JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antiproliferative and Chemopreventive Effects of Adlay Seed on Lung Cancer in Vitro and in Vivo

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This study examined the effects of different extracts of adlay seed on the growth of human lung cancer cells in vitro and in vivo. The data showed that a methanolic extract, but not a water extract, of adlay seed exerted an antiproliferative effect on A549 lung cancer cells by inducing cell cycle arrest and apoptosis. It was also found that tumor growth in vivo was inhibited by the methanolic extract in a dose-dependent manner. The chemopreventive effect of adlay seed on the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in A/J mice was also investigated. Groups of mice were pre-fed with different diets, followed by feeding with NNK-containing drinking water for 8 months. The results indicated that feeding with diet containing 30% of powdered adlay seed reduced the number of surface lung tumors by ∼50%. Taken together, these results indicate that the components of adlay seed exert an anticancer effect in vitro and in vivo and may be useful for the prevention of lung tumorigenesis.

KEYWORDS: Adlay seed; chemoprevention; lung tumorigenesis; NNK; apoptosis

INTRODUCTION

Adlay (*Coxi lachryma-jobi*) is a grass crop that has long been used in traditional Chinese medicine and as a nourishing food. The seed of adlay has been reported to exhibit anti-inflammatory, stomachic, diuretic, and antispastic effects in vivo and has been used in China for the treatment of warts, rheumatism, and neuralgia (*1*). Recent studies demonstrated that adlay seed could inhibit the growth of Ehrlich ascites sarcomas (*2*) and could increase the activity of cytotoxic T-lymphocytes and natural killer cells in experimental animals (*3*). However, the molecular mechanism by which adlay seed inhibits tumor development is largely unknown.

Recent study has indicated that the development of tumors is frequently associated with deregulation of cell cycle control (*4*). Three major classes of regulatory proteins that play important roles in the control of cell cycle progression are cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs). Progression of mammalian cell cycle from the G1 to S phase is mainly regulated by D- and E-type cyclins and CDK4, -6, and -2 (*5*−*7*). Conversely, A- and B-type cyclins and CDK2 are involved in the regulation of S, G2, and M phases (*8*−*10*). In addition, a group of negative regulatory proteins that may bind cyclin−CDK complexes and inhibit their kinase activity have been identified recently. These proteins are divided into the INK4 family, which includes $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$, and the CIP/KIP family, which includes $p21^{WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$ (*11*). Change of cell cycle regulatory gene expression is frequently found in human lung tumor tissues or cancer cell lines (*12*−*14*), and these cell cycle regulators may represent a new set of potential targets for anticancer drugs. Natural or synthesized agents that may specifically block cell cycle progression are thought to be useful for the treatment or prevention of lung cancer.

In this study, we tested the anticancer effect of adlay seed on lung cancer cell lines and investigated the molecular mechanism of this action. Additionally, we also evaluated the effect of adlay seed on NNK-induced lung tumorigenesis in experimental animals to clarify whether adlay seed exerts a chemopreventive effect in vivo.

MATERIALS AND METHODS

Cell Culture and Reagents. A549 human lung cancer cells were cultured in DMEM/F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37 °C. Antibodies against various cyclins (including D1, E, and A) and CDK2, -4, and -6 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Water and methanolic extracts of adlay seed were prepared as described previously (*15*).

MTT Assays. Cells (2×10^3 cells/well) were seeded in 96-well plates and grown overnight. Cells were then incubated in 10% FCS medium containing different amounts of water or methanolic extract of adlay seed for 48 h. After incubation, media were replaced with

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10.1021/jf021142a CCC: $25.00
Published on Web 05/09/2003

50 μL of [[3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide (MTT), 2 mg/mL] reagent and incubated in a 5% CO_2 incubator at 37 °C for another 3 h. Cells were harvested in 50 μL of DMSO, and absorbance was measured at 540 nm by using a microplate reader (Molecular Probes Inc., Eugene, OR).

Analysis of Cell Cycle Distribution. Cells were cultured in the absence or presence of a methanolic extract of adlay seed for 48 h in 10% FCS medium. Cells were fixed with 95% ethanol and stained with propidium iodide. Cell cycle distribution was analyzed by FACS flow cytometry (Becton Dickinson, Mountain View, CA) as previously described (*16*).

