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# Gastroprotective Activities of Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) on the Growth of the Stomach Cancer AGS Cell Line and Indomethacin-Induced Gastric Ulcers

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**ABSTRACT:** Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) seeds have long been used to treat warts, chapped skin, rheumatism, and neuralgia in traditional Chinese medicine (TCM). Recently, studies demonstrated its anti-inflammatory, antiproliferative, antitumor, and antiallergic activities. In the present study, we first report the gastroprotective effects of dehulled adlay (DA) seeds, which consist of bran (AB) and endosperm (AE). The DA ethanolic extract (DAE) was prepared, along with the AB and AE ethanolic extracts (ABE and AEE), and the inhibitory effects of these extracts were tested on the AGS gastric cancer cell line. Results indicated that the ABE showed better antiproliferative activity, and 19 compounds were purified from AB in a further phenolic-compound-guided separation. Among the isolated compounds, caffeic and chlorogenic acids significantly suppressed the growth of AGS cells. In addition, the antiulcer activity of DA was examined in an indomethacin-induced gastric lesion model. The ulcer index (UI) and oxidative biomarkers in animals decreased, while the non-protein sulfhydryl (NPSH) groups were elevated when given DA. This is the first investigation of antiulcer activity of adlay, and we demonstrated that the antioxidative-active phenolic acids in DA contribute to some portion of the gastroprotective effects.

**KEYWORDS:** Adlay, adlay bran, antiproliferation, phenolic compounds, antiulcer effect, dehulled adlay

## INTRODUCTION

Gastric hyperacidity and gastroduodenal ulcers are a very common global problem today and occur mainly in the stomach and proximal duodenum.[1] The leading cause of gastric ulcers is an infection by *Helicobacter pylori*,[1] while dietary habits, smoking, drinking, and consumption of non-steroidal anti-inflammatory drugs (NSAIDs) were also reported to elevate the risk of gastric ulcers.[2] The pathophysiology of these disorders focuses on the aggressive stress and defense mechanism of the stomach, such as hydrochloric acid (HCl) production, mucus secretion, non-protein sulfhydryl (NPSH) groups from the liver, and blood flow.[1] Recent research showed that the ingestion of NSAIDs promotes the production of reactive oxygen species (ROS) and was attributed to increasing oxidative stress in the human body.[3] ROS play critical roles in the development of acute gastric lesions induced by stress, ethanol, and NSAIDs.[3] In addition, lipid peroxidation of cell membranes induced by ROS causes DNA damage because of the free radicals, and cancer-causing mutations may occur.[4]

Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) seeds, also called Job's tears, are a component of traditional Chinese medicine (TCM) and have long been used as an anti-inflammatory agent to treat warts, chapped skin, rheumatism, and neuralgia.[5] A recent study showed that dehulled adlay (DA) modulated the microbiota in the intestinal tract of rats.[6] Also, the anti-inflammatory and antioxidative effects of adlay were elucidated *in vitro*.[7,8] Contents of various potent compounds in adlay seeds from different origins were quantified.[9] Several phenolic antioxidants were isolated from adlay seeds, and bioactive components in adlay seeds were found to be stable during processing.[10] Lignans and phenolic compounds were isolated from adlay hull (AH) in an assay-guided isolation.[8] Flavanone and several phenolic acids were isolated from anti-inflammatory fractions of adlay seeds.[11−13] Phenolic alcohol in the adlay testa (AT) was reported to possess antiallergic activity.[14] In addition, DA and adlay bran (AB) were shown to retard carcinogenesis through an anti-inflammatory pathway,[15,16] and ferulic acid was regarded as the active component in a further investigation.[17]

The above literature indicate that phenolic compounds in adlay are important bioactive components. Antioxidants, such as

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phenols and vitamin E, are believed to suppress the risk of gastrointestinal damage.[2,4] In the present study, the antiproliferative activities against the AGS gastric cancer cell line of extracts from different parts of DA were elucidated. Phenolic compounds were isolated from the AB alcoholic extracts. The antiulcer effect of DA was also reported. This is the first investigation of the gastroprotective activities of adlay seeds.

## MATERIALS AND METHODS

**Drugs and Reagents.** Adlay seeds were purchased from local farmers who planted Taichung Shuenyu no. 4 (TCS4) of *C. lachryma-jobi* L. var. *ma-yuen* Stapf in Taichung, Taiwan, in 2009. The AGS cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 2-thiobarbituric acid (TBA), butylated hydroxytolune (BHT), 1,1,3,3-tetraethoxypropane (TEP), indomethacin, trichloroacetic acid (TCA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), alcian blue, L-cysteine hydrochloride, sodium chloride (NaCl), sodium phosphotungstate tribasic hydrate, potassium carbonate ($K_2CO_3$), trizma base, magnesium chloride ($MgCl_2$), carboxymethyl cellulose (CMC), and the standards used in the assays were obtained from Sigma Chemicals (St. Louis, MO). Antibiotics, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Grand Island, NY). 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. Analytical-grade solvents used during the purification procedures, including methanol (MeOH), ethanol (EtOH), *n*-hexane (Hex), ethyl acetate (EA), 1-butanol (BuOH), acetone (ACE), dichloromethane ($CH_2Cl_2$), and dimethyl sulfoxide (DMSO) used for stock solution of in vitro studies, were purchased from Merck. Infrared (IR) spectra were recorded on a Nicolet Avatar 320 FTIR spectrophotometer (Thermo Electron, Akron, OH). Nuclear magnetic resonance (NMR) spectra were run in acetone-$d_6$, $CDCl_3$, or $CD_3OD$ on a Varian Unity Plus 400 or Varian Unity INOVA-500 (Varian, Palo Alto, CA). Electron impact (EI)−mass spectra (MS) were recorded on a Joel JMS-HX300 mass spectrometer, while electrospray ionization (ESI)−MS were run on a Finnigan MAT LCQ ion-trap mass spectrometer system (Thermoquest, San Jose, CA). The ingredients of the AIN-93 M diet were obtained from INC Biochemicals (Costa Mesa, CA).

**Extraction and Fractionation.** AH and AT were removed from adlay seeds with a grinder. The DA, AB, and adlay endosperm (AE) were further ground into a powder and soaked in 5-fold 95% EtOH (w/v) at room temperature for 48 h. The extracts were combined and concentrated under reduced pressure at 50 °C to give the DA ethanolic extract (DAE, 2.35% of DA), AB ethanolic extract (ABE, 8.09% of AB), and AE ethanolic extract (AEE, 2.65% of AE). The ABE was suspended in water, which was followed by sequential partitioning with Hex, EA, and BuOH to give the Hex-soluble fraction (ABE−Hex, 60.7% of ABE), EA-soluble fraction (ABE−EA, 7.2% of ABE), BuOH-soluble fraction (ABE−BuOH, 4.4% of ABE), and water-soluble fraction (ABE−$H_2O$, 27.2% of ABE) according to the reported literature.[17] In addition, the AB methanolic extract (ABM, 16.5% of AB), ABM−EA (14.3% of ABM), and ABM−BuOH (13.7% of ABM) were obtained as previously described.[7]

