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Antiproliferative Lactams and Spiroenone from Adlay Bran in Human Breast Cancer Cell Lines

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ABSTRACT: Two new lactams, coixspirolactam D (**1**) and coixspirolactam E (**2**), and a new spiroenone, coixspiroenone (**3**), together with seven known compounds, coixspirolactam A (**4**), coixspirolactam B (**5**), coixspirolactam C (**6**), coixlactam (**7**), coixol (**8**), ethyl dioxindole-3-acetate (**9**), and isoindol-1-one (**10**), and two neolignans, zhepiresionol (**11**) and ficusal (**12**), were isolated from the bioactive subfraction of adlay bran ethanolic extract (ABE). Compounds **9** and **10** are the first isolates from natural resources. The structures of new compounds were identified by spectroscopic methods, including infrared (IR) spectrum, 1D and 2D nuclear magnetic resonance (NMR), and mass spectrum (MS). All of the isolated compounds were tested for antiproliferative effects on MCF-7, MDA-MB-231, and T-47D cells. Results showed that compounds **1**, **3**, **4**, **6**, and **7** at 50 μM significantly inhibited MCF-7 cell proliferation by 30.2, 19.2, 21.0, 13.5, and 32.4%, respectively; compounds **2**, **4**, and **7** significantly inhibited T-47D cells at 50 μM by 20.7, 24.8, and 28.9%; and compounds **1**, **2**, and **12** significantly inhibited MDA-MB-231 cells at 50 μM by 47.4, 25.3, and 69.3%, respectively. In conclusion, ABE has antiproliferative activities, and this effect is partially related to the presence of lactams and spiroenone.

KEYWORDS: Chinese herb, adlay bran, lactams, spiroenone, neolignans, antiproliferative, MCF-7 cells, T-47D cells

INTRODUCTION

Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is a grass crop and mainly planted in India, Taiwan, Japan, and China.[1] The seeds of adlay are also known as Chinese pearl barley and soft-shelled Job's tears. Adlay seeds have long been used in traditional Chinese medicine (TCM) to treat inflammation, dysfunctions of the endocrine system, warts, chapped skin, rheumatism, and neuralgia and also as a nourishing food.[2] Dehulled adlay (DA) is believed to be beneficial to humans, and many processed products from adlay are manufactured as healthy foods or food supplements.[1] The DA is composed of about 8% bran and 92% endosperm, and previous studies showed that the adlay bran (AB) possesses anti-inflammatory, antiproliferative, and antiallergic activities better than the endosperm.[3−5]

In line with the medicinal uses of adlay, many studies have been performed to evaluate its effects. A series of studies on antitumor-related activities of adlay seeds have been reported.[6−14] Coixenolide,[6] fatty acids,[7] and a neutral lipid extract[12−14] have been claimed as its active components. Lee et al. reported that a methanolic extract of AB had antitumor and anti-inflammatory activities[3] and that cytotoxic lactams have been isolated.[4] Additionally, our previous study showed that the ethyl acetate (EA) fraction of AB ethanolic extract (ABE-EA) suppressed aberrant crypt foci (ACF) in dimethyl hydrazine (DMH)-induced colon carcinogenesis.[15] The modulatory effects in the endocrine system of adlay seeds have been reported in several papers.[16,17] In a continuation of this investigation, we herein report the isolation, structural elucidation, and antiproliferative activity on human breast cancer cells of various γ-lactams and spiroenone isolated from AB. A liquid chromatography (LC)−mass spectrum (MS) profile of ABE was also created as an analytical platform for the above components.

MATERIALS AND METHODS

General Experimental Procedures. The MCF-7, T-47D, MDA-MB-231, and H184B5F5/M10 cell lines were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Fetal bovine serum (FBS), minimum essential medium (MEM), MEM-α, Leibovitz's L-15, and RPMI-1640 medium were purchased from GIBCO (Grand Island, NY). Penicillin, streptomycin, glutamine, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), and other chemicals were obtained from Sigma (St. Louis, MO). Silica gel (230−400 mesh, Merck, Darmstadt, Germany), Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), and a semi-preparative Si column (LiChrosorb Si-60, Merck) were used for column chromatography. Dimethyl sulfoxide (DMSO) and other solvents (analytical grade) were purchased from Merck. Infrared (IR) spectra were recorded on a Nicolet Avatar 320 FTIR spectrophotometer (Thermo Electron, Akron, OH). Optical rotation was measured on a Jasco P-2000 polarimeter (Hachioji, Tokyo, Japan). Nuclear

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magnetic resonance (NMR) spectra were run in $CDCl_3$ or CD_3OD on a Varian unity INOVA-500 or VNMRS 600 (Varian, Palo Alto, CA). Electrospray ionization (ESI)−mass spectra (MS) were recorded on a Finnigan MAT LCQ ion-trap mass spectrometer system (Thermoquest, San Jose, CA).

Extraction and Fractionation. Adlay seeds (Taichung Shuenyu No. 4) were purchased from Taichung, Taiwan, 2009. The dried seeds were divided into the hull, testa, bran, and endosperm by a grinder and gently blown with an electric fan. A total of 25 kg of AB powder was soaked in 125 L of 95% ethanol at room temperature for 48 h. The ethanolic extract was combined and concentrated under reduced pressure at 50 °C to give 2834 g of ABE (10.9% of AB). The ABE was suspended in H_2O, which was followed by sequential partitioning with *n*-hexane (Hex), ethyl acetate (EA), and 1-butanol (BuOH) to give Hex-, EA-, BuOH- (ABE-BuOH, 42 g), and water-soluble fractions (ABE-H_2O, 258 g) according to the reported literature.[3] The ABE-Hex was then repartitioned with 70% ethanol/H_2O to extract the higher polar components in this fraction, and the 70% ethanol/H_2O fraction was combined with ABE-EA to give 1720 g of ABE-Hex (60.7% of ABE) and 204 g of ABE-EA (7.2% of ABE). The four fractions were screened for antiproliferative potency on MCF-7 and MDA-MB-231 cells.

Cell Lines and Culture. MCF-7, T-47D, and H184B5F5/M10 cells were cultured with 5% CO_2 atmosphere at 37 °C; MDA-MB-231 cells were cultured without CO_2 at 37 °C. The MCF-7 cells were cultured in MEM containing 10% heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate. The T-47D cells were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The H184B5F5/M10 cells were grown in MEM-α medium containing 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin as previously described.[18] The MDA-MB-231 cells were cultured in Leibovitz's L-15 containing 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.[12]

Assay for Antiproliferation. To determine the antiproliferative effect, MCF-7, T-47D, MDA-MB-231, or H184B5F5/M10 cells were assessed using the MTT assay as previously reported.[18] All of the test samples mentioned above were dissolved in DMSO, and the final concentration of DMSO was ≤0.1%. In addition, 0.1% DMSO was used as a blank vehicle. MCF-7, T-47D, MDA-MB-231, or H184B5F5/M10 cells were cultured in 96-well plates at a density of 1×10^5 cells/well and allowed to grow for 18−24 h. After this, the medium was replaced by FBS-free MEM, RPMI-1640, L-15, or MEM-α; then the various test samples in DMSO were added, and the cells were incubated for an additional 24 h. Next, MTT solution in FBS-free MEM, RPMI-1640, L-15, or MEM-α, which had been filtered through a 0.45 mm membrane, was added to each well (final concentration of 0.5 mg/mL). The plates were incubated under a 5% CO_2 atmosphere at 37 °C for 20 min. The medium with unreacted dye was removed. DMSO was added into each well to dissolve the MTT formazan crystals, and the samples were incubated at room temperature for 5−10 min. The absorbance at 570 nm was measured, and the viability of cells was calculated using the following equation: relative cell number (%) = (average absorbance of treated wells) × 100/(average absorbance of blank vehicle wells).