Immunoblotting. After treatments, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and harvested in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 1 mg/mL aprotinin, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin) for 20 min on ice. Cellular lysates were centrifuged at 12000*g* for 10 min, and protein concentrations of the lysates were determined by using a BCA protein assay kit (Pierce, IL). Proteins were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blots were blocked in 5% nonfat milk in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween-20 (TBST) overnight at 4 °C. After blocking, the blots were then washed in TBST, incubated with various primary antibodies for 2 h at room temperature, and incubated with peroxidase-conjugated secondary antibody for another 1 h. The blots were developed by using the ECL chemiluminescence system (Amersham Biosciences Corp., Piscataway, NJ) and were reprobed with actin antibody to confirm equal loading of proteins in each lane.

Apoptosis Assays. Cells were cultured in six-well plates in 10% FCS medium and were incubated with vehicle or different amounts of methanolic extract of adlay seed for 48 h. After incubation, detached cells were collected by centrifugation and attached cells were harvested by trypsinization. Cells were pooled and harvested for analysis of degradation of poly(ADP)-ribose polymerase (PARP). Cellular proteins extracted from pooled cells were subjected to SDS-PAGE and western blotting. The blots were probed with anti-PARP antibody to detect the native form (116 kDa) and the degradation form (85 kDa) of PARP.

Nude Mice Assays. All experiments on mice were performed according to the guidelines for our institute (*Guide for Care and Use of Laboratory Animals*, Kaohsiung Medical University). BALB/c-nu nude mice (8 weeks old) were housed in barrier facilities on a 12-h light/dark cycle and received food and water ad libitum. Tumors were induced by subcutaneous (sc) injection of A549 cells (2×10^6 cells in 0.1 mL of PBS) at one site of the right flank. Tumors (visualized as small nodules at the sites of injection) appeared ~20 days after injection, and the animals were randomly distributed into a control group, which received vehicle (5% methanol), or a methanolic extract-treated group, which received a high dose (3 mg/kg) or a low dose (1 mg/kg). Administration of vehicle or methanolic extract via ip injection was initiated from day 21 after cell inoculation. Animals were injected every day, and treatment was continued for 45 days. Tumor growth was measured every week, and tumor volume was calculated according to the formula (*17*)

$$\text{tumor volume} = \text{larger diameter} \times \text{small diameter}^2/2$$

Chemoprevention Experiments. A/J mice (8 weeks old, obtained from National Cheng Kung University) were housed in barrier facilities on a 12-h light/dark cycle and received food and water ad libitum. Mice were randomly distributed into group A, which was fed with normal diet, or groups B and C, which fed with diet containing 10 or 30% powdered adlay seed, respectively. Each group contains five experimental animals. Mice were pre-fed with different diets for 1 month, and treatment was initiated by administration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; 100 μg/mL) in drinking water. Consumption of diets and drinking water by each group of mice was recorded every week. The body weight was also measured every week. Experimental animals were continuously fed with diets and NNK-containing drinking water for 8 months. After treatment, mice were sacrificed and lungs were stained with a contrast medium (15% India ink in distilled water) by injecting the medium into the trachea. Lungs were then fixed in a fixation solution (100 mL of 70% alcohol, 10 mL of formaldehyde, and 5 mL of glacial acetic acid) (*18*). This solution permanently bleached the tumor white and preserved the lung indefinitely. The number of surface tumors was counted. This assay was repeated twice, and the results from two independent experiments were calculated.

