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Chemical and Biological Assessment of *Ziziphus jujuba* Fruits from China: Different Geographical Sources and Developmental Stages

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Supporting Information

ABSTRACT: Chinese date, the fruit of *Ziziphus jujuba* Mill., has thousands of years cultivation history, and about 700 cultivars of dates in China. Two types of dates are commonly found in the market: (i) fresh immature dates consumed as fruits, and (ii) dried mature dates used as Chinese medicines. Here, chemical and biological properties of these dates were revealed. Different sources of dates showed similar chemical profiles; however, the amounts of identified chemicals showed a great variation. The amount of nucleotides, flavonoids and polysaccharides in dates could be affected by its maturity and drying process. In parallel, the antioxidative functions of their extracts were compared. The date extracts protected PC12 cells against tBHP-induced cytotoxicity, and which also stimulated the transcriptional activity of antioxidant response element. The antioxidative effects were varied among different dates. The current results suggested the optimization of sources and specific usage of different maturity dates.

KEYWORDS: *Chinese date, Ziziphus jujuba Mill., source, maturity, HPLC-DAD-MS/MS, antioxidation, neuroprotection*

INTRODUCTION

Chinese date is the fruit of *Ziziphus jujuba* Mill., which is commonly known as Chinese jujube or red date. In an ancient Chinese book on herbal medicine named *Huangdi Neijing* (475−221 BC), the fruit of *Z. jujuba* was considered as one of five valuable fruits in China. In addition, *Shennong Bencao Jing* (300 BC−200 AD) recorded the date as one of the superior herbal medicines having the ability to prolong our life-span by nourishing blood, improving quality of sleep and regulating digestive system. Today, the fruit of *Z. jujuba* is considered as nutritious food, which contains a variety of nutrients, including carbohydrates, minerals, vitamins, sugars, and amino acids.[1,2] Being an herbal medicine or health food supplement, the pharmacological results indicated that the fruit possessed antioxidant activities,[3−5] immunological activities,[6−10] potential sedative effects,[11,12] treatment of jaundice in neonates,[13] and anti-inflammatory effects.[14] The aforementioned studies are mainly focused on two major domesticated fruits, namely *Z. mauritiana* Lam. (the Indian date) and *Z. jujuba* (the Chinese date). Besides, phyto-phamacology of the fruit has been summarized.[15,16]

Having a long history of cultivation, over 700 Chinese date cultivars from different sources have been found in China. Yet, the variation among them is not revealed. Besides, the price of the date is fluctuated due to their geographical sources.[17] In addition, two types of dates are commonly found in the market, including fresh immature dates that are eaten as fruits; and dried mature dates that are being used as Chinese medicinal herbs. However, it is not clear whether the immature date is exactly the same as mature date, both chemically and pharmacologically. On one hand, the main ingredients of Chinese date have been reported to contain nucleotides, flavonoids, and polysaccharides. Here, we aimed to reveal the variations among Chinese dates from different sources and maturities, chemically, and biologically. The chemical fingerprint and the amounts of major ingredients among various Chinese dates were determined. On the other hand, the possible biological function of date on neuronal cells against oxidation insult was also revealed and compared.

MATERIALS AND METHODS

Fruit and Chemical Materials. Twenty four fruit samples were collected from different geographical regions of China in November 2012. Among them, one sample from Cangzhou of Hebei province at two stages of maturity was collected. Classification of date: Immature date was collected in summer (September 2012) with a yellow-green background having mahogany-colored spots. Mature date was collected in autumn (October 2012) when its appearance became entirely red and the fruit began to soften and wrinkle. For dried mature dates, they were processed from fresh mature ones. Briefly, after the collection of fresh mature dates, the fruits were dried at 40 °C for 1 week. The water content of dried fruits under this condition was normally less than 5%. The materials were authenticated by one of the authors, Dr. Tina T. X. Dong, according to their morphological characteristics having the voucher numbers as: voucher no. 12-09-A for fresh immature date (A), voucher no. 12-10-A for fresh mature date (A), voucher no. 12-11-A for dried mature date (A), and voucher no.

