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Identification and Quantification of Phytochemical Composition and Anti-inflammatory, Cellular Antioxidant, and Radical Scavenging Activities of 12 *Plantago* Species

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Supporting Information

ABSTRACT: Twenty-eight seed samples of 12 *Plantago* species were investigated for their chemical compositions and anti-inflammatory, cellular antioxidant, and radical scavenging properties. A new UPLC-UV procedure was developed and applied to quantify acteoside and geniposidic acid, the characteristic constituents of the genus *Plantago*. The amounts of acteoside and geniposidic acid ranged from 0.07 to 15.96 mg/g and from 0.05 to 10.04 mg/g in the tested samples, respectively. Furthermore, 26 compounds were tentatively identified by UPLC/Q-TOF-MS analysis. The *Plantago* samples significantly differed in their phytochemical compositions. The extracts of *Plantago* seeds also showed inhibitory effects on LPS-induced IL-1β, IL-6, and COX-2 mRNA expression in RAW 264.7 mouse macrophage cells. Additionally, significant variations were observed among different samples on cellular antioxidant activities in HepG2 cells, as well as DPPH and hydroxyl radical scavenging capacities. The results from this study may be used to promote the use of the genus *Plantago* in improving human health.

KEYWORDS: *Plantago, UPLC, UPLC/Q-TOF-MS, acteoside, geniposidic acid, anti-inflammation, cellular antioxidant, radical scavenging*

INTRODUCTION

About 275 species of the genus *Plantago* L. (Plantaginaceae) are found all over the world, and 20 species are widely distributed in China.[1,2] The seeds and whole plants of *Plantago major*, *Plantago asiatica*, and *Plantago depressa* have been used in foods and traditional medicine since ancient times.[2] The genus *Plantago* has been shown to have anti-inflammatory, immunomodulatory, anticancer, and antioxidant activities.[3−5] The fresh leaves of *P. asiatica*, *P. major*, and *P. depressa* are consumed in fresh salads and soups.[2] The seeds of several species such as *P. asiatica* and *P. major* can also be used as snacks or in cakes and breads.[2,3]

To date, only a few *Plantago* species have been investigated for their chemical constituents and biological activities.[3,4,6] The majority of species including *Plantago arachnoidea*, *Plantago jehohlensis*, and *Plantago himalaica* have not been studied. It was also noted that *P. depressa* subsp., a subspecies of *P. depressa*, has not been investigated. There have been several phytological research studies on *Plantago minuta*[7] and phytochemical analysis of *Plantago cornuti*[6] and *Plantago camtschatica*,[8] but the health properties of these three species were not reported. There was no report on the cellular antioxidant activity for the seeds or aerial parts of several representative *Plantago* species, although free radical scavenging capacities of their aerial parts were studied.[1,3,5] In addition, the aerial parts of *Plantago lanceolata* were found to significantly inhibit COX-1, COX-2 and 12-LOX mRNA expressions,[3,9] but *Plantago* seeds have not been investigated for possible anti-inflammatory activities.

The aim of this study was to analyze the chemical compositions and anti-inflammatory and antioxidant properties of 28 seed samples belonging to 12 *Plantago* species grown in China. The results from this study may be used to promote better use of the genus *Plantago* for improving human health.

MATERIALS AND METHODS

Plant Materials. The seeds of 12 *Plantago* species were collected and identified by Dr. Linke Yin (Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences) and Dr. Haibo Yin (Liaoning University of Traditional Chinese Medicine) in 2011 and 2012 from different locations in China. Detailed information on the 28 samples is provided in Table 1.

Reagents. Fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), iron(III) chloride, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), dimethyl sulfoxide (DMSO), insulin, hydrocortisone, and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was obtained from J&K Scientific (Beijing, China). Williams' medium E (WME), Hanks' balanced salt solution (HBSS), L-glutamine, antibiotic−antimycotic, gentamicin, fetal bovine serum (FBS), TRIzol

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Table 1. Collection Information of *Plantago* Samples

sample	species	location	latitude and longitude	elevation (m)	collection time
PML-1	*P. major* L.	Turpan, Xinjiang Uygur Autonomous Region	89°11′36.48″E, 42°51′17.14″N	−93	Aug 2011
PML-2		Kuenes County, Xinjiang Uygur Autonomous Region	83°22′08.00″E, 43°25′27.40″N	948	Aug 2011
PML-3		Hejing County, Xinjiang Uygur Autonomous Region	84°49′47.40″E, 43°12′26.10′N	2715	Aug 2011
PML-4		Urumqi, Xinjiang Uygur Autonomous Region	87°32′47.22″E, 43°53′21.50″N	716	Aug 2011
PML-5		Shenyang, Liaoning Province			Sept 2011
PAL-1	*P. asiatica* L.	Yecheng County, Xinjiang Uygur Autonomous Region	77°28′25.88″E, 37°52′33.54″N	1353	July 2011
PAL-2		Anshan, Liaoning Province			Sept 2011
PAL-3		Jian, Jiangxi Province			June 2011
PAS-1	*P. arachnoidea* Schrenk	Urumqi County, Xinjiang Uygur Autonomous Region	87°03′09.35″E, 43°07′5.61″N	2289	Aug 2011
PAS-2		Tashenkuergan County, Xinjiang Uygur Autonomous Region	75°13′07.10″E, 37°46′59.10″N	3077	Aug 2011
PAS-3		Xinyuan County, Xinjiang Ili Kazak Autonomous Prefecture	83°34′51.20″E, 43°24′14.40″N	1089	Aug 2011
PAS-4		Hejing County, Xinjiang Uygur Autonomous Region	83°11′17.00″E, 43°03′05.91″N	2500	Aug 2012
PLL-1	*P. lanceolata* L.	Xinyuan County, Xinjiang Ili Kazak Autonomous Prefecture	83°22′08.00″E, 43°25′27.40″N	948	Aug 2011
PLL-2		Tokkuztara County, Xinjiang Uygur Autonomous Region	81°51′59.80″E, 43°36′31.70″N	709	Aug 2011
PLL-3		Dalian, Liaoning Province			Aug 2011
PDW-1	*P. depressa* Willd.	Hejing County, Xinjiang Uygur Autonomous Region	84°09′03.70″E, 43°01′43.40″N	2419	Aug 2011
PDW-2		Urumqi, Xinjiang Uygur Autonomous Region	87°32′47.22″E, 43°53′21.50″N	716	Aug 2011
PDW-3		Shenyang, Liaoning Province			Aug 2011
PDW-4		Tashenkuergan County, Xinjiang Uygur Autonomous Region	75°23′05.11″E, 37°34′13.27″N	3285	Aug 2012
PMP-1	*P. minuta* Pall.	Tokkuztara County, Xinjiang Uygur Autonomous Region	81°49′02.70″E, 43°37′22.60″N	710	Aug 2011
PMP-2		Urumqi, Xinjiang Uygur Autonomous Region	87°40′54.66″E,43°56′54.43″N	650	Aug 2011
PCG-1	*P. cornuti* Gouan.	Kuche County, Xinjiang Uygur Autonomous Region	85°15′37.26″E, 42°25′10.45″N	2135	Aug 2012
PCG-2		Hejing County, Xinjiang Uygur Autonomous Region	83°11′17.00″E, 43°03′05.91″N	2500	Aug 2012
PDS-1	*P. depressa* subsp. Turczaninowii	Yakeshi, Inner Mongolia Autonomous Region			Aug 2011
PCL-1	*P. camtschatica* Link.	Dalian, Liaoning Province			July 2011
PJK-1	*P. jehohlensis* Koidz.	Chaoyang, Liaoning Province			Sept 2011
PMS-1	*P. maritima* L. ssp. *ciliata* Printz	Altay, Xinjiang Uygur Autonomous Region	86°52′04.20″E, 47°48′53.80″N	506	Sept 2011
PHP-1	*P. himalaica* Pilger	Tashenkuergan County, Xinjiang Uygur Autonomous Region	75°25′14.95″E, 37°34′33.24″N	3537	Aug 2012