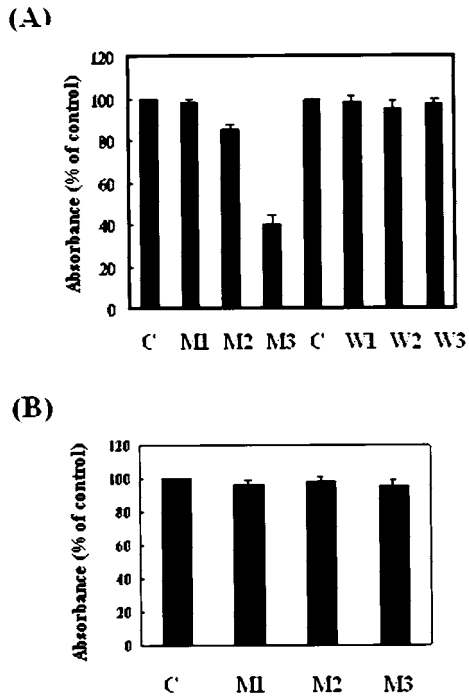

Figure 1. Effect of various extracts of adlay seed on the growth of A549 lung cancer cells and MRC-5 lung fibroblasts: (A) cells were cultured in methanolic extracts (M1, 33 μg/mL; M2, 100 μg/mL; M3, 330 μg/mL) or water extracts (W1, 33 μg/mL; W2, 100 μg/mL; W3, 330 μg/mL) for 48 h, and viable cell number was examined by MTT assays; (B) MRC-5 lung fibroblasts were incubated with different doses of methanolic extracts for 48 h, and viable cell number was examined by MTT assays. The experiments were repeated three times, and all determinations were done in triplicates. Results are shown as mean ± SE. The bars represent the standard error of the experiments.

RESULTS

Methanolic Extract of Adlay Seed at Low Doses Inhibits Proliferation of Lung Cancer Cells via Inhibition of Cyclin A Expression. To evaluate the inhibitory effect of different extracts of adlay seed on the proliferation of human lung cells, exponentially growing A549 cancer cells were cultured in 10% FCS medium containing different amounts of water or methanolic extract of adlay seed for 48 h and cell growth was determined by a MTT-based assay. The experiments were repeated three times, and all determinations were done in triplicates. As shown in **Figure 1A**, the methanolic extract, but not the water extract, inhibited the proliferation of A549 cells in a dose-dependent manner and the extract at 100 μg/mL blocked growth by 55−65% in these cells. We also examined the effect of this extract on MRC-5 human normal lung fibroblasts, and our results demonstrated that the methanolic extract of adlay seed did not show nonspecific inhibitory effect on MRC-5 cells (**Figure 1B**).

We next studied the mechanism by which the methanolic extract of adlay seed inhibited cell growth. Flow cytometric

Table 1. Inhibitory Effect of Methanolic Extract of Adlay Seed on Cell Cycle Progression of A549 Lung Cancer Cells

	cell cycle distribution (%)		
treatment	G0/G1	S	G2/M
vehicle	58.3	27.5	14.2
methanolic extract (100 μg/mL)	81.6	8.4	10.0

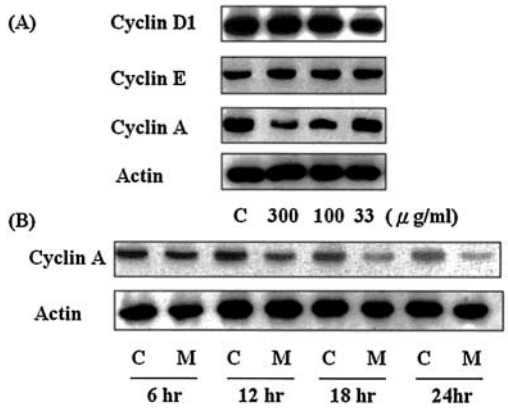

Figure 2. Effect of methanolic extracts of adlay seed on the expression of cell cycle regulatory proteins: (A) cells were treated with different doses of methanolic extracts for 24 h, and the expression of various cyclins was investigated by immunoblotting; (B) cells were incubated with vehicle (C) or 100 μg/mL methanolic extract (M) for different times, and the expression of cyclin A was determined by immunoblotting.

analysis was performed to investigate the effect of the extract on cell cycle distribution of A549 cells. Cells cultured in 10% FCS medium were incubated with 100 μg/mL of methanolic extract for 48 h. We found that the extract inhibited cell cycle progression at the G1/S transition (**Table 1**). Because the methanolic extract of adlay seed prevented S-phase entry in A549 cells, we studied whether G1/S-phase-related cell cycle regulatory proteins were changed after treatment of the extract. Whole cell lysates prepared from control or extract-incubated cells were subjected to SDS-PAGE and analyzed by immunoblotting. As shown in **Figure 2A**, we found that the extract could not affect cyclin D1 and E expression. On the contrary, the methanolic extract suppressed cyclin A expression in a dose-dependent manner. Additionally, our results also indicated that the methanolic extract inhibited cyclin A expression in a time-dependent manner (**Figure 2B**). We also investigated the effect of the methanolic extract on the expression of CDK2, -4, and -6, and our data indicated that CDK expression was not regulated by the extract (data not shown). These results suggest that the methanolic extract of adlay seed inhibits growth of lung cancer cells by suppressing the expression of cyclin A.