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12-11-B~X for sample B to X. The voucher specimens were deposited in the Center for Chinese Medicine at Hong Kong University of Science & Technology.

Uracil (1), cytidine (2), uridine (3), cGMP (4), xanthine (5), hypoxanthine (6), guanine (7), guanosine (8), cAMP (9), adenosine (10), and adenine (11) were purchased from Sigma (St. Louis, MO). (−)-catechin (12), procyanidin B2 (13), (−)-epicatechin (14), quercetin 3-*O*-galactoside (15), quercetin 3-*O*-rutinoside (16), quercetin 3-*O*-*β*-D-glucoside (17), kaempferol 3-*O*-rutinoside (18) and astilbin (internal standard; ISD) were purchased from Tauto Biotech (Shanghai, China). In addition, D-anhydrous glucose was purchased from Sigma. The purities of all marker chemicals were determined to be no less than 98% by normalization of peak areas, as revealed by HPLC-DAD. LC-MS acetonitrile was purchased from Merck (Darmstadt, Germany), and ultrapure water was prepared using a Milli-Q purification system (Molsheim, France). Other reagents used here were of analytical grade.

Preparation of Sample Solutions. After removing the kernel and chop into slices, the fruits were lyophilized to dryness. Then, the dried fruits were pulverized to homogeneous powders (40 mesh). The dried powder (50 g) was weighed accurately and boiled in 20 volume of water (v/w) for 1 h and extracted twice. After centrifugation (13 000 rpm, 10 min), the supernatant was stored at 4 °C and filtered through a 0.45 μm filter before injection into HPLC system for analysis. For biological analysis, the extract was dried under vacuum and stored at −80 °C. Before the treatment, the dried extract was redissolved and vortexed in room temperature.

Chromatographic Conditions and Instrumentation. Validated HPLC method was performed on an Agilent (Waldbronn, Germany) RRLC 1200 series system, which was equipped with a degasser, a binary pump, an autosampler, a diode array detector (DAD) and a thermo stated column compartment. The fruit extract was separated on Agilent ZORBAX SB-Aq 4.6 × 250 mm, 5 μm column. The mobile phase was composed of 10 mmol/L ammonium acetate (A) and acetonitrile (B) using the following gradient program: 0−10 min, isocratic gradient 99% (A), the flow rate was 0.5 mL/min; 10−40 min, linear gradient 99−96% (A), the flow rate was 0.5−0.8 mL/min; 40−45 min, linear gradient 96−90% (A), the flow rate was 0.8 mL/min; 45−60 min, linear gradient 90−70% (A), the flow rate was 0.8 mL/min. A pre-equilibration period of 10 min was used between each run. The column temperature was 25 °C: the injection volume was 8 μL: the wavelength was 260 nm. The contents of tested markers were calculated using their calibration curve with regarding to the dilution factor and expressed as microgram per gram of dried fruit weight.

UPLC-MS/MS Analysis of Flavonoids. The fruit extract was separated on Agilent Eclipse Plus C_{18} RRHD 1.8 μm, 2.1 × 50 mm column by an Agilent RRLC 1200 system. The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using the following gradient program: 0−3 min, isocratic gradient 90% (A), the flow rate was 0.30 mL/min; 3−4 min, linear gradient 90−89% (A), the flow rate was 0.30−0.35 mL/min; 4−15 min, isocratic gradient 89% (A), flow rate was 0.35 mL/min; 15−25 min, linear gradient 89−45% (A), the flow rate was 0.35−0.30 mL/min; 25−26 min, linear gradient 45−40% (A), the flow rate was 0.30 mL/min; 26−26.01 min, linear gradient 40−0% (A), the flow rate was 0.30 mL/min;, 26.01−27 min, isocratic gradient 0% (A), the flow rate was 0.30 mL/min. A pre-equilibration period of 3 min was used between each run. The column temperature was 25 °C. The injection volume was 5 μL. For the MS/MS analysis, an Agilent QQQ-MS/MS equipped with an ESI ion source was operated in negative ion mode. The drying gas temperature was 325 °C; Drying gas flow: 10 L/min; Nebulizer pressure: 35 psig; capillary voltage: 4000 V; delta electro multiplier voltage: 400 V. Two suitable transition pairs were chosen for acquisition in multiple reactions monitoring (MRM) mode for selected markers and internal standard astilbin. The fragmentor voltage and collision energy values were optimized to obtain the highest abundance. Agilent Mass Hunter workstation software version B.01.00 was used for data acquisition and processing.