**Cell Culture and Antiproliferation Assay.** The AGS cell line was cultured in DMEM containing 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin with a 5% $CO_2$ atmosphere at 37 °C.[18] To determine the antiproliferative effect, AGS cells were assessed using a MTT assay as previously described.[18] Test samples were dissolved in DMSO, and the final concentration of DMSO was ≤0.1%. AGS cells were cultured in 96-well plates at a density of $2 \times 10^3$ cells/well and allowed to grow for 24 h. After this, the medium was replaced with FBS-free DMEM, then various test samples were added, and cells were incubated for an additional 48 h. Next, a MTT solution in FBS-free DMEM, which had been filtered through a 0.45 mm membrane, was added to each well (to a final concentration of 1 mg/mL). The plates were incubated under a 5% $CO_2$ atmosphere at 37 °C for 4 h. The medium with unreacted dye was removed. DMSO (100 μL) was added to each well to dissolve the MTT formazan crystals, and the absorbance at 570 nm was measured. The viability of cells was calculated using the following equation: relative cell viability (%) = (average of absorbance of treated wells) × 100/(average of absorbance of untreated wells). DMSO at 0.1% was used as the blank vehicle.

**Isolation and Purification Procedures.** Silica gel, Sephadex LH-20 gel, Diaion gel, and preparative thin-layer chromatography (TLC) were used to separate the different subfractions, while semi-preparative high-performance liquid chromatography (HPLC) was used to purify the compounds. The ABE−EA and ABM−EA were eluted over a silica gel column with Hex/EA/MeOH gradients. The eluates with similar TLC patterns were combined and further chromatographed.

The 20−40% EA/Hex eluate from ABE−EA was separated by LH-20 using an EA/$CH_2Cl_2$ gradient. Subsequently, the 20−40% EA/$CH_2Cl_2$ fraction was separated by LH-20 with MeOH to eliminate long-chain components and was further chromatographed over a RP-18 column with a MeOH/$H_2O$ gradient. The 40% MeOH/$H_2O$ eluate was purified by a Si-60 column using EA/ACE/Hex at 1:2:3.5 as the mobile phase at a flow rate of 1.8 mL/min to yield compound **1** (1.2 mg). The 40−60% EA/$CH_2Cl_2$ fraction was further separated on a LH-20 column with MeOH/EA to yield compounds **11** (1.0 mg) and **13** (6.2 mg). The same fraction was also separated by RP-18, and the 10% MeOH/$H_2O$ eluate was further purified on a Si-60 column using EA/ACE/Hex at 1:2:2 as the mobile phase at a flow rate of 1.8 mL/min to yield compound **7** (4.1 mg). The 80% EA/$CH_2Cl_2$ fraction was further purified by RP-18 (MeOH/$H_2O$), and the 30−40% MeOH/$H_2O$ subfraction was eluted in LH-20 (MeOH) to yield compounds **5** (9.3 mg) and **3** (11.4 mg), respectively.

The 40−50% EA/Hex eluate from ABE−EA was separated by LH-20 (MeOH) and purified on a RP-18 semi-preparative column eluted by 80% MeOH/$H_2O$ at a flow rate of 3 mL/min. Subfractions from the LH-20 column were analyzed on TLC plates, and the low-polarity wastes were eliminated. Compounds **6** (3.1 mg), **8** (1.6 mg), **9** (4.5 mg), and **15** (2.7 mg) were collected with respective retention times ($t_R$) of 24.72, 8.74, 35.5, and 8.51 min.

ABM−EA and ABM−BuOH were suggested as antiproliferative agents as previously described.[7] The active fractions against lung and colorectal cancer cell lines were prepared to further investigate the chemical composition. The 40−50% EA/Hex fraction of ABM−EA was directly eluted by LH-20 (MeOH) to eliminate low-polarity components and injected into a Si-60 column with 60% EA/Hex at a flow rate of 3 mL/min to yield compound **2** (1.7 mg) ($t_R$ = 11.8 min). The same fraction was also chromatographed using silica gel (EA/Hex). The 20−30% EA/Hex subfraction was then further separated by silica gel (EA/$CH_2Cl_2$), and the 15% EA/$CH_2Cl_2$ eluate from the above procedure was further purified on a Si-60 column with 15% EA/$CH_2Cl_2$ at a flow rate of 3 mL/min to yield compounds **16**, **17**, and **18** as a mixed amorphous oil (20.6 mg) ($t_R$ = 8.6 min). The 50−60% EA/Hex fraction of ABM−EA was separated by LH-20 (MeOH) and purified by silica gel (EA/$CH_2Cl_2$). The 20−40% EA/$CH_2Cl_2$ subfraction was then chromatographed on a Si-60 column with 50% EA/$CH_2Cl_2$ at a flow rate of 3 mL/min to yield compound **10** (1.4 mg) ($t_R$ = 8.5 min). The 100% EA fraction of ABM−EA was purified by LH-20 (MeOH) and further separated by silica gel (EA/$CH_2Cl_2$). The 20% EA/$CH_2Cl_2$ subfraction was then purified on a Si-60 column with 20% EA/$CH_2Cl_2$ at a flow rate of 3 mL/min to yield compound **4** (3.0 mg) ($t_R$ = 7.8 min). The 60% EA/$CH_2Cl_2$ subfraction was then purified on a Si-60 column with 65% EA/$CH_2Cl_2$ at a flow rate of 3 mL/min to yield compound **19** (3.3 mg) ($t_R$ = 7.8 min). The 70% EA/$CH_2Cl_2$ subfraction was then purified on a

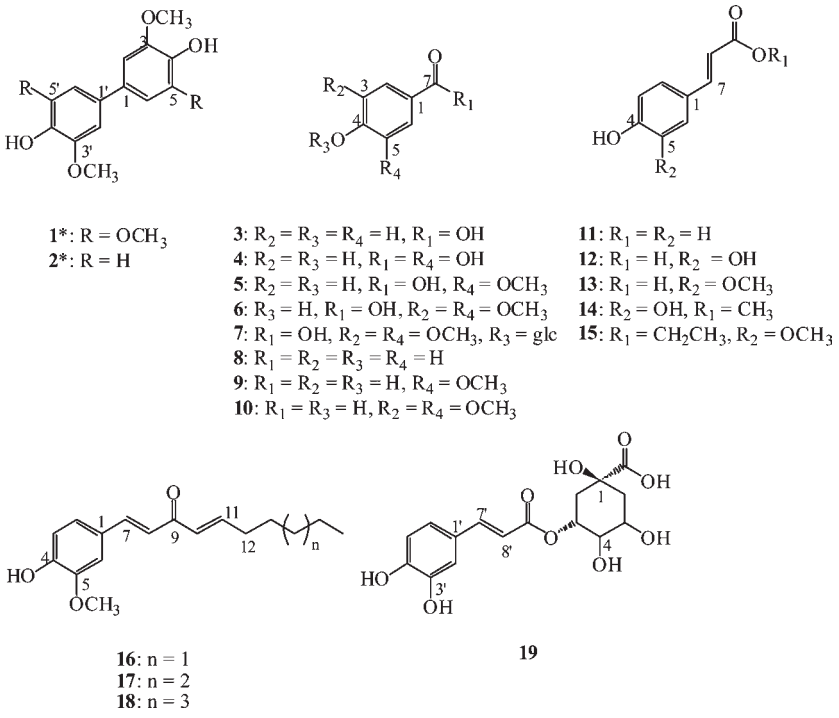


**Figure 1.** Structures of compounds **1**−**19**. (∗) Compound that was isolated for the first time from a natural source.