Induction of Apoptosis by Methanolic Extract of Adlay Seed. Because our results of MTT assays showed that significant reduction of cell viability was observed in cells treated with a high dose (300 μg/mL) of methanolic extract, we examined whether the extract can induce apoptosis in human lung cancer cells. We studied the induction of apoptosis in extract-treated cells by investigating the degradation of PARP. PARP is the first identified in vivo substrate for caspases, and its degradation is a typical marker of apoptosis. As shown in **Figure 3**, time-dependent degradation of PARP from 116 to 85 kDa was found in A549 cells treated with 300 μg/mL of methanolic extract. These results suggest that the methanolic extract of adlay seed at high dose can induce apoptosis in A549 cells.

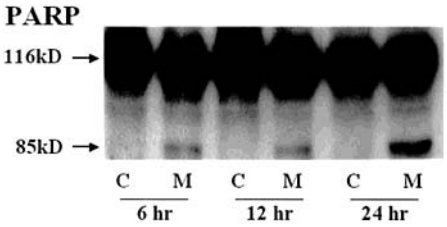

Figure 3. High dose of methanolic extract induces apoptosis in lung cancer cells. Cells were incubated with vehicle (C) or 330 μg/mL methanolic extract for different times, and PARP degradation, a biomarker for apoptotic cell death, was investigated by immunoblotting.

Table 2. Effect of Methanolic Extract of Adlay Seed on Tumor Growth in Nude Mice[a]

treatment	tumor volume (cm^3)	% of inhibition
vehicle ($n = 15$)	0.89 ± 0.18	
low dose ($n = 15$)	0.75 ± 0.06	16
high dose ($n = 15$)	0.33 ± 0.06	63

[a] Tumors were induced by subcutaneous (sc) injection of A549 cancer cells (2×10^6 cells in 0.1 mL of phosphate-buffered saline) at right flank. Animals were randomly distributed into the control group, which received vehicle (0.5% methanol), or the methanolic extract-treated group, which received a high dose (3 mg/kg) or a low dose (1 mg/kg) via ip injection every day for 45 days. Final tumor volume was calculated according to the formula given under Materials and Methods. Each group contains five experimental animals, and results from three independent experiments are expressed as mean ± SD.

Tumor Growth in Nude Mice Was Suppressed by the Methanolic Extract of Adlay Seed. We next tested the anticancer effect of methanolic extract in vivo. Tumors were induced by sc injection of A549 cells into nude mice. Mice were treated with vehicle (0.5% methanol), low-dose extract (1 mg/kg), or high-dose extract (3 mg/kg) via ip injection from 21 days after inoculation of tumor cells and continuously received treatment for 45 days. After experiments, mice were sacrificed and tumors were removed for calculation of tumor volume. Our results demonstrated that the methanolic extract at high dose suppressed tumor volume by 63% in nude mice (**Table 2**). Thus, the methanolic extract of adlay seed exerts an anticancer effect in vivo.

Feeding with Adlay Seed-Containing Diet Prevents Lung Tumorigenesis. Because the methanolic extract of adlay seed might inhibit proliferation and cause apoptosis of lung cancer cells, we addressed whether adlay seed might be a chemopreventive agent for lung tumorigenesis. A/J mice were prefed with diets containing different percentages of powdered adlay seed for 1 month, and lung tumor induction was initiated by administration of the tobacco-specific chemical carcinogen NNK in drinking water. Mice were fed with diets and NNK-containing water for 8 months. We found that the amounts of diet and the volumes of drinking water consumption in these three groups were similar (data not shown). After treatment, mice were sacrificed for analysis. Lungs were removed and stained with 15% India ink solution, and the number of surface lung tumors was calculated. Our data showed that uptake of adlay seed reduced lung tumors induced by NNK (**Figure 4**), and ~50% of reduction of lung tumors was observed in mice fed with diet containing 30% powdered adlay seed (**Table 3**). Organs from different experimental groups were obtained simultaneously for analysis. No significant differences of the weight of the organs were found (**Table 4**). In addition, no gross pathological changes were found in these organs (data not shown).