Determination of Total Polysaccharides. Total polysaccharide was determined with anthrone-sulfuric acid colorimetric method, as described previously.[18] The water extract was treated with four volumes of ethanol for precipitation at 4 °C. After incubation for 12 h, the precipitate was lyophilized to dryness. The powder was mixed in the solution of anthrone sulfate (dissolve 0.1 g of anthrone to 100 mL of 80% sulfuric acid, and mix well). The solution was mixed well, and the absorbance was measured against a prepared reagent blank at 625 nm with an UV-vis spectrophotometer. The amount of total polysaccharide was expressed as milligrams of D-anhydrous glucose (from a standard calibration curve) per 1 g dried fruit weight.

Cell Culture and Viability Test. Rat pheochromocytoma PC12 cell line was obtained from ATCC (Manassas, VA). The cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 6% fetal bovine serum and 6% horse serum at 37 °C in a water-saturated 7.5% CO_2 incubator. Reagents for cell cultures were purchased from Invitrogen Technologies (Carlsbad, CA). Cultured PC12 cells in 96-well plate (2×10^4 cells/well) were pretreated with the date extracts at different concentrations for 24 h before being exposed to *tert*-butyl hydroperoxide (tBHP) for 3 h, and the cell viability assay was performed with the addition of thiazolyl blue tetrazolium bromide (MTT; Sigma) in PBS at a final concentration of 0.5 mg/mL for 1 h. After the solution was removed, the purple precipitate inside the cells was resuspended in DMSO and then measured at 570 nm absorbance.[19] tBHP at various concentrations (0, 50, 100, 150, 200, 250, and 300 μM) served as a control for the cytotoxicity test.

Determination of ROS Formation. The determination of ROS level in cell cultures was performed according to previous report,[20] with minor modifications. Cultured PC12 cells (4×10^4 cells/well) in a 96-well plate were pretreated with different fruit extracts for 24 h and labeled by 100 μM DCFH-DA (Sigma) in HBSS for 1 h at 37 °C. Cultured were then treated with 100 μM tBHP for 1 h at 37 °C. The amount of intracellular tBHP-induced ROS was measured by fluorometric measurement with excitation at 485 nm and emission at 530 nm (Spectra max Gemini XS, Molecular Devices Corp., Sunnyvale, CA).

DNA Construction and Transfection. The vector, pGL4.37 [*luc2P*/ARE/Hygro], contains four copies of an antioxidant response element (ARE, 5′-TAGCTTGGAA ATGACATTGC TAATGGT-GAC AAAGCAACTT T-3′) that drives transcription of the luciferase reporter gene *luc2P* (*Photinus pyralis*) (Promega Corporation, Madison, WI). The cultured PC12 cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's guidelines. Under this condition, over 70% cells were successfully transfected, as determined by another control plasmid of having a galactosidase, under a cytomegalovirus enhancer promoter. The cells were used for experiments in 24 h after transfection.

Luciferase and Protein Assays. After drug treatment, luciferase assay was performed by a commercial kit (Tropix Inc., Bedford, MA). In brief, cultures were lysed by a buffer containing 100 mM potassium phosphate buffer (pH 7.8), 0.2% Triton X-100 and 1 mM dithiothreitol. The luminescent reaction was quantified in a Tropix TR717 Microplate Luminometer, and the activity was expressed as absorbance (up to 560 nm) per mg of protein. The luciferase activity was normalized by the fluorescent intensity of EGFP in the same amount of protein in each sample. Protein concentrations were measured routinely by Bradford's method with a kit from Bio-Rad Laboratories (Hercules, CA).