reagent, DMEM, and TrypLE Express (1×, no Phenol Red) were obtained from Gibco (Life Technologies, Carlsbad, CA, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was obtained from Millipore (Billerica, MA, USA). Human hepatocellular carcinoma (HepG2) and RAW 264.7 mouse macrophage cells were from the Chinese Academy of Sciences (Shanghai, China). Thirty percent hydrogen peroxide (H_2O_2), analytical grade methanol, acetone, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Sinopharm (Shanghai, China). HPLC grade formic acid, methanol, and acetonitrile were purchased from Merck (Darmstadt, Germany). Reference compounds, geniposidic acid (GA) and acteoside (AC), were purchased from Shanghai R&D Centre for Standardization of Chinese Medicines and Tauto Biotech (Shanghai, China), respectively. Ultrapure water was prepared using a Millipore ultra-Genetic polishing system with <5 ppb TOC and resistivity of 18.2 mΩ.

Sample Preparation. Air-dried seed samples were powdered to a particle size of 40 mesh. One gram of each sample was extracted with 10 mL of 60% methanol (v/v) in an ultrasonic bath for 30 min at ambient temperature and then centrifuged at 3082*g* for 10 min. The supernatant was collected and subjected to UPLC and UPLC/Q-TOF-MS analyses. Another set of 28 supernatants was prepared, and the solvents were removed using a nitrogen evaporator and a freeze-dryer under reduced pressure. The sample extracts were stored at −20 °C until bioactivity evaluations.

Method Development and Validation. A UPLC method was developed for quantitative analysis and validated for linearity, precision, accuracy, repeatability, and stability as follows. In brief, the standard compounds, GA and AC, were dissolved in 60% methanol to obtain the stock solution at a concentration of 1 mg/mL. A set of standard testing solutions was prepared by appropriate dilution of the stock solution with the mixture of 0.5% formic acid and methanol (9:1, v/v), containing 0.98−1000 μg/mL of GA and AC. The testing standard solutions were analyzed in triplicates. Calibration curves were constructed by plotting the peak areas versus concentrations of analytes. The limit of detection (LOD) and limit of quantification (LOQ) for the standard compounds were determined as 3- and 10-fold of the signal-to-noise (S/N) ratio, respectively. The intraday

precision was calculated using data collected at five different time points within a day, whereas interday precision was examined over 6 days at three concentrations (7.81, 62.50 and 500.00 μg/mL). Method repeatability was estimated by analysis of six independently prepared samples. For the stability test, the sample solution was analyzed over periods of 2, 4, 6, 8, 12, 24, 48 and 96 h, respectively. The accuracy of the analytical method was evaluated using the recovery test. Three concentration levels of GA (0.20, 0.25 and 0.30 mg) and AC (0.40, 0.50 and 0.60 mg) were spiked in 0.1 g of PAL-3 sample with known concentrations of the target compounds, respectively. The percent recoveries were calculated by the difference between the spiked and nonspiked samples using the calibration curves. All of the testing solutions were prepared using sample PAL-3. The relative standard deviation (RSD) of peak area was used for method validation.

Quantification of Geniposidic Acid and Acteoside by UPLC Analysis. The sample solution was separated using a UPLC system consisting of an Acquity UPLC H-Class system (Waters, Milford, MA, USA) and a Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm i.d.; 1.7 μm) kept at 40 °C. The sample extracts were diluted 10-fold with the mixture of 0.5% formic acid and methanol (9:1, v/v) (10 mg sample powder equivalents/mL). A binary mobile phase consisting of 0.5% formic acid (A) and acetonitrile (B) was used. Gradient elution was performed as follows: starting with 5% B, reaching 10% B in 1 min, 15% in 1 min, 20% in 1 min, 29% in 1 min, 35% in 1 min, and 40% in 1 min. The flow rate was 0.5 mL/min, and the injection volume was 5 μL. GA and AC in *Plantago* samples were detected at 254 nm and quantified using external standards.

Identification of Chemical Compositions by UPLC/Q-TOF-MS Analysis. *Plantago* seed samples were analyzed for their chemical compositions with a Waters Xevo G2 Q-TOF mass spectrometer (Milford, MA, USA). UPLC was performed using the same conditions for quantification except that the injection volume was 2 μL. Mass spectra were obtained with electrospray ionization (ESI) with a negative ion mode. A MS^E method (a scan model to get both parent and daughter ion information in one injection using tandem mass spectrometry) was used, and MS conditions were as follows: capillary voltage, 3.0 kV; sampling cone voltage, 40 V; source temperature, 120 °C; desolvation temperature, 450 °C; cone gas flow, 50.0 L/h; desolvation gas flow, 600.0 L/h; scan range of MS^1 and MS^2, from m/z 100 to 1500 for 6 min; and ramp collision energy, from 25 to 35 V. Data were collected and analyzed with Waters MassLynx v4.1 software.

Inhibition on IL-1β, IL-6, and COX-2 mRNA Expression. Nineteen *Plantago* sample extracts, and pure GA, AC, and their mixture were tested and compared under the same conditions. The sample extracts were redissolved in DMSO (100 mg/mL) and diluted to 0.1 mg/mL with culture medium. RAW 264.7 mouse macrophages were cultured in 6-well plates and reached the confluence of 80%. The cells were pretreated with culture medium containing samples (0.1 mg/mL) for 24 h. LPS was added at a concentration of 10 ng/mL, and cells were incubated in DMEM with 10% FBS and 1% antibiotic–antimycotic at 37 °C under 5% CO_2 for another 4 h. After induction, culture medium was removed and cells were collected for total RNA isolation and real-time PCR.[10]

RNA isolation and real-time PCR were performed according to a reported protocol.[11] Cells were washed once with phosphate buffer solution (PBS), and TRIzol reagent was added for total RNA isolation. An IScript advanced cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to reverse transcribe complementary DNA. Real-time PCR was performed on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Hercules, CA, USA) using AB Power SYBR Green PCR Master Mix (Applied Biosystems). Primers used in this study were as follows: IL-1β (forward, 5′-GTTGACG-GACCCCAAAAGAT-3′; reverse, 5′-CCTCATCCTGGAAGGTC-CAC-3′); IL-6 (forward, 5′-CACGGCCTTCCCTACTTCAC-3′; reverse, 5′-TGCAAGTGCATCATCGTTGT-3′); COX-2 (forward, 5′-GGGAGTCTGGAACATTGTGAA-3′; reverse, 5′-GCACGTT-GATTGTAGGTGGACTGT-3′). The mRNA amounts were normalized to an internal control, GAPDH mRNA (forward, 5′-AGGTGGTCTCCTCTGACTTC-3′; reverse, 5′-TACCAGGAAAT-GAGCTTGAC-3′). The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, and 46 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min.