Si-60 column with 70% EA/$CH_2Cl_2$ at a flow rate of 3 mL/min to yield compound **12** (7.1 mg) ($t_R$ = 9.4 min).

ABM−BuOH was eluted by LH-20 ($H_2O$/MeOH/EA). The MeOH fraction was further separated by silica gel with MeOH/$CH_2Cl_2$, and the 5−10% MeOH/$CH_2Cl_2$ subfraction was then purified on a Si-60 column with 20% EA/$CH_2Cl_2$ at a flow rate of 3 mL/min to yield compound **14** (1.2 mg) ($t_R$ = 4.7 min). The structures of compounds **1**−**19** (Figure 1) were identified by spectroscopic methods, including $^1$H NMR, IR, and ESI− or EI−MS analytical data.

*3,5,3′,5′-Tetramethoxybiphenyl-4,4′-diol* (***1***). Pale-yellow amorphous oil. ESI−MS *m*/*z* (%): 305 ([M − H]$^-$, 100). $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$: 3.97 (s, 3H, −$OCH_3$), 3.99 (s, 3H, −$OCH_3$), 4.00 (s, 3H, −$OCH_3$), 4.01 (s, 3H, −$OCH_3$), 6.76 (s, 4H, H-2, H-6, H-2′, H-6′), 7.60 (s, 1H, −OH), 7.87 (s, 1H, −OH).

*3,3′-Dimethoxybiphenyl-4,4′-diol* (***2***). Colorless amorphous solid. EI−MS *m*/*z* (%): 246 ($M^+$, 48), 231 ([M − $CH_3$]$^+$, 12), 143 (100), 115 (50), 66 (32). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3416, 1600, 1500. $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$: 3.93 (s, 6H, −$OCH_3$ × 2), 5.58 (brs, 2H, −OH × 2), 6.93 (d, *J* = 8.2 Hz, 2H, H-5, H-5′), 7.00 (brs, 2H, H-2, H-2′), 7.01 (d, *J* = 8.2 Hz, 2H, H-6, H-6′).

*4-Hydroxybenzoic Acid* (***3***). Pale-gray solid. ESI−MS *m*/*z* (%): 137 ([M − H]$^-$, 100). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3300−2500, 1683, 1607. $^1$H NMR ($CD_3OD$, 400 MHz) $\delta$: 6.69 (m, 2H, H-3, H-5), 7.71 (m, 2H, H-2, H-6).

*Protocatechuic Acid* (***4***). Pale-yellow crystal. EI−MS *m*/*z* (%): 168 ($M^+$, 100), 153 ([M − $CH_3$]$^+$, 70), 151 ([M − OH]$^+$, 14), 125 (18), 97 (18), 79 (3), 51 (4). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3300−2500, 1666. $^1$H NMR (acetone-$d_6$, 400 MHz) $\delta$: 3.91 (s, 3H, −$OCH_3$), 6.91 (d, *J* = 8.2 Hz, 1H, H-5), 7.56 (d, *J* = 1.5 Hz, 1H, H-2), 7.60 (dd, *J* = 8.2, 1.5 Hz, 1H, H-6).

*Vanillic Acid* (***5***). White solid. ESI−MS *m*/*z* (%): 167 ([M − H]$^-$, 100). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3300−2500, 1679. $^1$H NMR ($CD_3OD$, 400 MHz) $\delta$: 3.87 (s, 3H, −$OCH_3$), 6.74 (d, *J* = 8.4 Hz, 1H, H-5), 7.49 (dd, *J* = 8.0, 1.6 Hz, H-6), 7.56 (d, *J* = 1.6 Hz, 1H, H-2).

*Syringic Acid* (***6***). White solid. EI−MS *m*/*z* (%): 198 (M, 100). $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$: 3.88 (s, 3H, −$OCH_3$), 3.94 (s, 3H, −$OCH_3$), 7.19 (s, 2H, H-2, H-6).

*Glucosyringic Acid* (***7***). Gray solid. ESI−MS *m*/*z* (%): 359 ([M − H]$^-$, 100). $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$: 3.48 (m, 2H, H-6′$\alpha$, H-6′$\beta$), 3.87 (s, 3H, −$OCH_3$), 3.89 (s, 3H, −$OCH_3$), 5.53 (d, *J* = 7.0 Hz, 1H, H-1′), 7.29 (s, 2H, H-2, H-6).

*4-Hydroxybenzaldehyde* (***8***). Dark-yellow oil. ESI−MS *m*/*z* (%): 121 ([M − H]$^-$, 100). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3398, 1710, 1496. $^1$H NMR ($CD_3OD$, 400 MHz) $\delta$: 6.58 (d, *J* = 3.6 Hz, 2H, H-3, H-5), 7.38 (d, *J* = 3.6 Hz, 2H, H-2, H-6), 9.53 (s, 1H, −CHO).

*p-Vanillin* (***9***). White solid. ESI−MS *m*/*z* (%): 151 ([M − H]$^-$, 100). $^1$H NMR ($CD_3OD$, 500 MHz) $\delta$: 3.91 (s, 3H, −$OCH_3$), 6.93 (d, *J* = 8.0 Hz, 1H, H-5), 7.41 (d, *J* = 8.0, 1H, H-6), 7.42 (s, 1H, H-2), 9.73 (s, 1H, −CHO).

*Syringaldehyde* (***10***). Pale-yellow crystal. EI−MS *m*/*z* (%): 182 ($M^+$, 100), 181 ([M − H]$^+$, 58), 167 (14), 139 (14), 111 (14). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3290, 1670, 1607, 1585, 1513. $^1$H NMR (acetone-$d_6$, 400 MHz) $\delta$: 3.92 (s, 6H, −$OCH_3$ × 2), 7.24 (s, 2H, H-2, H-6), 9.80 (s, 1H, −CHO).

*p-Coumaric Acid* (***11***). White solid. ESI−MS *m*/*z* (%): 163 ([M − H]$^-$, 100). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3300−2500, 1663, 1600, 1509. $^1$H NMR ($CD_3OD$, 400 MHz) $\delta$: 6.27 (d, *J* = 16.0 Hz, 1H, H-8), 6.79 (d, *J* = 8.4 Hz, 2H, H-3, H-5), 7.44 (d, *J* = 8.4 Hz, 2, H-2, H-6), 7.58 (d, *J* = 16.0 Hz, 1H, H-7).