Isolation and Purification Process. The total ABE-EA fraction was subjected to silica gel column chromatography and elution with a Hex/EA/MeOH gradient solvent system to give five subfractions, namely, low polarity waste (20% EA/Hex eluate), ABE-EA-A (40% EA/Hex eluate), ABE-EA-B (70% EA/Hex eluate), ABE-EA-C (100% EA eluate), and ABE-EA-D (100% MeOH eluate). The ABE-EA-A and ABE-EA-B were combined (47.1 g, 24.5% of ABE-EA) and further chromatographyed over a Sephadex LH-20 column (MeOH) to give a lactam-containing fraction (LCF, 13.2 g) by the Dragendorff reagent.[17] The LCF subfraction was further purified over a Saphadex LH-20 column using an EA/CH_2Cl_2 gradient to yield the following subfractions: 5% EA/CH_2Cl_2 (LCF-A, 0.91 g, 6.82% of LCF), 10% EA/CH_2Cl_2 (LCF-B, 2.14 g, 16.22% of LCF), 20% EA/CH_2Cl_2 (LCF-C, 2.21 g, 16.65% of LCF), 40% EA/CH_2Cl_2 (LCF-D, 3.59 g, 33.28% of LCF), 60% EA/CH_2Cl_2 (LCF-E, 0.92 g, 6.96% of LCF), 80% EA/CH_2Cl_2 (LCF-F, 0.82 g, 6.22% of LCF), EA (LCF-G, 0.54 g, 5.11% of LCF), and methanol (LCF-H, 1.64 g, 12.42% of LCF). The 40−60% EA/CH_2Cl_2 subfractions from the LCF were regarded as the lactam-enriched fraction (LEF) as previously described[4] and were further purified by Sephadex LH-20 column chromatography (MeOH) and preparative high-performance liquid chromatography (HPLC) on a LiChrosorb Si-60 column (5 μm, Agilent, Santa Clara, CA) using EA/acetone/Hex = 1:2:5, 1:2:4, or 1:2:3 as the mobile phase. A total of 12 compounds, coixspirolactam D (**1**) (3.4 mg), coixspirolactam E (**2**) (3.0 mg), coixspiroenone (**3**) (2.7 mg), coixspirolactam A (**4**) (4.5 mg), coixspirolactam B (**5**) (3.2 mg), coixspirolactam C (**6**) (5.4 mg), coixlactam (**7**) (2.8 mg), coixol (**8**) (3.8 mg), ethyl dioxindole-3-acetate (**9**) (2.7 mg), isoindol-1-one (**10**) (1.8 mg), zhepiresionol (**11**) (1.8 mg), and ficusal (**12**) (2.4 mg), were isolated. Their structures are shown in Figure 1.

*Coixspirolactam D (**1**).* Colorless oil. $[\alpha]_D^{25}$ + 6.5 (*c* 0.14, MeOH). ESI−MS *m*/*z* (%): 244 (100, $[M-H]^-$). IR (KBr) ν_{max} (cm^{-1}): 3275, 1786, 1727, 1624, 1473, 1362, 1216. The ^1H and ^{13}C NMR data are presented in Table 1, and the key heteronuclear multiple-bond correlations (HMBCs) and nuclear Overhauser enhancement spectroscopy (NOESY) correlations are shown in Figure 2.

*Coixspirolactam E (**2**).* Colorless oil. $[\alpha]_D^{25}$ − 4.8 (*c* 0.20, MeOH). ESI−MS *m*/*z* (%): 244 (100, $[M-H]^-$). IR (KBr) ν_{max} (cm^{-1}): 3283, 1794, 1730, 1619, 1473, 1362, 1211. The ^1H and ^{13}C NMR data are presented in Table 1, and the key HMBCs and NOESY correlations are shown in Figure 2.

*Coixspiroenone (**3**).* Colorless oil. $[\alpha]_D^{25}$ ± 0 (*c* 0.20, MeOH). UV (MeOH) λ_{max}, nm (log ε): 243 (4.20). ESI−MS *m*/*z* (%): 255 (100, $[M+Na]^+$). IR (KBr) ν_{max} (cm^{-1}): 1711, 1659, 1624, 1196, 1105. The ^1H and ^{13}C NMR data are presented in Table 1, and the key HMBCs and NOESY correlations are shown in Figure 2.

*Coixspirolactam A (**4**).* Pale yellow oil. ESI−MS *m*/*z* (%): 202 (100, $[M-H]^-$). ^1H NMR ($CDCl_3$, 600 MHz) δ: 2.69 (1H, d, *J* = 17.4 Hz, H-8_{endo}), 3.15 (1H, d, *J* = 17.4 Hz, H-8_{exo}), 4.17 (1H, d, *J* = 9.0 Hz, H-10_{endo}), 4.58 (1H, d, *J* = 9.0 Hz, H-10_{exo}), 6.91 (1H, d, *J* = 8.4 Hz, H-7), 7.10 (1H, t, *J* = 8.4 Hz, H-6), 7.22 (1H, t, *J* = 8.4 Hz, H-5), 7.29 (d, *J* = 8.4 Hz, H-4), 7.38 (1H, brs, −NH).

*Coixspirolactam B (**5**).* Pale yellow oil. ESI−MS *m*/*z* (%): 216 (100, $[M-H]^-$). ^1H NMR ($CDCl_3$, 600 MHz) δ: 1.35 (3H, d, *J* = 6.6 Hz, H-12), 2.90 (1H, d, *J* = 18.0 Hz, H-8_{endo}), 3.00 (1H, d, *J* = 18.0 Hz, H-8_{exo}), 4.71 (1H, q, *J* = 6.6 Hz, H-11), 6.89 (1H, d, *J* = 7.8 Hz, H-7), 7.10 (1H, t, *J* = 7.8 Hz, H-6), 7.27 (1H, d, *J* = 7.8 Hz, H-4), 7.30 (1H, t, *J* = 7.8 Hz, H-5), 7.32 (1H, brs, −NH).

*Coixspirolactam C (**6**).* Pale yellow oil. ESI−MS *m*/*z* (%): 216 ($[M-H]^-$, 100). ^1H NMR ($CDCl_3$, 600 MHz) δ: 1.09 (3H, d, *J* = 6.6 Hz, H-12), 2.68 (1H, d, *J* = 17.4 Hz, H-8_{endo}), 3.31 (1H, d, *J* = 17.4 Hz, H-8_{exo}), 4.86 (1H, q, *J* = 6.0 Hz, H-11), 6.93 (1H, d, *J* = 7.8 Hz, H-7), 7.10 (1H, t, *J* = 7.8 Hz, H-5), 7.21 (1H, d, *J* = 7.8 Hz, H-4), 7.30 (1H, t, *J* = 7.8 Hz, H-6), 7.63 (1H, brs, −NH).