Cellular Antioxidant Activity (CAA) Analysis. The CAA values were examined following a previously reported method with slight modification.[12] The growth medium contained WME, 5% FBS, 10 mM Hepes at pH 7.4, 2 mM L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin. Treatment medium contained WME, HBSS (no Phenol Red), and 10 mM Hepes in pH 7.4. The sample extracts were redissolved in DMSO (100 mg/mL) and then diluted to 50 μg/mL with treatment medium. HepG2 cells were seeded in the growth medium at a density of 6×10^4 in 100 μL per well on a 96-well bottom-read microplate. Growth medium was added to the outside wells of the plate. After 24 h of culturing, the growth medium was discarded and the wells were washed with PBS. One hundred microliters of standard solution or sample extract solution plus 100 μL of 25 μM DCFH-DA in treatment medium were applied to the cells for 1 h at 37 °C. Then, 600 μM AAPH was added to the cells in 100 μL of HBSS. The fluorescence of the reaction mixture was measured using a Multimode Reader (TECAN, Männedorf, Switzerland) every minute for 1 h at 37 °C with an emission wavelength at 538 nm and an excitation at 485 nm. Each plate had three blank and three control wells. Blank wells contained cells treated with DCFH-DA. Control wells contained cells treated with DCFH-DA and AAPH. CAA value was expressed as micromoles of quercetin equivalents (QE) per gram *Plantago* seed powder.

Relative DPPH Radical Scavenging Capacity (RDSC). DPPH radical scavenging capacity was measured following a previously reported protocol.[13] Briefly, 100 μL of sample extracts (diluted 600-fold by 60% methanol from the original extract solutions), GA and AC solutions, trolox standard, or solvent was added into 100 μL of 200 μM $DPPH^•$ solution to induce antioxidant radical reaction. The absorbance of the reaction was determined at 515 nm every minute during 1.5 h of reaction. RDSC values were calculated using areas under the curve relative to trolox standard and expressed as micromoles of trolox equivalents (TE) per gram of *Plantago* seed powder. Reaction was conducted in triplicate.

Hydroxyl Radical Scavenging Capacity (HOSC). The HOSC values were estimated using a previously reported laboratory protocol.[14] The reaction mixture contained 30 μL of sample extracts, GA and AC solutions, trolox standard solution, or solvent, 170 μL of 9.28×10^{-8} M fluorescein, 60 μL of 3.43 M iron(III) chloride, and 40 μL 0.1990 M H_2O_2. The fluorescence of the reaction mixture was determined every minute for 6 h at ambient temperature, with an emission wavelength at 528 nm and an excitation wavelength at 485 nm. Reaction was conducted in triplicate, and HOSC values were reported as micromoles of TE per gram of *Plantago* seed powder.

Statistical Analysis. Data were reported as the mean ± SD for triplicate determinations. One-way ANOVA and Tukey's test were employed for different independent samples. Data evaluation was performed with SPSS for Windows (version rel. 16.0, SPSS Inc., Chicago, IL, USA). Significant differences were declared at $P < 0.05$. Cluster analysis was carried out by MATLAB (version 7.5 R2007b, The MathWorks, Natick, MA, USA).

RESULTS AND DISCUSSION

Optimization of UPLC Conditions. Different columns (Waters BEH C_{18}, HSS T_3, BEH Amide; 100 mm × 2.1 mm i.d., 1.7 μm) and mobile phases consisting of methanol–water and acetonitrile–water with different concentrations of formic acid in water (0.1, 0.3, and 0.5%, v/v) were compared. The BEH C_{18} column was more effective and gave better separation of GA and AC than the other two columns. To avoid the tautomeric rearrangements of iridoids,[15] the samples were dissolved in a mixture of 0.5% formic acid and methanol (9:1, v/v). Furthermore, other chromatographic variables, including column temperatures (25, 35 and 40 °C) and flow rates (0.4 and 0.5 mL/min), were also evaluated. When the flow rate was

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Table 2. Calibration Curves and Inter- and Intraprecision Data for Geniposidic Acid (GA) and Acteoside (AC)

	calibration curve	r^2	concn (μg/mL)	interday precision[a] (%)	intraday precision[a] (%)
GA	$y = 7781000x + 35740$	0.9996	7.81	0.25	0.30
			62.50	0.08	0.54
			500.00	0.04	0.76
AC	$y = 5317000x + 2105$	0.9999	7.81	0.06	0.84
			62.50	0.09	0.93
			500.00	0.08	1.58

[a]Expressed as relative standard deviation.

Table 3. Geniposidic Acid (GA) and Acteoside (AC) Contents, Relative DPPH$^\bullet$ Scavenging Capacity (RDSC), and HO$^\bullet$ Scavenging Capacity (HOSC) of *Plantago* Samples[a]