*Caffeic Acid* (***12***). Yellow crystal. EI−MS *m*/*z* (%): 180 ($M^+$, 100), 163 ([M − OH]$^+$, 28), 134 (33), 117 (10), 89 (15). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3390, 3300−2500, 1666, 1593, 1513. $^1$H NMR (acetone-$d_6$, 400 MHz) $\delta$: 6.26 (d, *J* = 16.0 Hz, 1H, H-8), 6.86 (d, *J* = 8.4 Hz, 1H, H-5), 7.05 (dd, *J* = 8.4, 2.0 Hz, 1H, H-6), 7.16 (d, *J* = 2.0 Hz, 1H, H-2), 7.53 (d, *J* = 16.0 Hz, 1, H-7).

*Ferulic Acid* (***13***). Gray crystal. ESI−MS *m*/*z* (%): 193 ([M − H]$^-$, 100). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3300−2500, 1699, 1596, 1508. $^1$H NMR ($CD_3OD$, 400 MHz) $\delta$: 3.90 (s, 3H, −$OCH_3$), 6.31 (d, *J* = 16.0 Hz, 1H, H-8), 6.90 (d, *J* = 8.4 Hz, 1H, H-5), 7.01 (s, 1H, H-2), 7.06 (dd, *J* = 8.4 Hz, 1H, H-6), 7.63 (d, *J* = 10.8 Hz, 1H, H-7).

*Methyl Caffeate* (***14***). Yellow crystal. EI−MS *m*/*z* (%): 194 ($M^+$, 100), 164 (7), 163 (97), 145 (11), 135 (14), 134 (18), 117 (9), 89 (14). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3389, 1717, 1606, 1515. $^1$H NMR

**Table 1. (A) DA-Containing Experimental Diets and (B) Effects on Body Weight, Feed Efficiency, and Liver Weight in Wistar Rats**

| (A) diet constituents | BL (g/kg of experimental diet) | NC (g/kg of experimental diet) | LDA (g/kg of experimental diet) | HDA (g/kg of experimental diet) |
|---|---|---|---|---|
| cornstarch | 397.49 | 397.49 | 329.20 | 192.59 |
| casein | 200.00 | 200.00 | 178.70 | 136.10 |
| dextrinized cornstarch | 132.00 | 132.00 | 132.00 | 132.00 |
| sucrose | 100.00 | 100.00 | 100.00 | 100.00 |
| soybean oil | 70.00 | 70.00 | 61.70 | 45.10 |
| alphacel, non-nutritive bulk | 50.00 | 50.00 | 50.00 | 50.00 |
| AIN-93 M mineral mix | 35.00 | 35.00 | 35.00 | 35.00 |
| L-cystine | 10.00 | 10.00 | 10.00 | 10.00 |
| AIN-93 vitamin mix | 3.00 | 3.00 | 3.00 | 3.00 |
| choline bitartrate | 2.50 | 2.50 | 2.50 | 2.50 |
| *tert*-butyhydroquinone (TBHQ) | 0.01 | 0.01 | 0.01 | 0.01 |
| DA | | | 100.00 | 300.00 |

| (B) | groups[a] | | | |
|---|---|---|---|---|
| parameters[b] | BL | NC | LDA | HDA |
| body weight (g) | | | | |
| initial | 346 ± 18 | 325 ± 14 | 335 ± 23 | 331 ± 7 |
| final | 466 ± 25 | 439 ± 21 | 451 ± 44 | 465 ± 28 |
| feed intake (g/day) | 23.5 ± 0.0 | 21.1 ± 1.1 | 21.9 ± 3.0 | 24.2 ± 1.2 |
| feed efficiency[c] (%) | 19.6 ± 7.1 | 20.8 ± 1.9 | 20.3 ± 3.0 | 21.3 ± 3.3 |
| liver weight (g) | 10.8 ± 0.4 | 9.9 ± 0.9 | 10.6 ± 1.6 | 10.8 ± 1.1 |

[a] BL, control group; NC, negative control group; LDA, low-dose DA group; and HDA, high-dose DA group. [b] All values are presented as the mean ± SD ($n$ = 8). [c] Feed efficiency = (body weight gained/total feed intake) × 100.

($CD_3OD$, 400 MHz) $\delta$: 3.73 (s, 1H, $-OCH_3$), 4.87 (brs, 1H, −OH), 6.24 (d, $J$ = 15.9 Hz, 1H, H-8), 6.77 (d, $J$ = 8.2 Hz, 1H, H-5), 6.92 (dd, $J$ = 8.2, 1.6 Hz, 1H, H-6), 7.04 (d, $J$ = 1.6 Hz, 1H, H-2), 7.53 (d, $J$ = 15.9 Hz, 1H, H-7).

*Ethyl Ferulate (**15**).* Pale-yellow solid. ESI−MS $m/z$ (%): 221 ($[M-H]^-$, 100). $^1$H NMR ($CD_3OD$, 600 MHz) $\delta$: 1.29 (t, $J$ = 8.4 Hz, 3H, $-CH_3$), 3.88 (s, 3H, $-OCH_3$), 4.22 (q, $J$ = 6.6 Hz, 2H, $-CH_2-$), 6.34 (d, $J$ = 15.6 Hz, 1H, H-8), 6.79 (d, $J$ = 8.4 Hz, 1H, H-5), 7.06 (d, $J$ = 7.8 Hz, 1H, H-6), 7.17 (s, 1H, H-2), 7.59 (d, $J$ = 16.2 Hz, 1H, H-7).

*(E)-1-(4-Hydroxy-3-methoxyphenyl)dec-4-en-3-one (**16**), (E)-1-(4-hydroxy-3-methoxyphenyl)undec-4-en-3-one (**17**), and (E)-1-(4-hydroxy-3-methoxyphenyl)dodec-4-en-3-one (**18**).* Yellow oil. EI−MS $m/z$ (%): 304 (M of compound **18**, 2), 290 (M of compound **17**, 3), 276 (M of compound **16**, 44), 205 (55), 151 (20), 137 (100), 119 (10), 99 (13), 55 (23). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3411, 2928, 2856, 1666, 1626, 1515. $^1$H NMR ($CDCl_3$, 100 MHz) $\delta$: 0.89 (t, $J$ = 7.2 Hz, 3H, H-16), 1.29−1.24 (m, $n$H, H-14), 1.42 (m, 2H, H-13), 2.13 (t, $J$ = 7.0 Hz, 2H, H-8), 2.15 (t, $J$ = 7.0 Hz, 2H, H-7), 2.18 (q, $J$ = 6.8 Hz, 2H, H-12), 3.86 (s, 3H, $-OCH_3$), 5.46 (s, 1H, −OH), 6.07 (dt, $J$ = 15.6, 2.0 Hz, 1H, H-10), 6.66 (dd, $J$ = 7.2, 2.0 Hz, 1H, H-6), 6.69 (d, $J$ = 2.0 Hz, 1H, H-2), 6.80 (dt, $J$ = 15.6, 6.8 Hz, 1H, H-11), 6.81 (d, $J$ = 7.2 Hz, 1H, H-5).