*Coixlactam (**7**).* Pale yellow oil. ESI−MS *m*/*z* (%): 228 (100, $[M+Na]^+$). ^1H NMR ($CDCl_3$, 500 MHz) δ: 2.82 (1H, dd, *J* = 16.5, 7.8 Hz, H-4_{eq}), 3.07 (1H, dd, *J* = 16.5, 4.5 Hz, H-4_{ax}), 3.68 (3H, s, $-OCH_3$), 3.79 (1H, dd, *J* = 7.8, 4.5 Hz, H-3), 6.84 (1H, d, *J* = 8.0 Hz, H-8), 7.00 (1H, t, *J* = 8.0 Hz, H-7), 7.20 (1H, t, *J* = 8.0 Hz, H-6), 7.21 (1H, d, *J* = 8.0 Hz, H-5), 7.33 (1H, brs, −NH).

*Coixol (**8**).* Pale yellow oil. ESI−MS *m*/*z* (%): 164 (100, $[M-H]^-$). ^1H NMR ($CDCl_3$, 500 MHz) δ: 3.78 (3H, s, $-OCH_3$), 6.39 (1H, dd, *J* = 8.4, 1.8 Hz, H-6), 6.89 (1H, d, *J* = 1.8 Hz, H-4), 6.96 (1H, d, *J* = 8.4 Hz, H-7).

*Ethyl Dioxindole-3-acetate (**9**).* Pale yellow oil. ESI−MS *m*/*z* (%): 258 (100, $[M+Na]^+$). IR (KBr) ν_{max} (cm^{-1}): 3370, 1731, 1711, 1620, 1520, 1469, 1192. The ^1H and ^{13}C NMR data are presented in Table 1.

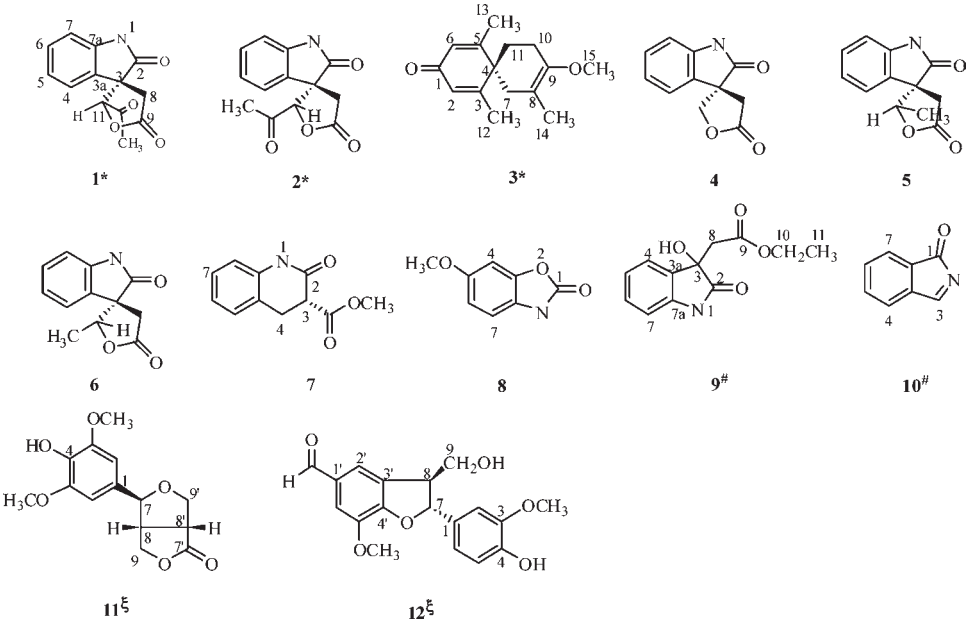

Figure 1. Structures of compounds **1**−**12**. (∗) Compounds that were previously undocumented. (#) Compounds that were isolated from a natural resource in this study for the first time. (ζ) Compounds that were isolated from adlay for the first time in this study.

Table 1. ^{1}H and ^{13}C NMR Spectral Data (500 and 125 MHz) for Compounds 1, 2, and 3 in $CDCl_3$, while That of Compound 9 Was in CD_3OD (600 and 150 MHz)[a]

	1		2		3		9	
compound carbon number	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	7.76 br s		7.89 br s			168.1 s		
2		172.2 s		172.4 s	6.05 s	126.6 d		180.9 s
3		53.4 s		53.7 s		158.0 s		74.8 s
3a		128.9 s		127.6 s				131.7 s
4	7.25 d (7.5)	122.6 d	7.12 d (7.5)	122.7 d		52.4 s	7.34 d (7.5)	130.9 d
5	7.12 t (7.5)	123.7 d	7.27 t (7.5)	123.4 d		163.3 s	7.02 t (7.5)	123.5 d
6	7.30 t (7.5)	129.8 d	7.03 t (7.5)	130.1 d	6.05 s	126.6 d	7.23 t (7.5)	125.2 d
7	6.90 d (7.5)	110.5 d	6.91 d (7.5)	110.8 d	2.66 s	43.2 t	6.87 d (7.5)	111.2 d
7a		140.4 s		140.1 s				143.6 s
8	3.09 d (18.0)$_{exo}$ 2.88 d (18.0)$_{endo}$	39.1 t	3.29 d (17.5)$_{exo}$ 2.74 d (17.5)$_{endo}$	39.7 t		119.9 s	3.04 dd (15.0, 13.0)	42.9 t
9		176.7 s		175.0 s		168.1 s		170.4 s
10					3.16 t (8.4)	34.2 t	3.89 q (4.8)	61.6 t
11	4.88 s	86.6 d	5.05 s	86.8 d	2.07 t (8.4)	35.7 t	1.00 t (7.3)	14.1 q
12		205.0 s		202.7 s	1.97 s	20.2 q		
13	2.35 s	28.4 q	2.06 s	28.0 q	1.97 s	20.2 q		
14					1.87 s	16.3 q		
15					3.74 s	51.4 q		

[a] d, doublet; t, triplet; s, singlet; br s, broad singlet; dd, doublet of doublet; q, quartet. Chemical shifts are in δ (ppm). The coupling constants (J) are given in parentheses (Hz).

*Isoindol-1-one (**10**).* Pale yellow oil. ESI−MS *m*/*z* (%): 132 (100, $[M + H]^+$). ^{1}H NMR (CD_3OD, 500 MHz) δ: 7.15 (1H, t, J = 7.5 Hz, H-6), 7.18 (1H, t, J = 7.5 Hz, H-5), 7.42 (1H, d, J = 7.5 Hz, H-4), 7.92 (1H, d, J = 1.5 Hz, H-3), 8.06 (1H, dd, J = 7.5, 1.5 Hz, H-7).

*Zhepiresionol (**11**).* Colorless amorphous powder. ESI−MS *m*/*z* (%): 279 (100, $[M - H]^-$). ^{1}H NMR (CD_3OD, 500 MHz) δ: 3.08 (1H, m, H-8), 3.42 (1H, m, H-8′), 3.89 (6H, s, $-OCH_3 \times 2$), 4.18 (1H, dd, J = 3.8, 9.0 Hz, H-9′α), 4.33 (1H, t, J = 9.5 Hz, H-9α), 4.37 (1H, t, J = 9.0 Hz, H-9′β), 4.49 (1H, dd, J = 9.5, 7.6 Hz, H-9β), 4.57 (1H, d, J = 7.0 Hz, H-7) 5.51 (1H, br s, 1H, −OH), 6.55 (2H, s, H-2, H-6).