Statistical Analysis. The hierarchical cluster analysis of samples was performed using SPSS software (version 13.0, SPSS). Individual data was expressed as Mean ± standard deviation (SD). Statistical tests were performed with *t*-test (version 13.0, SPSS). Statistically significant changes were classified as significant (*) where $p < 0.05$, more significant (**) where $p < 0.01$ and highly significant (***) where $p < 0.001$.

RESULTS

Chemical Analysis of Dates from Different Sources. The major production sites of Chinese dates are Shanxi, Shaanxi, Hebei, Shandong, Henan, Ningxia, Gansu, and

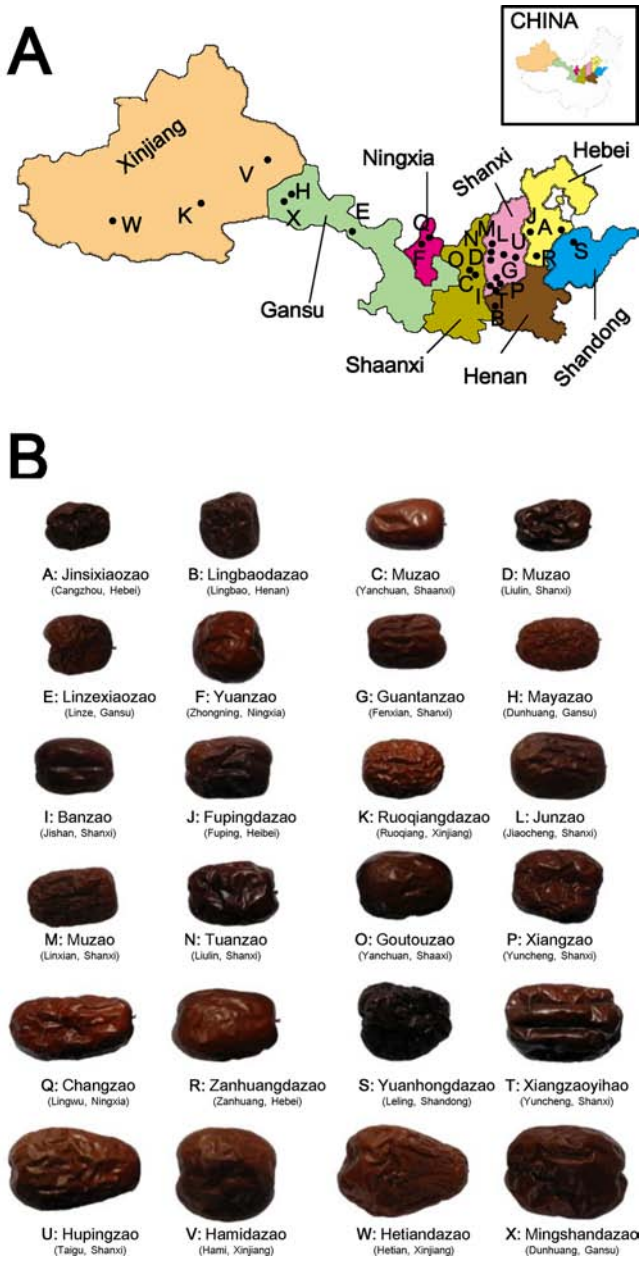

Figure 1. The geographical locations and photographs of Chinese dates used in this experiment. (**A**): Samples of Chinese dates were collected from different regions of China. The insert shows the location of different provinces in China. (**B**): Photographs of 24 Chinese dates (A to X) used in this experiment. The terms A to X correspond to the notations as indicated in (**A**). These dates were collected at mature stage. Bar: 1 cm.