sample	GA (mg/g seed powder)	AC (mg/g seed powder)	RDSC (μmol TE/g)	HOSC (μmol TE/g)
PML-1	5.21o ± 0.10	8.01k ± 0.15	84.40ghi ± 2.91	507.69jk ± 6.05
PML-2	5.20o ± 0.09	7.77jk ± 0.09	85.73ghi ± 4.81	419.66gh ± 16.87
PML-3	1.61hi ± 0.06	1.07b ± 0.02	58.83cd ± 2.13	388.36fgh ± 21.19
PML-4	3.34m ± 0.08	7.41j ± 0.20	88.46ij ± 3.69	586.05m ± 7.14
PML-5	0.35bc ± 0.02	1.33b ± 0.01	31.19b ± 0.83	228.21bc ± 10.80
PAL-1	10.04r ± 0.16	10.69n ± 0.16	129.85l ± 2.14	840.52n ± 12.98
PAL-2	3.21m ± 0.08	6.00h ± 0.25	59.68cd ± 2.44	275.70cd ± 12.85
PAL-3	2.53k ± 0.05	4.73fg ± 0.15	55.75c ± 0.62	307.51de ± 14.37
PAS-1	1.26g ± 0.04	4.04e ± 0.15	87.03hij ± 3.93	516.95kl ± 28.19
PAS-2	1.93j ± 0.05	4.48ef ± 0.16	93.24ij ± 4.03	386.39fgh ± 18.93
PAS-3	1.87ij ± 0.05	5.85h ± 0.15	87.23hij ± 3.40	504.49jk ± 9.14
PAS-4	0.43bc ± 0.03	0.07a ± 0.00	27.94ab ± 0.45	57.18a ± 3.53
PLL-1	1.54h ± 0.08	3.45d ± 0.11	116.54k ± 1.92	490.73ijk ± 26.90
PLL-2	2.82l ± 0.10	6.91i ± 0.14	118.58kl ± 5.61	499.53ijk ± 15.79
PLL-3	4.99o ± 0.11	1.51b ± 0.02	74.76efgh ± 4.29	320.35de ± 6.13
PDW-1	4.49n ± 0.08	15.96o ± 0.20	150.23m ± 1.59	780.90n ± 30.56
PDW-2	0.47cd ± 0.01	5.15g ± 0.16	81.28fghi ± 2.10	399.83fgh ± 16.44
PDW-3	1.07fg ± 0.00	8.09k ± 0.01	82.32ghi ± 3.99	579.21lm ± 16.87
PDW-4	0.30abc ± 0.03	1.49b ± 0.05	74.84efgh ± 0.99	365.49efg ± 11.44
PMP-1	0.93ef ± 0.01	7.77jk ± 0.03	98.38ij ± 2.07	448.00hij ± 23.43
PMP-2	7.41q ± 0.12	1.19b ± 0.02	18.00a ± 0.17	117.13a ± 4.99
PCG-1	0.46cd ± 0.00	4.47ef ± 0.01	73.48efg ± 5.20	356.60ef ± 7.20
PCG-2	0.19ab ± 0.00	4.25ef ± 0.02	67.69cde ± 0.43	196.87b ± 17.65
PDS-1	0.19ab ± 0.01	5.79h ± 0.17	99.13j ± 0.80	441.53hi ± 24.44
PCL-1	0.05a ± 0.00	0.23a ± 0.01	18.45a ± 0.26	86.31a ± 2.77
PJK-1	6.58p ± 0.11	8.77l ± 0.16	75.33efgh ± 2.71	271.12cd ± 12.07
PMS-1	2.25k ± 0.06	10.17m ± 0.10	69.20def ± 3.04	263.80cd ± 7.87
PHP-1	0.71de ± 0.03	2.10c ± 0.05	26.05ab ± 0.15	68.06a ± 2.92

[a]Data expressed as mean values ± SD ($n = 3$). Within each column, values with the same letter are not significantly different ($p \leq 0.05$). TE stands for trolox equivalents.

0.5 mL/min with 40 °C column temperature, the analytes were baseline-separated with sharp peak shape. Compared with the traditional HPLC, the UPLC has higher sensitivity with a shorter analytical time. Under the present conditions, the target compounds were well separated within 6 min (Figure S1 in the Supporting Information), a much shorter analysis time than attainable results with the previously reported HPLC method.[16,17]

Method Validation. The UPLC method for quantitative analysis was validated for linearity, precision, accuracy, repeatability, and stability. The calibration curves for GA and AC showed excellent linear regression (Table 2). The LOD and LOQ for GA were 6.51 and 19.53 ng/mL and those for AC were 9.77 and 39.06 ng/mL, respectively. The established method showed a good reproducibility for the quantification of the two analytes with intra- and interday variations of less than 0.25 and 1.58%, respectively. The RSDs of peak areas were

Table 4. Characterization and Occurrence of Compounds in *Plantago* Samples

	RT (min)	compd	mass	calcd mass	error (ppm)	UV λ_{max} (nm)	formula	occurrence
1	0.83	shanzhiside	391.1259	391.1240	4.9	235	$C_{16}H_{24}O_{11}$	PAS-; PLL-1,2,3; PMP-1; PMS-1; PHP-1
2	0.95	arborescosidic acid	373.1115	373.1135	−5.4	nd[a]	$C_{16}H_{22}O_{10}$	PLL-1
3	1.13	geniposidic acid	373.1127	373.1135	−2.1	239	$C_{16}H_{22}O_{10}$	all samples
4	1.43	decaffeoylverbascoside	461.1649	461.1659	−2.2	294	$C_{20}H_{30}O_{12}$	PAS-1,2,3
5	1.57	salidroside	299.1148	299.1131	5.7	275	$C_{14}H_{20}O_{7}$	PAS-1,2; PDW-1,2,3; PCG-1,2; PCL-1; PDS-1
6	1.68	ferulic acid	193.0494	193.0501	−3.6	281	$C_{10}H_{10}O_{4}$	PML-4,5
7	1.76	gentiopicroside	355.1012	355.1029	−4.8	242/295	$C_{16}H_{20}O_{9}$	PML-2,3; PDW-1,4; PCG-1,2; PDS-1
8	2.00	ampelopsin 7-glucoside	481.0968	481.0982	−2.9	294	$C_{21}H_{22}O_{13}$	PAL-3; PAS-1,2,4; PDW-1,2,3,4; PCG-1,2; PCL-1; PDS-1
9	2.29	calodendroside A	627.1547	627.1561	−2.2	290	$C_{27}H_{32}O_{17}$	PML-1,4; PAL-1
10	2.44	ampelopsin 3′-glucoside	481.0971	481.0982	−2.3	294	$C_{21}H_{22}O_{13}$	PAS-1,2,4; PLL-1,2,3; PDW-1,2,3,4; PCG-1,2; PCL-1; PDS-1
11	2.56	alpinoside	415.1239	415.1240	−0.2	250	$C_{18}H_{24}O_{11}$	PLL-1,2,3; PMP-2
12	2.70	plantainoside D	639.1903	639.1925	−3.4	328	$C_{29}H_{36}O_{16}$	PAL-1,2; PLL-1,2,3
13	2.77	picraquassioside C	597.2208	597.2183	4.2	243	$C_{28}H_{38}O_{14}$	PAS-1,2,3; PDW-1,2,3,4; PCG-1,2; PCL-1
14	2.86	unknown	785.2136	785.2140	−0.5	251/340	$C_{34}H_{42}O_{21}$	PLL-1,2
15	2.93	xeractinol	465.1043	465.1033	2.2	293	$C_{21}H_{22}O_{12}$	PML-3
16	3.10	ombuoside	637.1783	637.1769	2.2	nd	$C_{29}H_{34}O_{14}$	PLL-1,2
17	3.16	plantagoside	465.1022	465.1033	−2.4	288	$C_{21}H_{22}O_{12}$	PML-1,2,4,5; PAL-1,2,3; PDW-2; PJK-1; PDS-1; PHP-1
18	3.23	plantamajoside	639.1786	639.1773	2.0	327	$C_{29}H_{36}O_{16}$	PML-2,3; PAL-2,3; PAS-4; PMP-2; PHP-1
19	3.27	forsythoside B	755.2392	755.2399	−0.9	330	$C_{34}H_{44}O_{19}$	PLL-1,2,3
20	3.31	unknown	312.1909	312.1923	−1.4	nd	$C_{15}H_{27}N_{3}O_{4}$	PML-1,2,4,5; PAL-1,2,3; PCL-1; PJK-1
21	3.39	stachysoside G/H	669.2024	669.2031	−1.0	312	$C_{30}H_{38}O_{17}$	PMP-1; PMS-1
22	3.42	plantagoguanidinic acid	224.1413	224.1399	6.2	nd	$C_{11}H_{19}N_{3}O_{2}$	all samples except PLL-1,2,3, PMP-1, and PMS-1
23	3.53	acetoside	623.1961	623.1976	−2.4	330	$C_{29}H_{36}O_{15}$	all samples
24	3.77	isoactecide	623.1976	623.1976	0.6	328/329	$C_{29}H_{36}O_{15}$	all samples except PAS-4
25	3.85	methylhesperidin	623.1976	623.1976	0.6	328/329	$C_{29}H_{36}O_{15}$	PML-1,2,4; PAL-1,2; PAS-1,2,3; PLL-1,2,3; PDW-1,2,3,4; PMP-1,2; PCG-1,2; PDS-1; PMS-1; PHP-1
26	3.92	lavandulifolioside	755.1998	755.1976	2.9	251/338	$C_{34}H_{44}O_{19}$	PLL-1,2,3
27	4.03	astilbin/smitilbin	449.1075	449.1084	−2.0	287	$C_{21}H_{22}O_{11}$	PML-3,4; PAL-1,3
28	4.56	martynoside	651.2306	651.2289	2.6	328	$C_{31}H_{40}O_{15}$	PAS-1,2,3; PLL-1,2,3; PHP-1
29	4.63	unknown	421.2077	421.2074	0.7	nd	$C_{19}H_{34}O_{10}$	PML-1,2,3,4,5; PAL-1,2,3; PAS-1,2,3; PDW-1,2,3,4; PCG-1,2; PCL-1; PDS-1
30	5.82	unknown	1119.5592	1119.5587	2.5	nd	$C_{54}H_{88}O_{24}$	PLL-1,2,3