*Ficusal (**12**).* Colorless amorphous powder. UV (MeOH) λ_{max}, nm (log ε): 233 (1.76), 288 (0.94). ESI−MS *m*/*z* (%): 329 (100, $[M - H]^-$). ^{1}H NMR (acetone-d_6, 500 MHz) δ: 3.66 (1H, m, H-8), 3.82 (3H, s, 3-OCH_3), 3.92 (3H, s, 5′-OCH_3), 3.90−3.94 (2H, m, H-9), 5.69 (1H, d,

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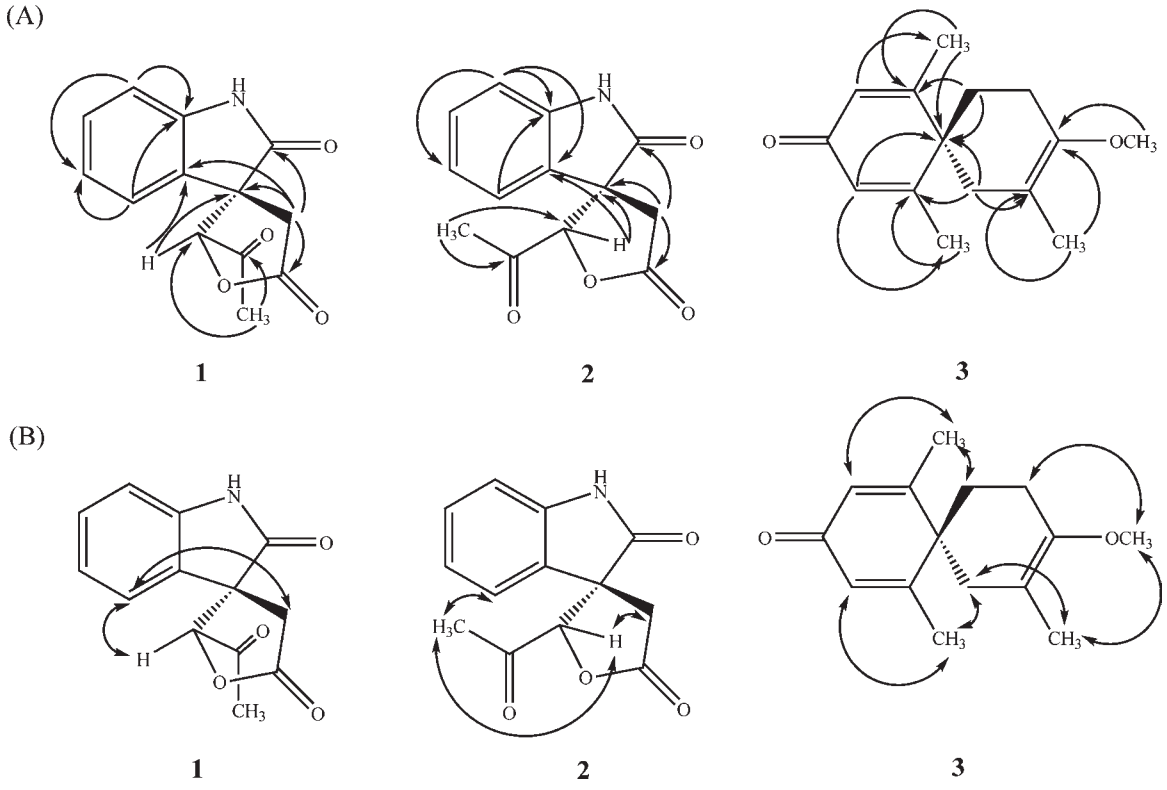

Figure 2. Key (A) HMBCs (→) and (B) NOESY correlations (↔) of compounds **1**, **2**, and **3**.

J = 7.0 Hz, H-7), 6.82 (1H, d, *J* = 8.0 Hz, H-5), 6.89 (1H, dd, *J* = 8.0, 2.4 Hz, H-6), 7.05 (1H, d, *J* = 2.4 Hz, H-2), 7.42 (1H, s, H-6′), 7.52 (1H, s, H-2′), 7.67 (1H, br s, −OH), 9.83 (1H, s, H-7′).

LC−MS Analysis. HPLC analysis was performed using a Finnign MAT pump (P4000, Finnign) with a ultraviolet (UV) detector (UV2000, Finnign). Gradient elution was performed with 0.01% formic acid aqueous solution (v/v, pH 3.30, A) and 50% acetonitrile (ACN) in methanol (v/v, B) at a constant rate of 0.3 mL/min through an ODS-3 (250 × 4.6 mm, 5 μm) reverse-phase column (Inertsil). Initial starting conditions were 20% B, at 0−10 min; increase from 20 to 40% B, at 10−30 min; increase from 40 to 60% B, at 30−40 min; increase from 60 to 75% B, at 40−50 min; increase from 75 to 78% B, at 50−60 min; increase from 78 to 80% B, at 60−70 min; increase from 80 to 100% B, at 70−80 min; and finally, decrease from 100 to 20% B, at original conditions. The absorbance was set at 280 nm. This system was coupled with a Finnigan MAT LCQ ion-trap mass spectrometer system (Finnigan MAT, San Jose, CA), which was operated in the ESI mode. An aliquot of the bioactive fraction (10 mg/mL, 20 μL) was directly introduced into the column through the autosampler (Finnigan MAT AS3000), with nitrogen being used as the nebulizing and drying gas. The operating parameters used were as follows: a gas temperature of 250 °C, a spray needle voltage of 5 kV, a nebulizer pressure of 60 psi, and an auxiliary gas pressure of 30 psi. An ion trap containing helium damping gas was introduced following the protocols of manufacturer. The mass spectra were acquired in a *m*/*z* range of 100−1000 with 5 microscans and a maximum ion injection time of 200 min. The isolated compounds were confirmed with a retention time (t_R) and were qualified by MS using either a negative or positive mode.

Statistical Analysis. The bar values are present as the mean ± standard deviation (SD) from three independent experiments. The results among groups were analyzed by analysis of variance (ANOVA) and Student's *t* test. A *p* value of <0.05 is considered to show a significant difference.

RESULTS AND DISCUSSION

Effects of ABE and the Various Separated Fractions on the Growth of Human Breast Cancer Cells. Adlay seeds have long been used for treating tumor and endocrine system disorders in TCM.[2] The antiproliferative effects of adlay on various tumor cells,[3,6−15] including estrogen receptor (ER)-negative human breast cancer cells, MDB-MB-231, have been explored.[12] The influence of adlay seeds on hormone secretion has also been investigated.[16,17] Nonetheless, the effects of AB on endocrine-related cancer cells remain unclear. Human breast cancer cells that express ER and prostate cancer cells that express androgen receptor (AR) are closely involved with cells that secrete hormone; they are regarded as benign but possibly may turn into a malignant tumor.[18] Our previous study demonstrated that AB shows more potent antiproliferative activity on cancer cells than DA.[3−5] In a further study of the antiproliferation of AB on MCF-7 cells, which is an ER-positive metastatic adenocarcinoma cell line with wild-type p53 protein, on T-47D cells, which is an ER-positive ductal carcinoma but with a mutant p53 protein, and on MDA-MB-231 cells, which is an ER-negative carcinoma, involved evaluation of the antiproliferative effects of various subfractions and isolated compounds from ABE. In these experiments, H184B5F5/M10 cells are normal human breast epithelia cells used as a control.