*Chlorogenic Acid (**19**).* Pale-yellow solid. EI−MS $m/z$ (%): 354 ($M^+$, 27), 336 (47), 180 (93), 163 (100), 162 (41), 60 (38), 57 (24). IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3500, 3300−2500, 1694, 1715, 1614, 1519. $^1$H NMR ($CD_3OD$, 400 MHz) $\delta$: 2.03 (dd, $J$ = 13.9, 2.0 Hz, 1H, H-$2_{ax}$), 2.07 (dd, $J$ = 12.8, 3.8 Hz, 1H, H-$6_{ax}$), 2.17 (dd, $J$ = 13.9, 2.0 Hz, 1H, H-$2_{eq}$), 2.20 (dd, $J$ = 12.8, 3.8 Hz, 1H, H-$6_{eq}$), 3.73 (dd, $J$ = 5.2, 2.0 Hz, 1H, H-4), 4.16 (ddd, $J$ = 8.4, 2.0, 2.0 Hz, 1H, H-3), 5.32 (m, 1H, H-5), 6.26 (d, $J$ = 15.9 Hz, 1H, H-8′), 6.77 (d, $J$ = 8.4 Hz, 1H, H-5′), 6.96 (dd, $J$ = 8.4, 2.0 Hz, 1H, H-6′), 7.05 (d, $J$ = 2.0 Hz, 1H, H-2′), 7.56 (d, $J$ = 15.9 Hz, 1H, H-7′).

**HPLC Analysis of ABE, ABE−EA, and ABE−BuOH.** Active isolated compounds were analyzed by HPLC on a Hitachi L-6200 intelligent pump and Hitachi L-7455 photodiode array detector according to Chung et al.[17] Briefly, gradient elution was performed with a 2% acetic acid aqueous solution (v/v, A) and 0.5% acetic acid in acetonitrile (v/v, B) at a constant rate of 1 mL/min through a C18 (150 × 4.6 mm, 5 $\mu$M) reverse-phase column (Kanto Chemical). Initial starting conditions were 5% B, 0−10 min for B to increase from 5 to 10%, 10−40 min for B to increase from 10 to 40%, 40−55 min for B to increase from 55 to 80%, 60−65 min for B to increase from 80 to 100%, 65−70 min for B to decrease from 100 to 50%, 70−75 min for B to decrease from 50 to 30%, 75−80 min for B to decrease from 30% to 10%, and finally, 80−85 min for B to decrease from 10 to 5% at original conditions. The absorbencies at 280 nm were recorded, and the concentration of each analyzed compound was calculated by calibration curves from reference standards purchased from Sigma.

**Animal Diets and NSAID-Induced Gastric Ulcer Model.** The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University. A total of 32 male Wistar rats (5 weeks of age) were purchased from the Animal Center of the College of Medicine at National Taiwan University (Taipei, Taiwan). Animals were individually housed in plastic cages in a pathogen-free room at 23 ± 2 °C and 50 ± 10% relative humidity with a 12 h light/dark cycle and were fed a standard AIN-93 M diet[19] and water *ad libitum* before the experimental period began. Ingredients in the experimental diets were substituted with DA as previously described.[10,15] Rats were weighed, and food consumption was recorded twice a week. The rats were randomly divided into four groups: a blank group (BL) and a negative control group (NC) comprised of 8 rats each maintained on a standard AIN-93 M diet, a low-dose DA group (LDA) consisting of

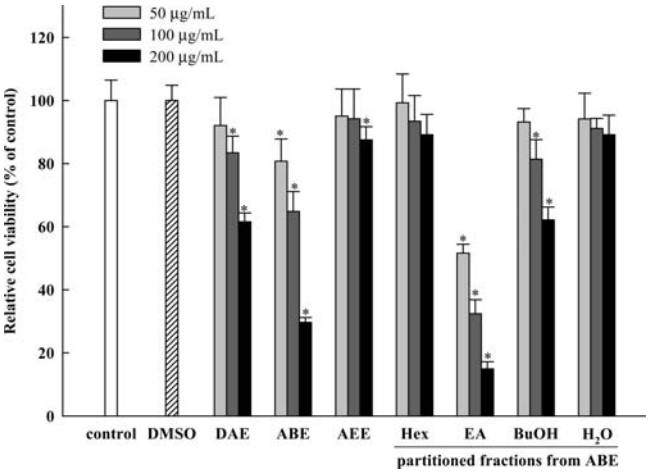


**Figure 2.** Effects of DAE, ABE, AEE, and partitioned fractions from ABE on cell proliferation in the AGS cell line. Cells were cultured with or without test samples for 48 h and examined using a MTT assay. The results are expressed as a percentage of living cells cultured in the presence of the test samples relative to a parallel culture that receives no treatment. Each bar represents the mean ± SD ($n$ = 3), and bars with different letters differ significantly. (*) $p < 0.05$ compared to the control group.

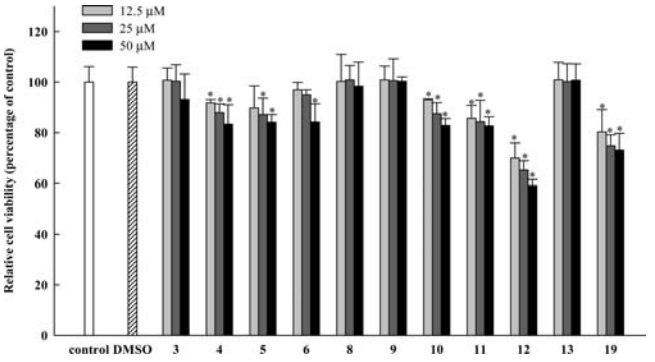


**Figure 3.** Effects of isolated compounds on cell proliferation in the AGS cell line. Cells were cultured with or without test samples for 48 h and examined using a MTT assay. The results are expressed as a percentage of living cells cultured in the presence of the test samples relative to a parallel culture that received no treatment. Each bar represents the mean ± SD ($n$ = 3), and bars with different letters differ significantly. (*) $p < 0.05$ compared to the control group.

8 rats on a 10% DA-substituted AIN-93 M diet, and a high-dose DA group (HDA) consisting of 8 rats on a 30% DA-substituted AIN-93 M diet (Table 1A). Animals were maintained on the test diets for 4 weeks and then starved for 36 h. To induce gastric ulcers, a previously described method was used with a few modifications.[2] Indomethacin at 30 mg/kg of body weight was dissolved in a 0.5% CMC aqueous solution, which was used to induce gastric ulcers, while the BL group was given 0.5% of the CMC vehicle. After treatment with indomethacin or vehicle for 7 h, rats were sacrificed. The stomach, liver, and serum were obtained and stored at −20 °C for further analysis.