Xinjiang provinces (Figure 1A). Twenty-four fruit samples (denoted from A to X) at mature stage from these regions were collected (Figure 1B): the size of fruit increased accordingly from A to X. Morphologically, different dates from geographical locations in China showed variation in size significantly. The quality of dates could be closely related to their chemical constituents, and which could be assessed by a chemical pattern recognition method. In order to clarify the chemical properties of different dates, the chromatograms were generated. The major cyclic nucleotide (cAMP; peak 6) found in all fruits was used as the reference standard in the fingerprints, and seven common peaks from the chromatograms were identified

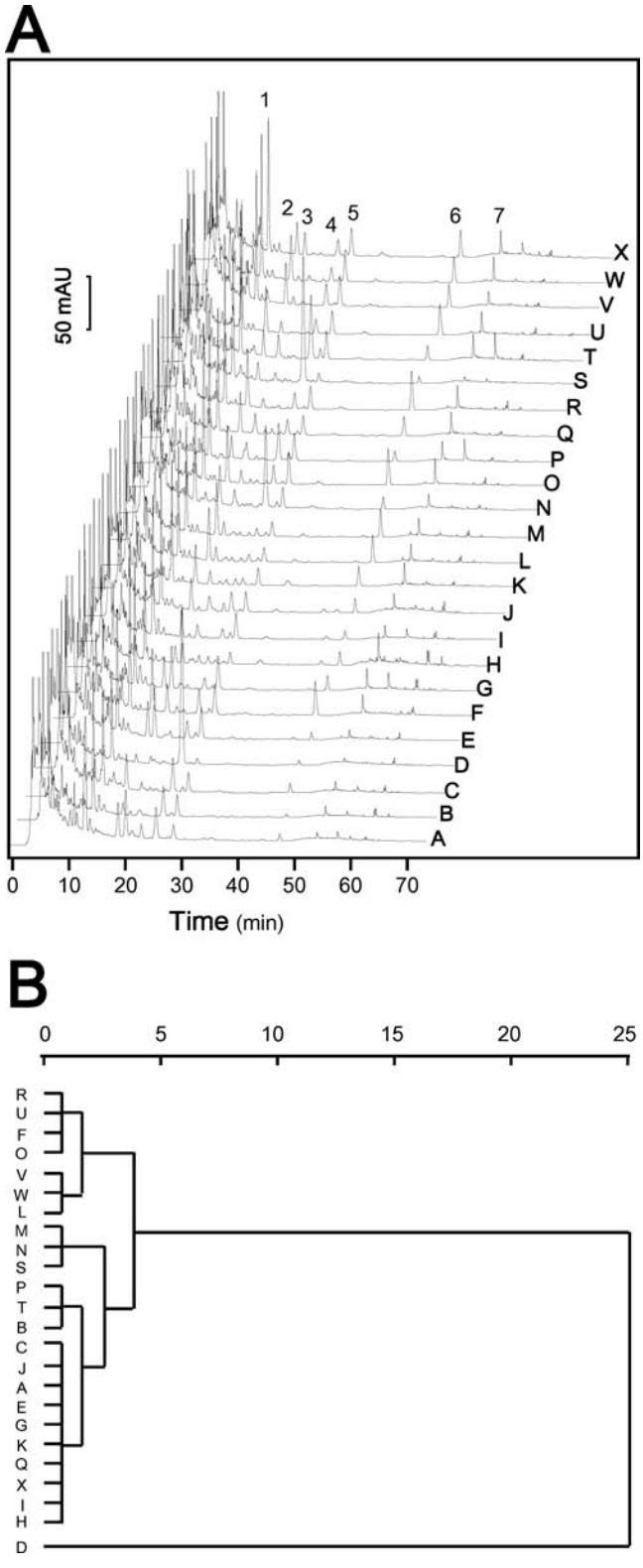

Figure 2. HPLC fingerprint of different sources of Chinese dates. (**A**): HPLC fingerprints of 24 dates' water extracts were performed, as described in Materials and Methods. The effluent was monitored at 260 nm. The terms A to X corresponding to different samples were shown in Figure 1. The notation peaks 1−7 were defined as common peaks, and the peak 6 (cAMP) served as the reference peak. (**B**): Hierarchical clustering analysis was performed from the HPLC fingerprints of 24 dates. The loading plot was performed with the original peak areas of seven common peaks as input data. The scale here is rescaled distance cluster combine.