As can be seen in Figure 3, ABE-EA and ABE-BuOH show significant antiproliferative effects on MCF-7 and MDA-MB-231 cells. Our previous studies have demonstrated that ABE-EA shows more potent activities than other fractions;[3,10,11] at the same time, the quantitative proportion of ABE-EA in ABE is much greater than ABE-BuOH. Therefore, ABE-EA was used for further fractionation. Subfractions ABE-EA-A−ABE-EA-D showed

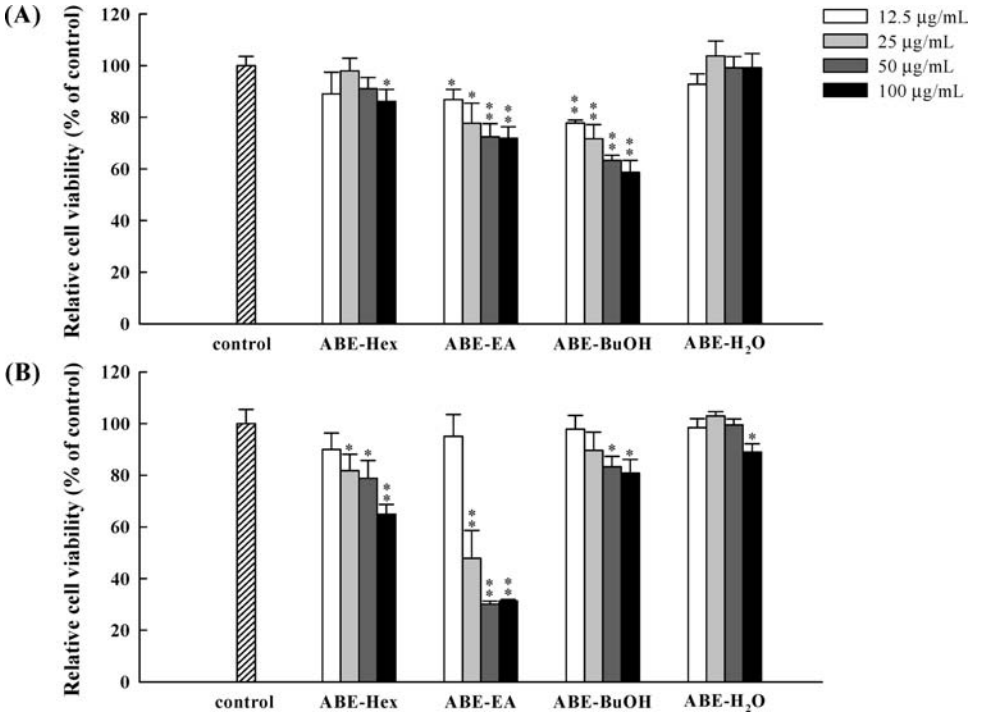

Figure 3. Effects of ABE and partitioned fractions on the cell proliferation in (A) MCF-7 and (B) MDA-MB-231 cell lines. Cells were cultured with or without test samples for 24 h and examined using the MTT assay. The results are expressed as a percentage of living cells cultured in the presence of the test samples relative to a parallel culture that did not receive any treatment. Each bar represents the mean ± SD (n = 3). (∗) $p < 0.05$ compared to the control group. (∗∗) $p < 0.01$ compared to the control group.

significant inhibitory effects on human breast cancer cell lines at concentrations of 25−100 μg/mL compared to the control (Figure 4). However, the ABE-EA-C and ABE-EA-D also suppressed the viability of H184B5F5/M10 cells at 100 μg/mL. On the basis of the above result, ABE-A and ABE-B (40−70% EA/Hex eluate) were selected to isolate the antiproliferative components. LCF, Drogendorff's reagent positive subfraction, was obtained and subjected to further purification.

Compound Isolation and Structural Elucidation. The LCF was further purified by Sephedex LH-20 column chromatography (CC) and semi-preparative HPLC over a Si-60 column to yield three new compounds, coixspirolactam D (**1**), coixspirolactam E (**2**), and coixspiroenone (**3**), along with seven known lactams, coixspirolactam A (**4**),[4] coixspirolactam B (**5**),[4] coixspirolactam C (**6**),[4] coixlactam (**7**),[4] coixol (**8**),[19] ethyl dioxindole-3-acetate (**9**),[20] and isoindol-1-one (**10**),[21] as well as two lignan derivatives, zhepiresionol (**11**)[22] and ficusal (**12**).[23] Compounds **9** and **10** are the first isolates from natural resources. Each known compound was identified by NMR and MS spectroscopic analyses and a comparison to authentic samples or published data. Among them, compounds **9** and **10** have been synthesized[20,21] and, here, were isolated for the first time from natural resources. Compounds **11** and **12** were isolated for the first time from adlay.

Coixspirolactam D (**1**) was isolated as a colorless oil and gave a *quasi*-molecular ion at m/z 244 ($[M - H]^-$). The IR spectrum displayed absorption bands for amino (3275 cm^{-1}), γ-lactone carbonyl (1786 cm^{-1}), γ-lactam carbonyl (1727 cm^{-1}), and aromatic (1624 and 1473 cm^{-1}) groups.[4] The ^{1}H NMR spectral data (Table 1) showed signals for a acetyl methyl group [δ_H 2.35 (3H, s, H-13)], two methylene protons [δ_H 3.09 (1H, d, J = 18.0 Hz, H_{exo}-8) and 2.88 (1H, d, J = 18.0 Hz, H_{endo}-8)], an oxygenated methine proton [δ_H 4.88 (1H, s, H-11)], one set of 1,2-disubstituted benzene protons [δ_H 7.30 (1H, t, J = 7.5 Hz, H-6), 7.25 (1H, d, J = 7.5 Hz, H-4), 7.12 (1H, t, J = 7.5 Hz, H-5), and 6.90 (1H, d, J = 7.5 Hz, H-7)], and one D_2O exchangeable N−H proton [δ_H 7.76 (1H, br s)]. The ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra indicated the presence of 13 carbons, including an acetyl group [δ_C 205.0 (s) and 28.4 (q)], two carbonyls (δ_C 176.7 and 172.2), four aromatic methines [δ_C 129.8, 123.7, 122.6, and 110.5], two quaternary sp^2 carbons (δ_C 140.4 and 128.9), one oxygenated methine (δ_C 86.6), one methylene (δ_C 39.1), and one quaternary sp^3 carbon (δ_C 53.4). These results suggested that compound **1** had the same lactam structure as coixspirolactam B (**5**),[4] with the exception of one additional acetyl group at C-11. The planar structure and acetyl location were further confirmed by the HMBCs (Figure 2) from H-4 to C-5, C-6, C-3, C-3a, and C-7a; from H-7 to C-3a, C-5, C-6 and C-7a; from H-8 to C-2, C-3, C-3a, C-11, and C-9; from H-11 to C-2, C-3, and C-3a; and from H-13 to C-11 and C-12. The presence of the NOESY correlation (Figure 2) between H-4 and H-11 determined the relative configuration of C-11 to be in a similar orientation as coixspirolactam B (**5**).[4] From the above evidence, the structure of compound **1** was elucidated as shown in Figure 1 and named coixspirolactam D.