**Determination of the Gastric Ulcer Index (UI), TBA Reactive Substances (TBARS), NPSH, and Pathological Observations of the Stomachs.** Total blood was collected before the stomach was surgically removed by an abdominal incision. After being rinsed in phosphate-buffered saline (PBS), the stomach was opened and extended, so that it could be photographed. Areas with erosion were

**Table 2. Contents of Phenolic Acids and Aldehydes in Different Parts of Adlay Seeds**

| | concentrations of phenolic compounds (μg/g of sample)[a] | | |
|---|---|---|---|
| compounds | ABE | ABE−EA | ABE−BuOH |
| protocatechuic acid (**4**) | ND[b] | 89 ± 7 | ND |
| vanillic acid (**5**) | 10 ± 3 | 1093 ± 72 | 318 ± 27 |
| syringic acid (**6**) | ND | 5 ± 0 | ND |
| syringaldehyde (**10**) | 2 ± 0 | 1042 ± 57 | 374 ± 26 |
| *p*-coumaric acid (**11**) | ND | 1428 ± 93 | 512 ± 38 |
| caffeic acid (**12**) | ND | 457 ± 27 | 203 ± 15 |
| chlorogenic acid (**19**) | ND | 203 ± 21 | 112 ± 7 |

[a] All values are presented as the mean ± SD ($n$ = 3). ABE, adlay bran ethanolic extract; ABE−EA, ethyl acetate-soluble fraction of ABE; and ABE−BuOH, 1-butanol-soluble fraction of ABE. [b] ND = not detectable.

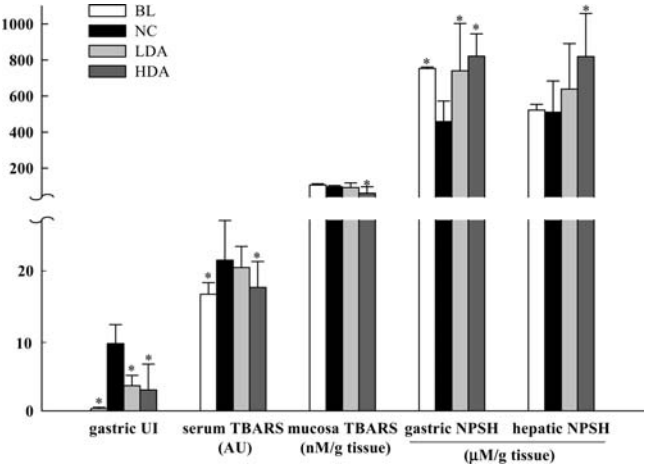


**Figure 4.** Influence on the UI, serum and mucosal TBARS, and mucosal and hepatic NPSH groups of indomethacin-treated Wistar rats administrated DA. Each bar represents the mean ± SD ($n$ = 8), and bars with different letters differ significantly. The break omits 28−40. (*) $p < 0.05$ compared to the NC group. BL, blank group; NC, negative control group; LDA, low-dose DA group; and HDA, high-dose DA group.

analyzed by Image software designed by the National Institute of Health (NIH, Bethesda, MD) as the UI ($mm^2$).[20] The mucus was removed by a slide immediately after being photographed. Then, the tissues were fixed in 10% formalin and stored at 4 °C. The fixed samples were dehydrated and defatted using various concentrations of EtOH, then embedded in paraffin, and exterminated to 3−4 μm section slices. Paraffin in the slices was eliminated, and the sections were stained with hematoxylin and eosin (H&E). Histopathological changes were observed using an optical microscope. Mucus or liver tissue (0.2 g) was mixed with 2 mL of 0.15 M NaCl with 0.002% BHT solution or 2 mL of a 0.02 M ethylenediaminetetra acetic acid (EDTA)−$Na_2$ solution and then homogenized in a blender, and the homogenates were stored at −20 °C for further analysis.

Serum and gastric mucosal TBARS were examined as previously described.[21] Briefly, 20 μL of serum was mixed with 4 mL of $^1/_{12}$ N sulfuric acid aqueous solution and allowed to stand at room temperature for 5 min after adding 0.5 mL of a 10% sodium phosphotungstate aqueous solution. The supernatants were removed after centrifugation at 1570*g* for 15 min, and the pellets were mixed with 4 mL of double-distilled (dd) water and 1 mL of a 0.67% TBA solution. The samples were placed in a water bath at 95 °C for 60 min. After cooling, 5 mL of

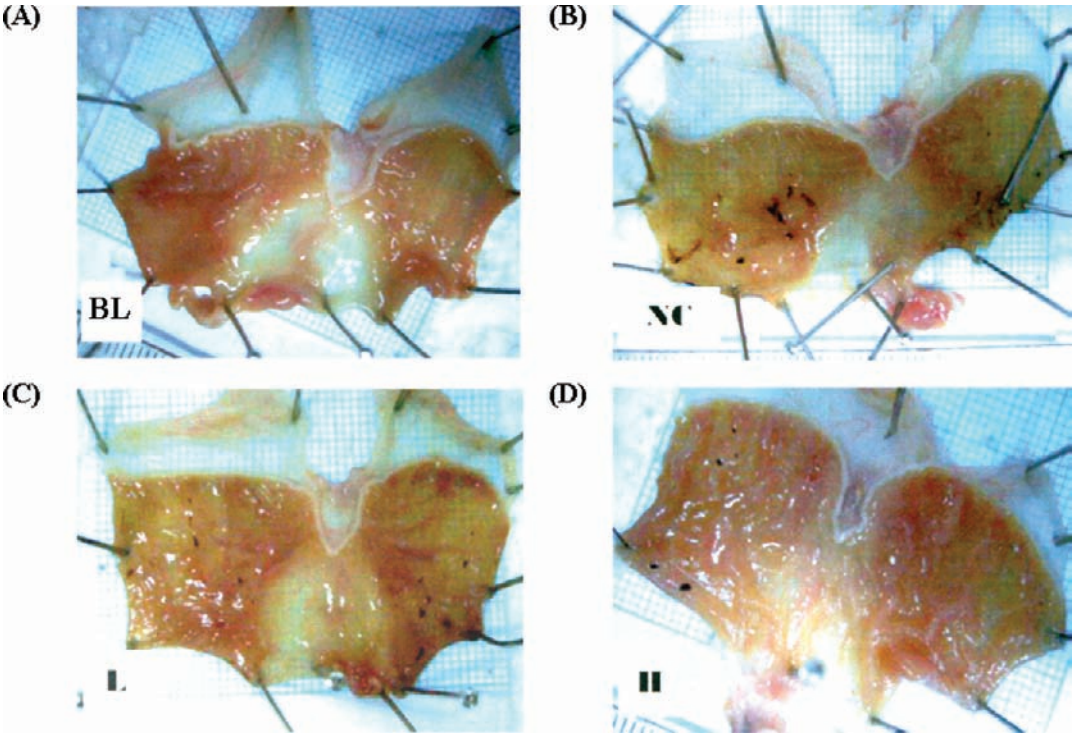


**Figure 5.** Pathological changes in the stomachs of male Wistar rats with indomethacin-induced gastric ulcers: (A) blank group, (B) negative control group, (C) LDA group, and (D) HDA group.

BuOH was added to the test samples and centrifuged at 1570*g* for 15 min. The supernatants were measured by a fluorescence spectrometer (F-4500, Hitachi, Japan) (with excitation at 515 nm and emission at 553 nm), and the results were recorded as absorbance units (AU). Likewise, 0.2 mL of the gastric mucus homogenate was mixed with 0.2 mL of 1.15% KCl/3 mM EDTA−$Na_2$, 3 mL of 1% $H_3PO_4$, 0.3 mL of 0.3% BHT/EtOH, and 1 mL of a 0.6% TBA solution. Test samples were placed in a water bath at 100 °C for 60 min after being vortex-mixed. BuOH (2 mL) was added to cooled samples, and the absorbance of the BuOH fraction was measured at 532 nm. The calibration curve was established using various concentrations of TEP solutions. Results were recorded as nanomoles per gram of tissue.