[a]nd, not detected.

found to be 3.67% for GA and 4.59% for AC, suggesting an excellent repeatability. Stability was 0.53 and 4.64% for GA and AC within 96 h at room temperature, respectively. The accuracy of the analytical method was evaluated at three levels (80, 100, and 120%) for the two analytes in three replicates. Spike recoveries of GA and AC ranged from 98.89 to 101.06%, and the RSD values were all <3.57%, indicating that the improved UPLC-UV method was reliable and accurate for analyzing the two bioactive compounds in the genus *Plantago*. The procedure was utilized to examine GA and AC contents in the 28 *Plantago* seed samples.

Sample Analysis. Phenylpropanoid glycosides and iridoids are two major chemical constituents of the genus *Plantago* and can be used as valuable taxonomic markers.[6,8] As a representative of phenylpropanoid glycoside and iridoid, respectively, AC and GA are widely distributed in the genus *Plantago* (Figure S2 in the Supporting Information).[6,8] Previous studies also showed that they are primary contributors for the known bioactivities of *Plantago* species.[16,17] In this study, the two important compounds were quickly quantified using the UPLC-UV method in 6 min.

AC and GA were detected in all 28 *Plantago* seed samples, which further supported their taxonomic importance (Table 3). The contents of AC and GA in all samples ranged from 0.07 to 15.96 mg/g and from 0.05 to 10.04 mg/g, respectively. The amount of AC in *P. depressa* varied the most (1.49−15.96 mg/g). PDW-1 (*P. depressa*) and PAL-1 (*P. asiatica*) had the greatest contents of AC and GA among all samples, respectively. Nearly all samples contained more AC than GA, except for PML-3, PAS-4, PLL-3 and PMP-2.

Among the 28 samples, *P. jehohlensis*, *P. arachnoidea* and *P. depressa* subsp. seeds were investigated for the first time, and *P. himalaica* was found in China for the first time. *P. jehohlensis* had the greatest amount of AC at 8.77 mg/g, whereas *P. himalaica* had a low AC concentration of 2.10 mg/g. Several less investigated species, *P. cornuti*, *P. camtschatica*, *P. depressa* subsp. and *P. himalaica*, contained trace amounts of GA. The contents of GA and AC may provide new evidence for chemotaxonomy of *Plantago* species in China.

P. depressa and *P. asiatica* grown in Xinjiang Uygur Autonomous Region might be a good source of AC and GA, respectively. In general, the samples grown in Xinjiang had greater AC contents than those from other areas. Some previous papers suggested that concentrations of GA and AC were highly related to temperature, light, and atmosphere. Tamura et al. reported that relatively low temperatures could increase the contents of AC but reduce iridoid glucoside concentrations.[18,19] Higher CO_2 density could improve the AC