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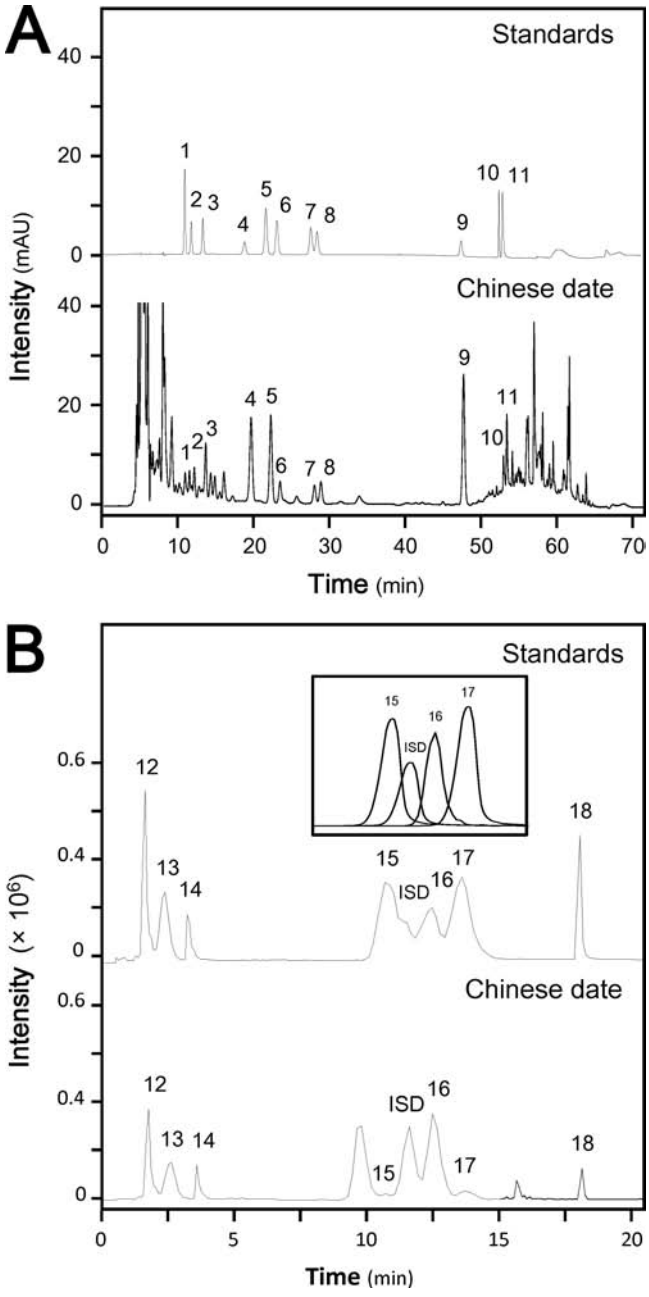

Figure 3. Typical LC chromatograms of marker chemicals in the water extracts of dates. (**A**): The representative HPLC chromatograms of mixed standards and date extract. The HPLC condition was described in Materials and Methods. The effluent was monitored at 260 nm. The notations are (1) uracil, (2) cytidine, (3) uridine, (4) cGMP, (5) xanthine, (6) hypoxanthine, (7) guanine, (8) guanosine, (9) cAMP, (10) adenosine and (11) adenine. (**B**): The representative LC-QQQ-MS/MS chromatograms of mixed standards and date extract. The LC condition was described in the Materials and Methods. The identification of (−)-catechin (12), procyanidin B2 (13), (−)-epicatechin (14), quercetin 3-*O*-galactoside (15), quercetin 3-*O*-rutinoside (16), quercetin 3-*O*-*β*-D-glucoside (17), kaempferol 3-*O*-rutinoside (18) and astilbin (internal standard, ISD) were determined by a MS detector.