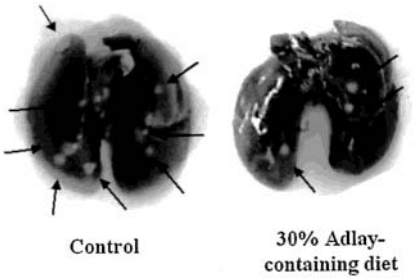

Figure 4. Surface lung tumors induced by NNK are reduced by feeding with adlay diet. A/J mice were treated as described in **Figure 4**. After treatment, mice were sacrificed and lungs were stained with a contrast medium (15% India ink in distilled water) by injecting the medium into the trachea. Lungs were then fixed in a fixation solution (100 mL of 70% alcohol, 10 mL of formaldehyde, and 5 mL of glacial acetic acid). Surface lung tumors are indicated by arrows.

Table 3. Chemopreventive Effect of Adlay Seed on NNK-Induced Lung Tumorigenesis[a]

treatment group	no. of surface lung tumors
A	7.2 ± 0.8
B	6.0 ± 0.5
C	3.6 ± 0.3

[a] A/J mice were randomly distributed into group A, which was fed with normal diet, or groups B and C, which were fed with diet containing 10 or 30% powdered adlay seed, respectively. Each group contained five experimental animals. Mice were pre-fed with different diets for 1 month, and treatment was initiated by administration of NNK (100 μg/mL) in drinking water. Animals were continuously fed with diets and NNK-containing drinking water for 8 months. The number of surface tumors was counted. This assay was repeated twice, and the results from two independent experiments were calculated.

Table 4. Comparison of the Organ Weight in Experimental Animals Fed with Different Diets[a]

treatment group	heart (g)	liver (g)	kidney (g)	spleen (g)
A	0.103 ± 0.026	0.978 ± 0.238	0.285 ± 0.055	0.073 ± 0.008
B	0.107 ± 0.015	1.010 ± 0.096	0.311 ± 0.027	0.080 ± 0.012
C	0.106 ± 0.019	1.088 ± 0.063	0.324 ± 0.014	0.082 ± 0.015

[a] Group A, normal diet; group B, 10% powdered adlay seed-containing diet; group C, 30% powdered adlay seed-containing diet; data shown as mean ± SD. No gross pathological change was observed in any groups.

DISCUSSION

In the present study, we studied the anticancer effect of adlay seed in vitro and in vivo. Our results demonstrate that a methanolic extract of adlay seed may inhibit proliferation and induce apoptosis in human lung cancer cells and suppress tumor growth in nude mice. The active components in the methanolic extract are unclear at present. However, previous studies have demonstrated that several classes of compound including phenolic compounds (such as vanillic acid, syringic acid, and *trans-p*-coumaric acid) and flavonoids (such as naringenin and tricin) are found in adlay seed (*19*, *20*). In addition, a chemical substance named coixenolide isolated from adlay seed has been reported to exert an anticancer effect in vitro (*21*, *22*). It is possible that these components may act synergistically to inhibit proliferation and induce apoptosis in cancer cells. However, it should be noted that concentrations of these compounds are low in adlay seed and may not be enough to exhibit potently an anticancer effect as observed in this study. Therefore, experiments are now being undertaken to identify the active components in the methanolic extract.

More importantly, our results demonstrate for the first time that uptake of adlay seed may prevent lung tumorigenesis. This chemopreventive action could be achieved by several mechanisms. First, the components that exert antiproliferative and apoptosis-inducing activities in lung cancer cells may directly inhibit tumor growth in experimental animals. Second, several components found in adlay seed are known to increase cytotoxic T and NK cells and may strengthen the immunity to against tumor development (*3*). Third, a number of components with potent antioxidant activity have also been identified in adlay seed. Because tobacco-specific carcinogens may induce oxidative damage to promote the development of lung cancer (*23*, *24*), it is rational to speculate that these antioxidant compounds may protect against oxidative damage and inhibit tumorigenesis. Taken together, our results suggest that the uptake of adlay seed may suppress growth of cancer cells in vitro and in experimental animals and may prevent lung cancer development induced by tobacco-specific carcinogens.

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Received for review November 21, 2002. Revised manuscript received March 21, 2003. Accepted March 21, 2003. This study was supported by Grants NSC 89-2316-B-037-005, NSC 90-2316-B-037-001, and NSC 91-2113-M-037-010 from the National Science Council of the R.O.C.

JF021142A