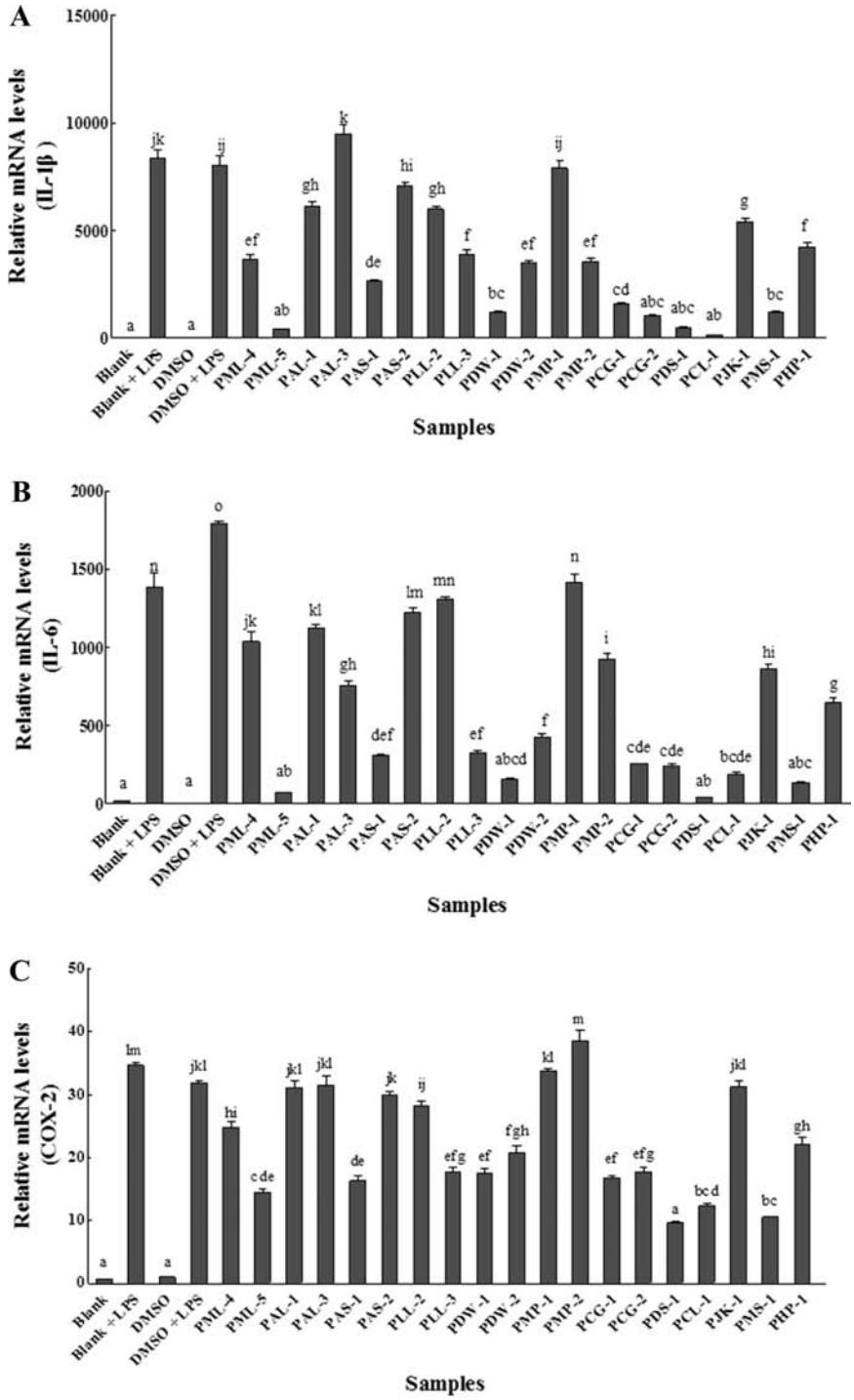

Figure 1. Effects of *Plantago* samples on (A) IL-1*β*, (B) IL-6, and (C) COX-2 mRNA expressions in RAW 264.7 mouse macrophage cells. PML-4,5; PAL-1,3; PAS-1,2; PLL-2,3; PDW-1,2; PMP-1,2; PCG-1,2; PDS-1; PCL-1; PJK-1; PMS-1; and PHP-1 stand for the 19 *Plantago* seed samples of *P. major*, *P. asiatica*, *P. arachnoidea*, *P. lanceolata*, *P. depressa*, *P. minuta* Pall., *P. cornuti* Gouan, *P. depressa* subsp. Turczaninowii., *P. camtschatica* Link., *P. jehohlensis* Koidz., *P. maritima*, and *P. himalaica* Pilger. species, respectively. The final concentration was 100 *μ*g *Plantago* seeds extract/mL in the initial culture medium. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters reflect significant differences ($P < 0.05$).

contents,[20] whereas low nitrogen supply and light intensity strongly inhibited the accumulation of AC in leaves.[18] Altitudes with different UV intensities and temperatures also could influence the concentrations of phenylpropanoid glycosides.[21] These previous papers could partially explain the observations of the variation of GA and AC contents for *P. depressa* and *P. asiatica* grown in Xinjiang Uygur Autonomous Region.

Identification of Chemical Compounds in *Plantago* Seeds. *Plantago* was reported to be rich in phenylethanoid glycosides, iridoids, flavonoids and phenolic acids.[1,3,6,19,22] In the present study, the chemical compositions in the 28 *Plantago* seed samples were characterized by UPLC/Q-TOF-MS analysis. The representative total ion current chromatograms of six selected *Plantago* species are shown in Figure S3

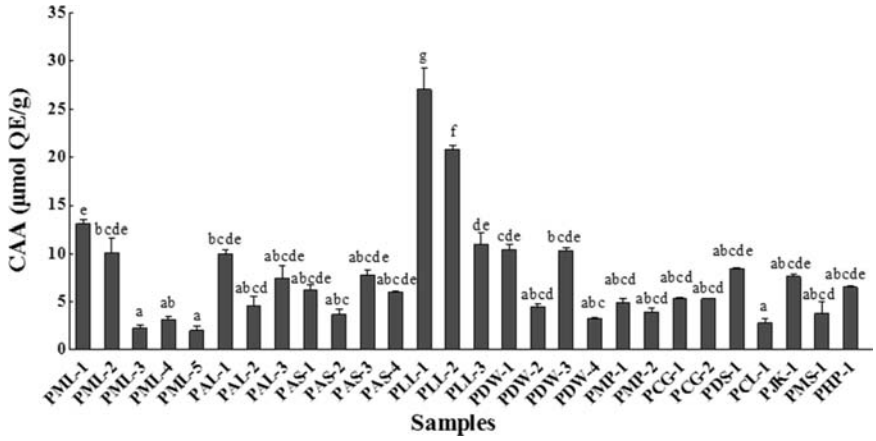

Figure 2. Cellular antioxidant activity (CAA) of *Plantago* samples. PML1−5, PAL1−3, PAS1−4, PLL1−3, PDW1−4, PMP1-2, PCG1-2, PDS-1, PCL-1, PJK-1, PMS-1, and PHP-1 represent 28 *Plantago* seed samples. Data are reported as QE per gram of *Plantago* seed powder basis and as mean ± SD (n = 3). QE stands for quercetin equivalents. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters reflect significant differences ($P < 0.05$).

(Supporting Information). A total of 30 compounds was detected in the 60% methanol extract of the 28 *Plantago* seed samples (Table 4). Twenty-six compounds were tentatively identified on the basis of accurate MS data and secondary MS fragmentation profiles. As an example, the fragmentation pathway proposed for plantamajoside (peak 18) is shown in Figure S4 (Supporting Information). There were still four compounds remaining unknown. The retention times, maximum absorption UV wavelength, accurate MS data, and occurrence in *Plantago* seed samples of the detected compounds are summarized in Table 4.

Nearly all *Plantago* species showed similar chemical profiles except *P. lanceolata*. Arborescosidic acid (**2**), forsythoside B (**19**), lavandulifolioside (**26**) and two unknown compounds (**14** and **30**) were detected only in *P. lanceolata*. Alpinoside (**11**) was detected in *P. lanceolata* seeds for the first time. Forsythoside B (**19**) was previously detected in *P. asiatica* and *P. depressa*,[23] but was not detected in the seeds of these two species in the present study. Interestingly, plantagoguanidinic acid (**22**), a guanidine compound reported only in *P. asiatica*,[24] was detected in 23 of the tested seed samples. Compound **20** was deduced to possess a structure similar to that of plantagoguanidinic acid (**22**) by its molecular formula.

On the basis of the total ion current profiles, cluster analysis was employed to effectively discriminate these species (Figure S5 in the Supporting Information). *P. lanceolata*, *P. minuta*, and *P. maritima* were clustered and separated with other species. *P. major*, *P. asiatica*, *P. jehohlensis* and *P. himalaica* were in a relatively tight cluster, supporting the observation that *P. jehohlensis* and *P. himalaica* showed chemical profiles similar to those of *P. major* and *P. asiatica*. In addition, *P. arachnoidea*, *P. depressa*, *P. depressa* subsp., *P. cornuti* and *P. camtschatica* formed a cluster, providing new evidence for a close relationship between *P. depressa* subsp. and *P. depressa*. This study revealed the relationships of 12 Chinese *Plantago* species and provided valuable information for the less studied species.