Compound **2** had the same molecular ion peak at m/z 244 ($[M - H]^-$). The similar IR and ^{1}H and ^{13}C NMR spectra showed that compound **2** possessed the same γ-lactam structure, with the only difference being the orientation of the acetyl group. The HMBCs (Figure 2) of compound **2** exhibited correlations as follows: H-8/C-2, C-3, C-3a, C-9, and C-11 and H-11/C-2, C-3, C-3a, C-8, C-9, C-12, and C-13. The NOESY experiments found correlations between H-4 [δ_H 7.12 d (7.5)] and acetyl

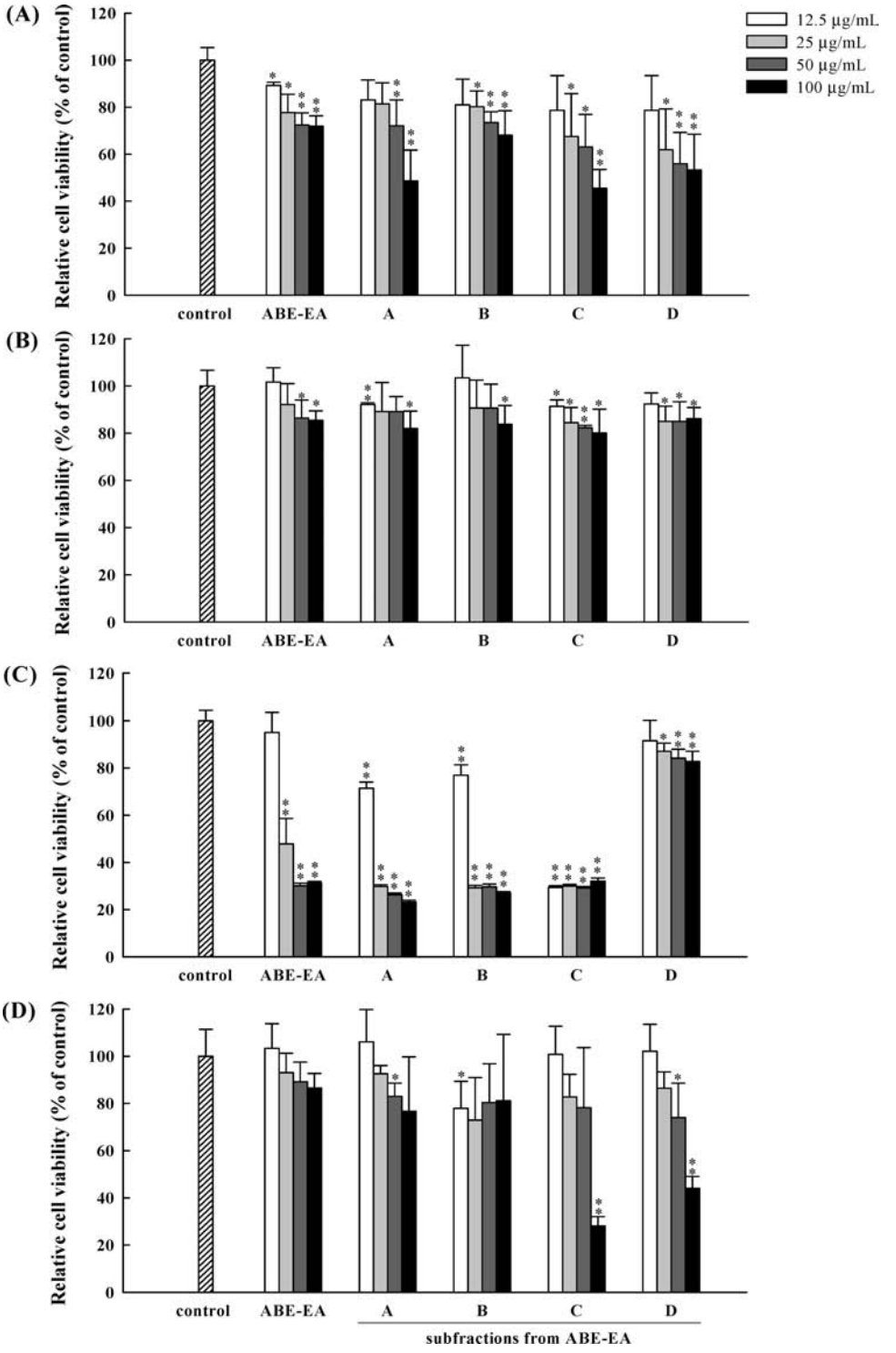

Figure 4. Effects of ABE-EA and its subfractions on the cell proliferation in (A) MCF-7, (B) T-47D, (C) MDA-MB-231, and (D) H184B5F5/M10 cells. Cells were cultured with or without test samples for 24 h and examined using the MTT assay. The results are expressed as a percentage of living cells cultured in the presence of test samples relative to a parallel culture that did not receive any treatment. Each bar represents the mean ± SD ($n = 3$). (*) $p < 0.05$ compared to the control group. (**) $p < 0.01$ compared to the control group.

(δ_H 2.06 s) and H-8$_{endo}$. Because of the shielding effect from the aromatic ring, the acetyl group was in a higher field than the corresponding protons in compound **1**. The evidence confirmed that acetyl and phenyl groups are on the same side. Therefore, the structure of compound **2** was established as a C-11 epimer of compound **1** and named coixspirolactam E.

Coixspiroenone (**3**) was isolated as a colorless oil. The molecular formula was suggested to be $C_{15}H_{20}O_2$ by ESI−MS, The combination of the molecular formula, ^{13}C NMR, and DEPT data confirmed compound **3** with six indices of hydrogen deficiency. The IR and UV spectra showed the presence of an α, β-unsaturated carbonyl (ν_{max}, 1711 and 1659 cm^{-1}; and λ_{max},