A previously described method[22] was used to examine the NPSH in the gastric mucus and liver. Briefly, 1 mL of gastric mucus or liver homogenate was mixed with 0.8 mL of dd water and 0.5 mL of a 50% TCA solution. After vortex-mixing for 2 min, the solutions were centrifuged at 1570*g* for 20 min. Supernatants (0.5 mL) were mixed with 1.0 mL of 0.4 M Tris-buffer (pH 8.9) and colored by 25 $\mu$L of a 0.01 M DTNB solution. The absorbance at 412 nm was recorded, and the results are presented as micromoles per gram of tissue.

**Statistical Analysis.** All values are presented as the mean ± standard deviation (SD). Results of the various groups were analyzed by an analysis of variance (ANOVA) and Duncan's multiple-range test. $p$ values of <0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

**Antiproliferative Activities of DAE, ABE, and Partitioned Fractions from ABE and AEE.** To evaluate the antiproliferative effects on AGS cells of DA, DAE, ABE, and AEE were prepared. DAE inhibited 38.5% of AGS cell viability at 200 $\mu$g/mL, and ABE suppressed 71.0% of cell growth under treatment with 200 $\mu$g/mL, while AEE showed lower activity (Figure 2). The results demonstrated that AB contributes to a major part of the antiproliferative effect of DA. The inhibitory activities of the partitioned fractions were also examined against AGS cells. The suppressive 50% inhibitory concentration ($IC_{50}$) of ABE−EA was <100 $\mu$g/mL, while ABE−BuOH inhibited 37.9% of cell growth at 200 $\mu$g/mL (Figure 2). The results showed that ABE−EA and ABE−BuOH possessed higher activities than the other fractions, which means that abundant bioactive components exist in these fractions.

Components with medium-high polarity in adlay seeds, such as lignans, flavonoids, and phenolic acids, were reported to possess antiproliferative and antitumor activities.[7,8,16,17] The targeted isolation of phenolic compounds of adlay seeds, such as phenolic acids, flavonoids, and lignans, was performed in several studies.[8,12,13] The bioactive compounds in adlay seeds were analyzed in various investigations.[11−15,17] According to the above observations, the EA and BuOH fractions can be regarded as the bioactive fractions in DA. To further investigate the medium-high polarity chemical composition of AB, ABM was extracted in addition to ABE as previously described.[7,17]

**Structure Identification and Antiproliferative Activities and Contents of Phenolic Compounds Isolated from AB.** ABE−EA and ABM−EA were regarded as the bioactive fractions for several biofunctions[7,17] and were separated in the present study. A total of 19 compounds were isolated from the bioactive fractions of AB (Figure 1), including 2 diphenyl compounds of 3,5,3′,5′-tetramethoxybiphenyl-4,4′-diol (**1**)[23] and 3,3′-dimethoxybiphenyl-4,4′-diol (**2**),[24] 9 phenolic acids of 4-hydroxybenzoic acid (**3**),[25] protocatechuic acid (**4**),[25] vanillic acid (**5**),[26] syringic acid (**6**),[8] glucosyringic acid (**7**),[27] coumaric acid (**11**),[12] caffeic acid (**12**),[28] ferulic acid (**13**),[8] and chlorogenic acid (**19**),[28] 3 phenolic aldehydes of 4-hydroxybenzaldehyde (**8**),[25] *p*-vanillin (**9**),[26] and syringaldehyde (**10**),[26] 2 phenolic acid esters of methyl caffeate (**14**)[29] and ethyl ferulate

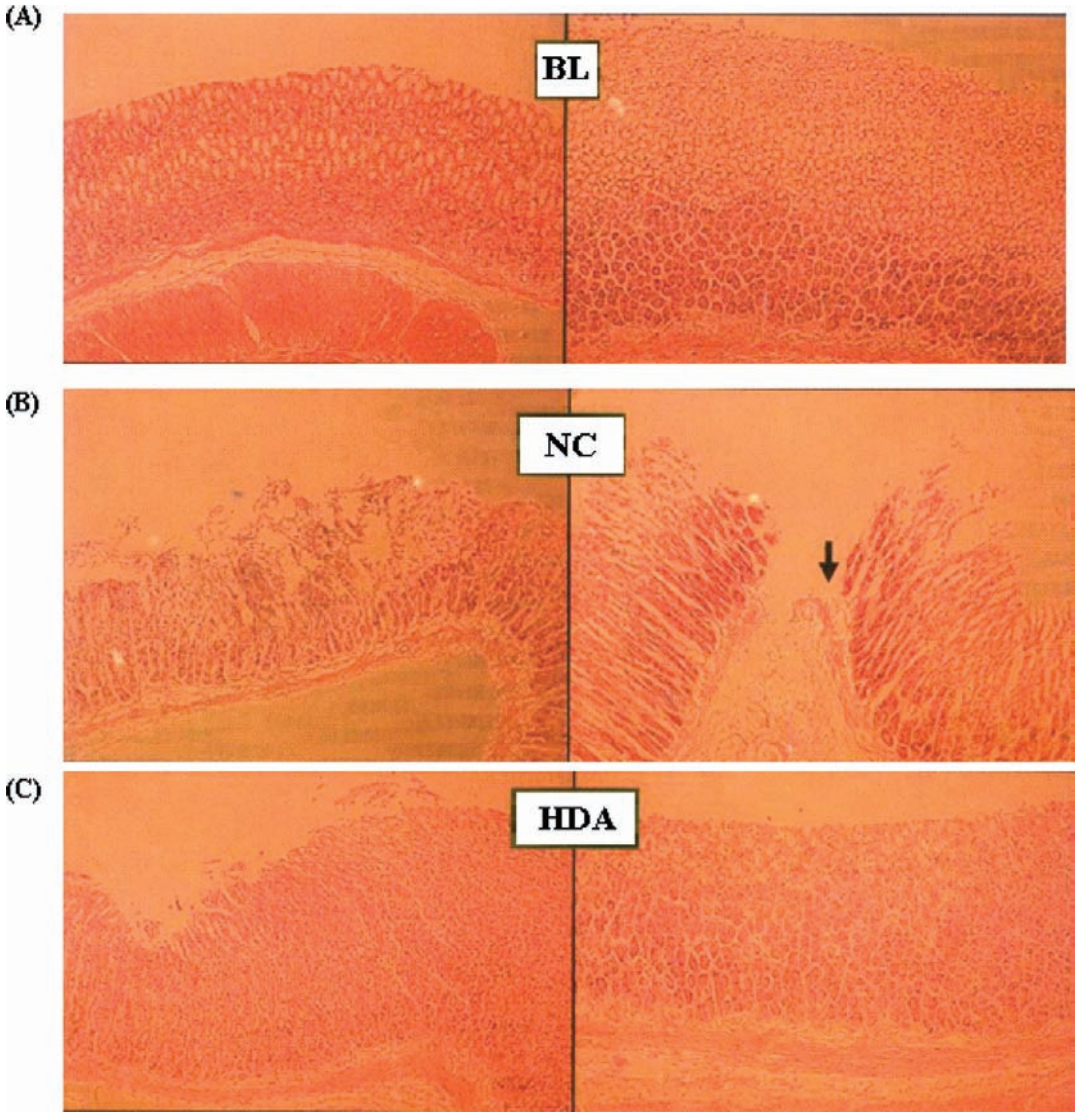


**Figure 6.** Histopathological observations of H&E-stained sections in the stomach of Wistar rats: (A) blank group, (B) negative control group, and (C) HDA group (left, 60×; right, 100×). The black arrow indicates erosion because of ulceration.