Effects on IL-1β, IL-6, and COX-2 mRNA Expression. Chronic inflammation has been recognized as a risk factor for a number of chronic diseases such as autoimmune diseases and atherosclerosis.[25] To determine the anti-inflammatory activity for the seeds of the12 *Plantago* species, IL-1β, IL-6, and COX-2 mRNA expressions in LPS-stimulated RAW 264.7 mouse macrophage cells were examined.

Nineteen *Plantago* samples significantly differed in their inhibitory activities of IL-1β, IL-6 and COX-2 mRNA expressions. As shown in Figure 1A, all samples greatly inhibited IL-1β mRNA expression except PAL-3 and PMP-1 seed extracts. *P. camtschatica* seed extract showed the strongest suppression on IL-1β mRNA expression. All samples inhibited IL-6 mRNA expression (Figure 1B), and *P. depressa* subsp., an unreported species, showed the strongest inhibitory activity. Nearly all samples suppressed COX-2 mRNA expression except those belonging to *P. asiatica*, *P. minuta* and *P. jehohlensis* (Figure 1C). *P. depressa* subsp. had the strongest inhibitory effect, and *P. maritima* and *P. camtschatica* also exhibited significant anti-inflammatory activities. In summary, seeds of *Plantago* species, especially *P. depressa* subsp., might be potentially used in anti-inflammatory nutraceuticals and functional foods.

Cellular Antioxidant Activity. The CAA of different samples is shown in Figure 2, indicating significant differences in CAA values of the 28 *Plantago* seed samples. *P. lanceolata* exhibited the greatest CAA. It was remarkable that *P. himalaica* had a stronger CAA than *P. maritima* and *P. camtschatica*, indicating a potential application of *P. himalaica* as a dietary source for natural antioxidants. Species collected from different locations also exhibited different cellular antioxidant activities. PLL-1, collected from Xinjiang, showed a greater CAA than other samples of the same species from different locations. Similar results were also observed in other species including *P. major*, *P. asiatica* and *P. depressa*. Together, these data suggested the possible effects of growing conditions on health properties of *Plantago* seeds. This study gave a first insight into the cellular antioxidant activities of the genus *Plantago*, providing useful information for further studies and uses of the botanicals.

DPPH Radical Scavenging Capacities. In the present study, the free radical scavenging capacity of the *Plantago* seed extracts was investigated against DPPH$^{\bullet}$. As shown in Table 3, all seed extracts showed significant DPPH$^{\bullet}$ scavenging capacities (RDSC). PDW-1 had the greatest DPPH$^{\bullet}$ scavenging capacity, followed by PAL-1. Also noted was that the same species samples collected from different growing locations might differ in their DPPH$^{\bullet}$ scavenging capacities.

Hydroxyl Radical Scavenging Capacity. The HOSC values of the 28 samples are shown in Table 3. To avoid solvent interference in the assay, 40% acetone was used to redissolve

the seed extracts. PDW-1 extract showed the strongest HOSC among all tested samples, followed by PAL-1 extract. It was notable that *P. depressa* subsp. seed extract showed greater HOSC than those of *P. jehohlensis* and *P. himalaica*, suggesting the possible effects of growing conditions on HOSC of the seed extracts. To the best of our knowledge, the present study investigated the DPPH and HO radical scavenging activities of *P. depressa* subsp., *P. arachnoidea*, *P. minuta*, *P. cornuti*, *P. camtschatica*, *P. jehohlensis* and *P. himalaica* seeds for the first time.

Synergistic Antioxidant and Anti-inflammatory Effects of Geniposidic Acid and Acteoside. To investigate the contributions of GA and AC and their possible synergistic effects in the overall DPPH$^{\bullet}$ and HO$^{\bullet}$ scavenging capacities, cellular antioxidant activity, and anti-inflammatory activity, pure compounds and their mixture solutions in the same concentrations as in the PAL-1 and PDW-1 seed extracts were investigated under the same condition. As shown in Figures 3 and 4, there was no difference between AC and the mixtures on RDSC and HOSC, whereas GA gave no contribution on radical scavenging capacities. The results indicated that AC contributed significantly to the DPPH$^{\bullet}$ and HO$^{\bullet}$ scavenging capacities in PAL-1 and PDW-1, which were consistent with the observations from previous studies.[26,27] However, GA possessed weak cellular antioxidant activity with AC as a major contributor. Anti-inflammatory tests also showed similar trends in inhibiting IL-1β, IL-6, and COX-2 mRNA expressions in PAL-1 and PDW-1. No synergistic effect was observed between the two components on DPPH$^{\bullet}$ and HO$^{\bullet}$ scavenging capacities, CAA, and anti-inflammatory activity under the experimental conditions. According to the UPLC/Q-TOF-MS analysis, other minor phenylethanoids or flavonoids might have contributed to the overall antioxidant and anti-inflammatory activities of *Plantago* seeds.

Correlations of Antioxidant and Anti-inflammatory Activities and Chemical Compositions. The RDSC and HOSC values of *Plantago* seed extracts showed a positive correlation with AC contents (R = 0.736, 0.709; P < 0.001, respectively). The HOSC value was significantly correlated with the RDSC value (R = 0.889, P < 0.001). Meanwhile, no correlation between HOSC value and GA content was observed, which was consistent with previous reports that iridoids showed a weak correlation with radical scavenging capability against DPPH$^{\bullet}$.[26] Cellular antioxidant activity and inhibition on IL-1β, IL-6 and COX-2 mRNA expression of *Plantago* seed samples showed no significant correlation with GA or AC concentration. The data suggested that GA contributed to the overall CAA and anti-inflammatory activity of PAL-1 and PDW-1. Taken together, it was possible that cell metabolites of GA and AC or minor constituents might contribute to the cellular antioxidant and anti-inflammatory activities of *Plantago* seeds.

The RDSC and HOSC assays have been widely used to assess the antioxidant capacity of foods, phytochemicals, and botanical extracts.[1,28,29] Recently, several CAA assays utilizing HepG2, erythrocytes, or red blood cells, it is possible to evaluate cell-based antioxidant activity in vitro in a cost-effective way.[12,30,31] This study showed that CAA values of the 28 seed samples were weakly correlated with RDSC (R = 0.530, P < 0.001) and HOSC values (R = 0.387, P < 0.001), which were consistent with reports that a poor correlation existed between CAA assay and oxygen radical absorbance (ORAC) assay.[30,32] CAA is a peroxyl radical induced cellular oxidative stress model,