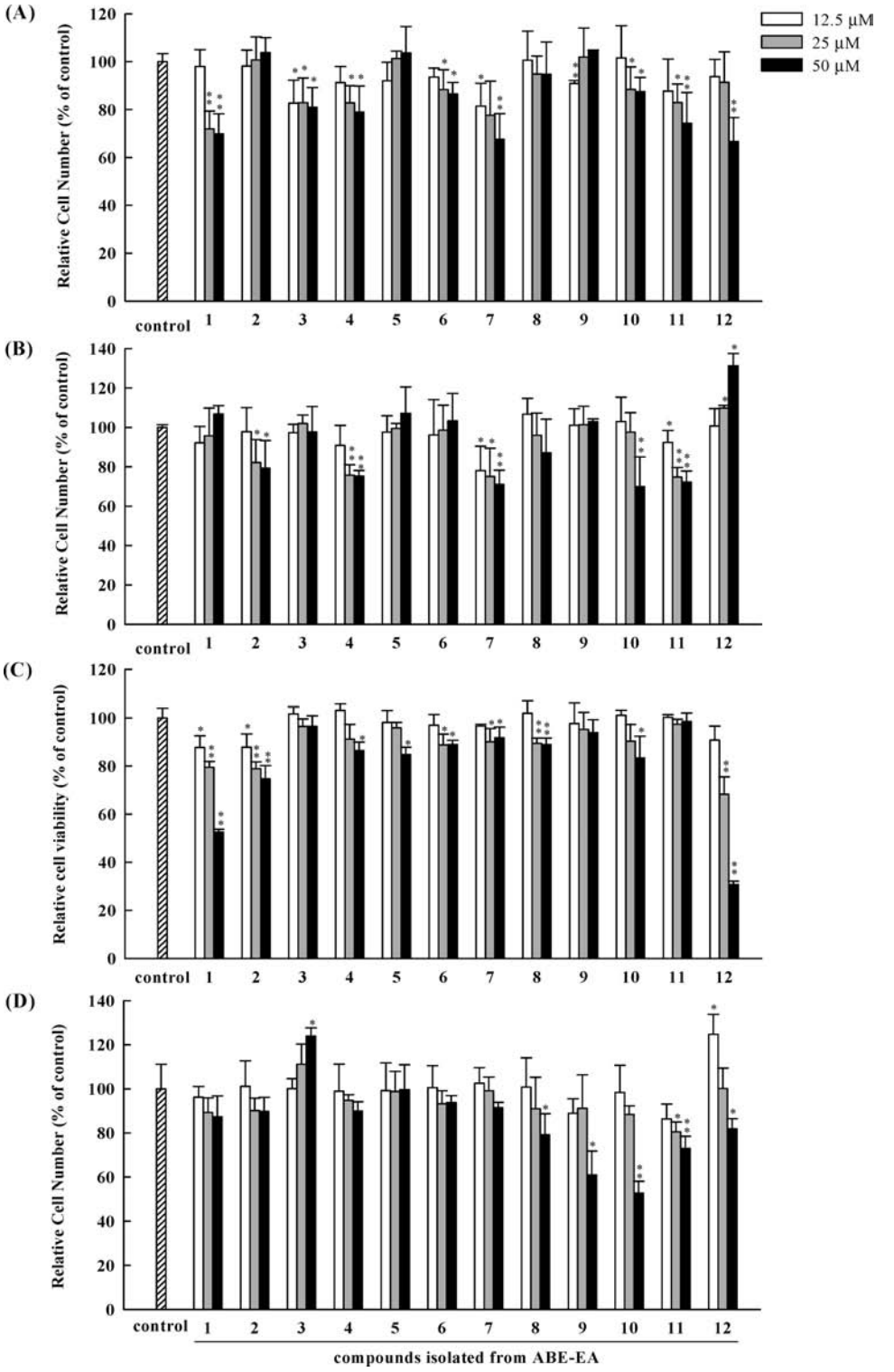

Figure 5. Effects of isolated compounds on the cell proliferation of (A) MCF-7, (B) T-47D, (C) MDA-MB-231, and (D) H184B5F5/M10 cells. The cells were cultured with or without test samples for 24 h and examined using the MTT assay. The results are expressed as a percentage of living cells cultured in the presence of test samples relative to a parallel culture that did not receive any treatment. Each bar represents the mean ± SD ($n = 3$). (∗) $p < 0.05$ compared to the control group. (∗∗) $p < 0.01$ compared to the control group.

243 nm, respectively). The ^{1}H and ^{13}C NMR (Table 1) showed three olefinic tertiary methyl groups [δ_H 1.97 (6H, s), δ_C 20.2 q; δ_H 1.87 (3H, s), δ_C 16.3 q], two dienone conjugated α-position olefinic protons [δ_H 6.05 (2H, s), δ_C 126.6 d], one methoxy group [δ_H 3.74 (3H, s), δ_C 51.4 q], and three methylene groups, including two mutual coupling methylene groups [δ_H 2.07 and 3.16 (2H each, t), δ_C 35.7 and 34.2 t, respectively] and one singlet methylene group [δ_H 2.66 (2H, s), δ_C 43.2 t], identified by COSY and HMQC spectra. The HMBC spectrum indicated correlations as follows: H-14 [δ_H1.87 (3H, s)]/C-7 (δ_C 43.2 t),

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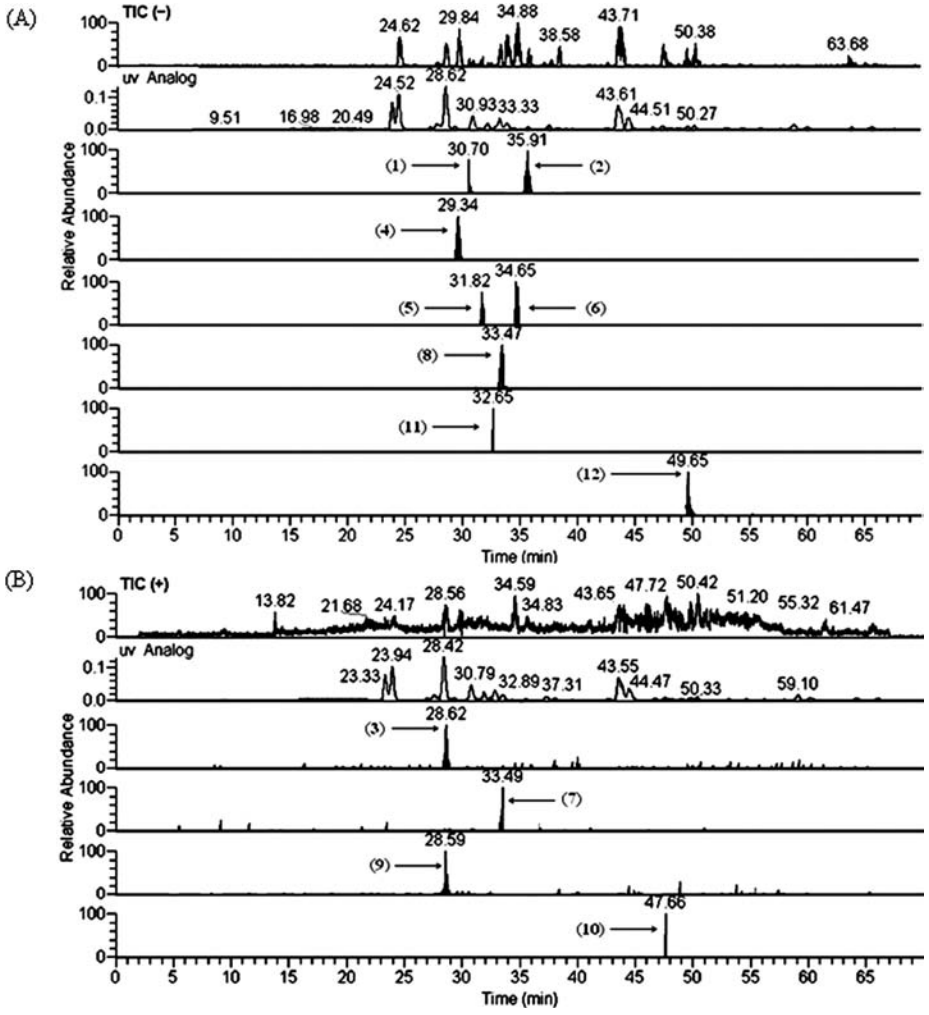

Figure 6. LC−MS analytical spectrum of (A) total negative-ion currents and UV chromatograms and (B) total positive-ion currents and UV chromatograms of ABE-EA-LEF and the compounds coixpirolactam D (**1**), coixspirolactam E (**2**), coixspiroenone (**3**), coixspirolactam A (**4**), coixspirolactam B (**5**), coixspirolactam B (**6**), coixspirolactam C (**7**), coixol (**8**), ethyl dioxindole-3-acetate (**9**), isoindol-1-one (**10**), zhepiresionol (**11**), and ficusal (**12**).