(**15**),[30] and 3 phenolic aliphatic compounds of (*E*)-1-(4-hydroxy-3-methoxyphenyl)dec-4-en-3-one (**16**), (*E*)-1-(4-hydroxy-3-methoxyphenyl)undec-4-en-3-one (**17**), and (*E*)-1-(4-hydroxy-3-methoxyphenyl)dodec-4-en-3-one (**18**). Each known compound was identified by NMR and MS spectroscopic analyses and compared to published data. Among them, compounds **1** and **2** were previously synthesized[23,24] and, herein, were isolated for the first time from a natural source.

The antiproliferative activities against AGS cells of several isolated compounds were examined (Figure 3). After treatment with various doses of isolated phenolic compounds for 48 h, compounds **4**, **5**, **6**, **10**, **11**, **12**, and **19** showed significant suppressive effects on cell growth with respective inhibitory percentages of 16.7, 15.9, 15.8, 17.1, 17.3, 41.0, and 27.9% at 50 $\mu$M. A previous investigation reported the antiproliferative effects of compounds **4** and **12**,[18] but $IC_{50}$ values were much higher than those of our investigation. Active compounds mentioned above were analyzed by HPLC. The contents of compounds **4**, **5**, **6**, **10**, **11**, **12**, and **19** were 89, 1093, 5, 1042, 1428, 457, and 203 $\mu$g/g of ABE−EA, and the contents of compounds **5**, **10**, **11**, **12**, and **19** were 318, 374, 512, 203, and 112 $\mu$g/g of ABE−BuOH, while compounds **4** and **6** were not detected (Table 2). ABE−EA and ABE−BuOH were suggested as active fractions from ABE, but the active phenolic acids did not seem abundant in ABE (Table 2). The above results demonstrated that phenolic acids may be important in the ability of adlay seeds to suppress the growth of AGS cells, but other active components need to be studied further.

**Effects of DA on Animals with Indomethacin-Induced Gastric Ulcers.** Rats administrated DA were treated with indomethacin to induce gastric ulcer as previously described.[2] The AIN-93 M standard[19] used in the experiment was modified, while 10 and 30% DA contents were integrated into ANI-93 M ingredients as previously described, including crude fat, crude protein, and a nitrogen-free extract (NFE), to ensure that the calories were the same for all experimental groups[10,15] (Table 1A). After 4 weeks of the experimental period, the final body weights, average food intake levels, feed efficiencies, and liver weights did not differ significantly among all groups (Table 1B). The results demonstrated that DA did not retard normal growth of the

animals. The UI decreased with administration of DA, which was significantly suppressed compared to the NC group (Figure 4). Numbers of bleeding clots on the surface of the stomach of the LDA and HAD groups were fewer than those of the NC group, while they were not seen in the BL group (Figure 5). Stomachs from the BL, NC, and HAD groups were investigated by histopathological observations. The results showed that the erosion was severe in the NC group, while it was inhibited when DA was given (Figure 6). The antiulcer effects of phenolic acids in an indomethacin-induced model were elucidated in a previous study, and the results showed that caffeic acid at a dose of 50 mg/kg of body weight significantly inhibited lesions.[2] Caffeic acid was not detectable in ABE (Table 2), which suggested that many active components in DA, including caffeic acid, contributed to antiulcer capacity.

To investigate the mechanism of the antiulcer effects, several indicators were analyzed in the present study. Serum TBARS was elevated in the NC group compared to the BL group and was lower in the HDA group compared to the NC group, while that in the LDA group was not significant (Figure 4). Mucosal TBARS did not show significant differences among the BL, NC, and LDA groups but was lower in the HDA group (Figure 4). The gastric NPSH was lower in the NC group compared to the BL group, while DA raised the NPSH to a normal status compared to the NC group (Figure 4). The hepatic NPSH did not change when treated with indomethacin but was elevated in the HDA group compared to the NC and BL groups (Figure 4). Recent studies revealed a relationship between oxidative stress and gastric ulceration, and excessive stress leads to consumption of the internal antioxidative barrier.[1,2,4] Our results showed that DA suppressed levels of serum and mucosal TBARS, while it increased gastric and hepatic NPSH, which means that the antioxidative capacity of DA is mainly responsible for its antiulcer activity.

In conclusion, we demonstrated the gastroprotective effects of DA. AB showed better antiproliferative activity against AGS cells than AE. In addition, DA inhibited indomethacin-induced gastric ulcers through antioxidative pathways. Caffeic acid in DA is one of the compounds indicative of a gastroprotective agent.

## AUTHOR INFORMATION

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## ABBREVIATIONS USED

AB, adlay bran; ABE, adlay bran ethanolic extract; ABM, adlay bran methanolic extract; ACE, acetone; AE, adlay endosperm; AEE, adlay endosperm ethanolic extract; AH, adlay hull; ANOVA, analysis of variance; AT, adlay testa; AU, absorbance units; BHT, butylated hydroxytoluene; BL, blank group; BuOH, 1-butanol; $CH_2Cl_2$, dichloromethane; CMC, carboxymethyl cellulose; DA, dehulled adlay; DAE, dehull adlay ethanolic extract; dd, double-distilled; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EA, ethyl acetate; EDTA, ethylenediaminetetra acetic acid; EI, electron impact; ESI, electrospray ionization; EtOH, ethanol; FBS, fetal bovine serum; H&E, hematoxylin and eosin; HCl, hydrochloric acid; HDA, high-dose dehulled adlay group; Hex, *n*-hexane; $IC_{50}$, 50% inhibitory concentration; $K_2CO_3$, potassium carbonate; HPLC, high-performance liquid chromatography; IACUC, Institutional Animal Care and Use Committee; IR, infrared spectra; LDA, low-dose dehulled adlay group; $MgCl_2$, magnesium chloride; MeOH, methanol; MS, mass spectrum; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NaCl, sodium chloride; NC, negative control group; ND, not detectable; NFE, nitrogen-free extract; NIH, National Institute of Health; NMR, nuclear magnetic resonance; NPSH, non-protein sulfhydryl; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SD, standard deviation; TBA, 2-thiobarbituric acid; TBARS, 2-thiobarbituric acid reactive substances; TBHQ, *tert*-butyhydroquinone; TCA, trichloroacetic acid; TCM, traditional Chinese medicine; TCS4, Taichung Shuenyu no. 4; TEP, 1,1,3,3-tetraethoxypropane; TLC, thin-layer chromatography; $t_R$, retention time; UI, ulcer index

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