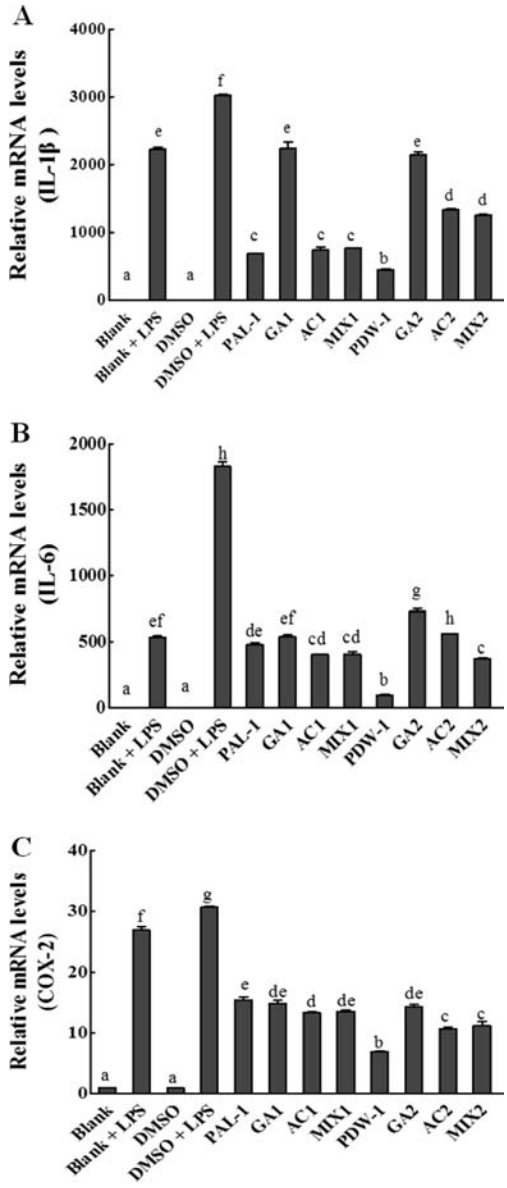

Figure 3. Effects of PAL-1, PDW-1, geniposidic acid, and acteoside on (A) IL-1β, (B) IL-6, and (C) COX-2 mRNA expressions in RAW 264.7 mouse macrophage cells. PAL, *P. asiatica*; PDW, *P. depressa*; GA, geniposidic acid; AC, acteoside. MIX1 and MIX2 are mixtures of GA1 and AC1 and GA2 and AC2, respectively. GA1, AC1, and MIX1 were made at the same GA, AC, and GA+AC concentrations as PAL-1. GA2, AC2, and MIX2 were made at the same GA, AC, and GA+AC concentrations as PDW-1. The vertical bars represent the standard deviation (n = 3) of each data point. Different letters reflect significant differences (P < 0.05).

and the oxidative damage may be induced by several mechanisms and vary with chemical assays employed including RDSC, HOSC and ORAC.[33] It has been reported that CAA is highly related to some factors including the ability to pass through cell membranes, solubility in incubation medium, and lipophilicity.[30,32,34] Therefore, the CAA assay combined with the RDSC and HOSC assays could provide a comprehensive evaluation of the antioxidant activity of a selected food factor or samples.

In summary, the present study developed an efficient and precise UPLC-UV procedure for quantifying acteoside and geniposidic acid in *Plantago* species. A total of 26 compounds

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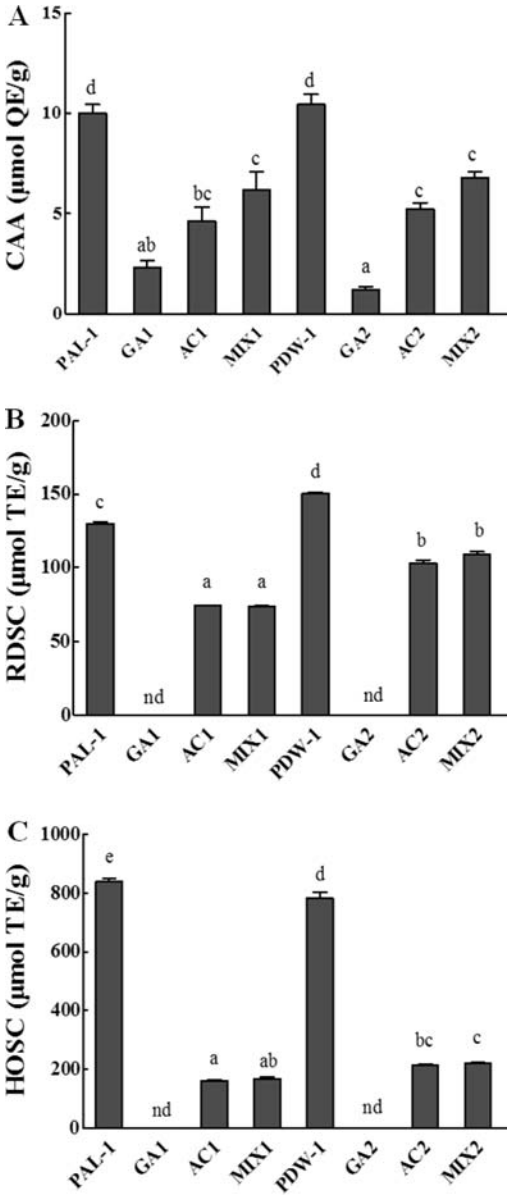

Figure 4. Effects of PAL-1, PDW-1, geniposidic acid, and acteoside on (A) Ccellular antioxidant activity (CAA), (B) relative DPPH• scavenging capacity (RDSC), and (C) HO• scavenging capacities (HOSC). PAL, *P. asiatica*; PDW, *P. depressa*; GA, geniposidic acid; AC, acteoside. MIX1 and MIX2 are mixtures of GA1 and AC1 and GA2 and AC2, respectively. GA1, AC1, and MIX1 were made at the same GA, AC, and GA+AC concentrations as PAL-1. GA2, AC2, and MIX2 were made at the same GA, AC, and GA+AC concentrations as PDW-1. QE and TE stand for quercetin equivalents and trolox equivalents, respectively. The vertical bars represent the standard deviation ($n = 3$) of each data point. Different letters reflect significant differences ($P < 0.05$).

were tentatively identified in *Plantago* seeds by UPLC/Q-TOF-MS analysis. The present study also evaluated and compared the anti-inflammatory effects and the scavenging capacities against DPPH and HO radicals for 12 *Plantago* species and gave the first insight into the cellular antioxidant activity of *Plantago* species. The results of the present study provided valuable evidence for the potential use of *Plantago* seeds as a good dietary source of anti-inflammatory components and natural antioxidants for improving human health.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AC, acteoside; GA, geniposidic acid; CAA, cellular antioxidant activity; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical; RDSC, relative DPPH radical scavenging capacity; HOSC, hydroxyl radical scavenging capacity; RSD, relative standard deviation; LOD, limit of determination; LOQ, limit of quantification; TE, trolox equivalents; QE, quercetin equivalents; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, dichlorofluorescin; AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; WME, Williams' medium E; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; PBS, phosphate buffer solution; HepG2, human hepatocellular carcinoma; ORAC, oxygen radical absorbance; PCR, polymerase chain reaction; ESI, electrospray ionization; UPLC, ultraperformance liquid chromatography; TOF, time of flight; UV, ultraviolet; MS, mass spectrometry

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