C-8 (δ_C 119.9 s)], and C-9 (δ_C 168.1 s); H-12 (H-13) [δ_H1.97 (6H, s)]/C-2 (C-6) (δ_C 126.6 d), C-3 (δ_C 158.0 s), C-4 (δ_C 52.4 s), and C-5 (δ_C 163.3 s); methoxyl group [δ_H 3.74 (3H, s)]/C-9 (δ_C 168.1 s); H-7 (δ_H 2.66 s)/C-3, C-4, C-5, C-8, C-9, and C-11 (δ_C 35.7 t); and H-11 [δ_H 2.07 t (8.4)]/C-3, C-4, C-5, C-7, and C-10. These spectral data suggested that compound **3** had a spirodienone structure (Figure 1). For the anisotropic effect, the chemical shifts of C-3 and C-5 appeared at δ_C 158.0 s and δ_C 163.3, respectively. The structure of compound **3** was further confirmed by NOESY experiments, with NOE correlations between H-12 (13) and H-2 (6), H-7, and H-11; between H-14 and H-7; and between the methoxyl group and H-10, which was a novel compound, named coixspiroenone (Figure 1).

Compounds That Suppress Human Breast Cancer Cell Line Growth. All of the isolated compounds were tested for antiproliferative effects by the MTT assay. The results indicated that MCF-7 cells were significantly inhibited by compounds **1**, **3**, **4**, **6**, and **7** at 50 μM, with the percentage of inhibition being 30.2, 19.2, 21.0, 13.5, and 32.4%, respectively. Furthermore, compounds **2**, **4**, and **7** significantly inhibited T-47D cells at 50 μM, with the percentage of inhibition being 20.7, 24.8, and 28.9%, respectively. Also, the MDA-MB-231 cells were significantly inhibited at 47.4, 25.3, and 69.3% by compounds **1**, **2**, and **12** at 50 μM (Figure 5). Compounds **9**−**12** influenced the growth of H184B5F5/M10 cells at concentrations of 50 μM, while at the same concentrations, compounds **10** and **11** significantly inhibited the growth of MCF-7 and T-47D cells. The overall influence of compounds **9**−**12** in terms of retarding the growth of H184B5F5/M10 cells needs further study.

Lactams have been previously reported as an antiproliferative agent for human cancer cells in some literature.[24−26] Meegan et al. found that β-lactam-type molecular scaffolds inhibited breast cancer cell proliferation.[24] Davis et al. isolated two caprolactams, pestalactam A and pestalactam B, that suppress MCF-7 cell growth.[25] Hormone-dependent human breast and renal cancer cells were also founds to be inhibited by a γ-lactam-based histone deacetylase inhibitor.[26] As shown in Figure 5, the compounds isolated in the present study had more potency against MCF-7 and MDA-MB-231 cells than T-47D cells. The results are consistent with those in Figure 4. P53 DNA repair might be one of the targeted pathways associated with this inhibition. Although compounds **9**−**12** suppress the growth of MCF-7, MDA-MB-231, and T-47D cells, they also influence the viabilities of H184B5F5/M10 cells. Compound **3**, a new spiroenone, significantly inhibited

MCF-7 cell growth and slightly stimulated H184B5F5/M10 cells growth.

Overall, in the present study, 12 compounds were isolated from ABE-EA, with 6 of them suppressing the growth of MCF-7, MDA-MB-231 or T-47D cells. Compounds **4** and **7** inhibited MCF-7, MDA-MB-231, and T-47D cells; compounds **1**, **3**, and **6** suppressed MCF-7 cells; compound **2** retarded T-47D cell growth; and compounds **1**, **2**, and **12** inhibited MDA-MB-231 cells. However, their action mechanisms remain to be further investigated.

LC−MS Analysis. LC−MS was applied to analyze and confirm the isolated compounds from ABE-EA. The total ion current (TIC) (lane 1) and UV chromatogram (lane 2) of LC−MS for the bioactive fraction are shown in Figure 6. Peaks were identified for coixspirolactam D (**1**) (t_R, 30.70 min; $[M - H]^-$ m/z, 244), coixspirolactam E (**2**) (t_R, 35.91 min; $[M - H]^-$ m/z, 244), coixspirolactam A (**4**) (t_R, 29.34 min; $[M - H]^-$ m/z, 202), coixspirolactam B (**5**) (t_R, 31.82 min; $[M - H]^-$ m/z, 216), coixspirolactam C (**6**) (t_R, 34.65 min; $[M - H]^-$ m/z, 216), coixol (**8**) (t_R, 33.47 min; $[M - H]^-$ m/z, 164), zhepiresionol (**11**) (t_R, 32.65 min; $[M - H]^-$ m/z, 279), and ficusal (**12**) (t_R, 49.65 min; $[M - H]^-$ m/z, 329) in a negative-ion mode. Peaks were identified for coixspiroenone (**3**) (t_R, 28.62 min; $[M + Na]^+$ m/z, 255), coixlactam (**7**) (t_R, 33.49 min; $[M + Na]^+$ m/z, 228), ethyl dioxindole-3-acetate (**9**) (t_R, 28.59 min; $[M + Na]^+ = 258$), and isoindol-1-one (**10**) (t_R, 47.66 min; $[M + H]^+ = 132$) in a positive-ion mode. From the above results, it is clear that the spirolactams (compounds **1**, **2**, and **4**−**6**) can be appropriately qualified in negative-ion mode and that spiroenone (compound **3**) needs the positive-ion mode. Overall, LC−MS analysis can be used as an analytical platform for these compounds.

In conclusion, we identified two new spirolactams, one new spiroenone, seven known γ-lactams, and two known neolignans from the bioactive fractions of ABE-EA in the present study. On the basis of antiproliferative effects on human breast cancer cells, spirolactams and spiroenone may be regarded as contributing to the antiproliferative effect of the ABE-EA. To confirm these active compounds, a LC−MS method was established as an analytical platform.

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ABBREVIATIONS USED

AB, adlay bran; ABE-EA, ethyl acetate fraction of adlay bran ethanol extract; ABE-Bu, 1-butanol fraction of adlay bran ethanol extract; ABE-H_2O, water fraction of adlay bran ethanol extract; ABE-Hex, *n*-hexane fraction of adlay bran ethanol extract; ACF, aberrant crypt foci; ACN, acetonitrile; ANOVA, analysis of variance; AR, androgen receptor; BuOH, 1-butanol; CC, column chromatography; DA, dehulled adlay; DEPT, distortionless enhancement by polarization transfer; DMH, dimethyl hydrazine; DMSO, dimethyl sulfoxide; EA, ethyl acetate; ER, estrogen receptor; ESI, electrospray ionization; FBS, fetal bovine serum; Hex, *n*-hexane; HPLC, high-performance liquid chromatography; HMBC, heteronuclear multiple-bond coherence; IR, infrared; LC, liquid chromatography; LCF, lactam-containing fraction; MEM, minimum essential medium; MS, mass spectra; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; LEF, lactam-enriched fraction; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; SD, standard deviation; TCM, traditional Chinese medicine; TIC, total ion current; t_R, retention time; UV, ultraviolet

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