XOD, xanthine oxidase.

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