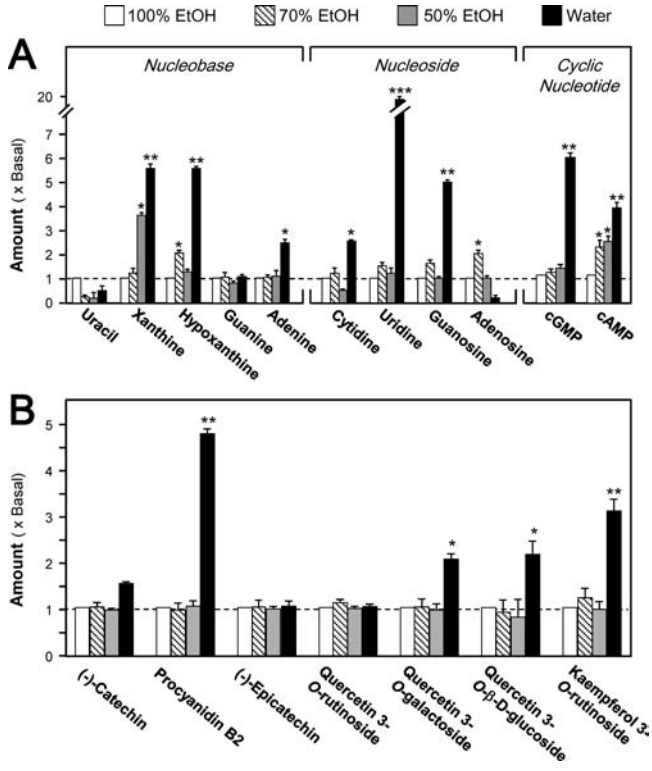

Figure 4. Optimization of different solvents in extracting date. Water, 50% ethanol, 70% ethanol and 100% ethanol were used as extracting solvents for 50 g powder of dates. The tested nucleotides and its derivatives, including uracil, cytidine, uridine, cGMP, xanthine, hypoxanthine, guanine, guanosine, cAMP and adenine were shown in (A). The flavonoids, including (−)-catechin, procyanidin B2, (−)-epicatechin, quercetin 3-*O*-galactoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-*β*-D-glucoside and kaempferol 3-*O*-rutinoside were shown in (B). The determination of these chemicals was described in Figure 3. Values are expressed in μg/g of dried fruit weight, and they are in mean ± SD, where $n = 4$. Statistical comparison was made with 100% EtOH extract; $^{*}p < 0.05$; $^{**}p < 0.01$.

(Figure 2A). The fingerprints were confirmed with high stability and reproducibility. In the hierarchical clustering analysis by Pearson correlation, the 24 batches of dates were divided into three main clusters (Figure 2B): the sources of R, U, F, O, V, W, L (notation as shown in Figure 1B) grouped as cluster one; M, N, S, P, T, B, C, J, A, E, G, K, Q, X, I, H grouped as cluster two; and D singly as cluster three. The geographical sources of dates did not show a close relationship at all regardless they were collected from neighboring provinces (Figure 2B).

An HPLC-DAD method was developed to quantify 11 chemical markers of nucleobase, nucleoside and cyclic nucleotide in water extracts of dates (Figure 3A). For flavonoids, an established LC/MS method was employed to quantify 7 individual flavonoids in the date extracts (Figure 3B). The chemical structures of these markers and method validation results were demonstrated in Supporting Information (SI) Figure S1 and Table S1−S5.

In order to find the optimal extracting conditions, different extraction solvents (water, 50% ethanol, 70% ethanol and 100% ethanol) were tested. As for the tested 18 chemicals, water was found to be a superior extracting solvent (Figure 4A, B). The extraction cycle was tested for the efficiency (SI, Figure S2). Here, the extraction of two cycles was routinely used, and over 95% extracting efficiency was achieved.

By using the established analytic methods, the amounts of 18 chosen chemicals were determined in water extracts from different sources of dates, i.e. total 24 batches of dates. In order

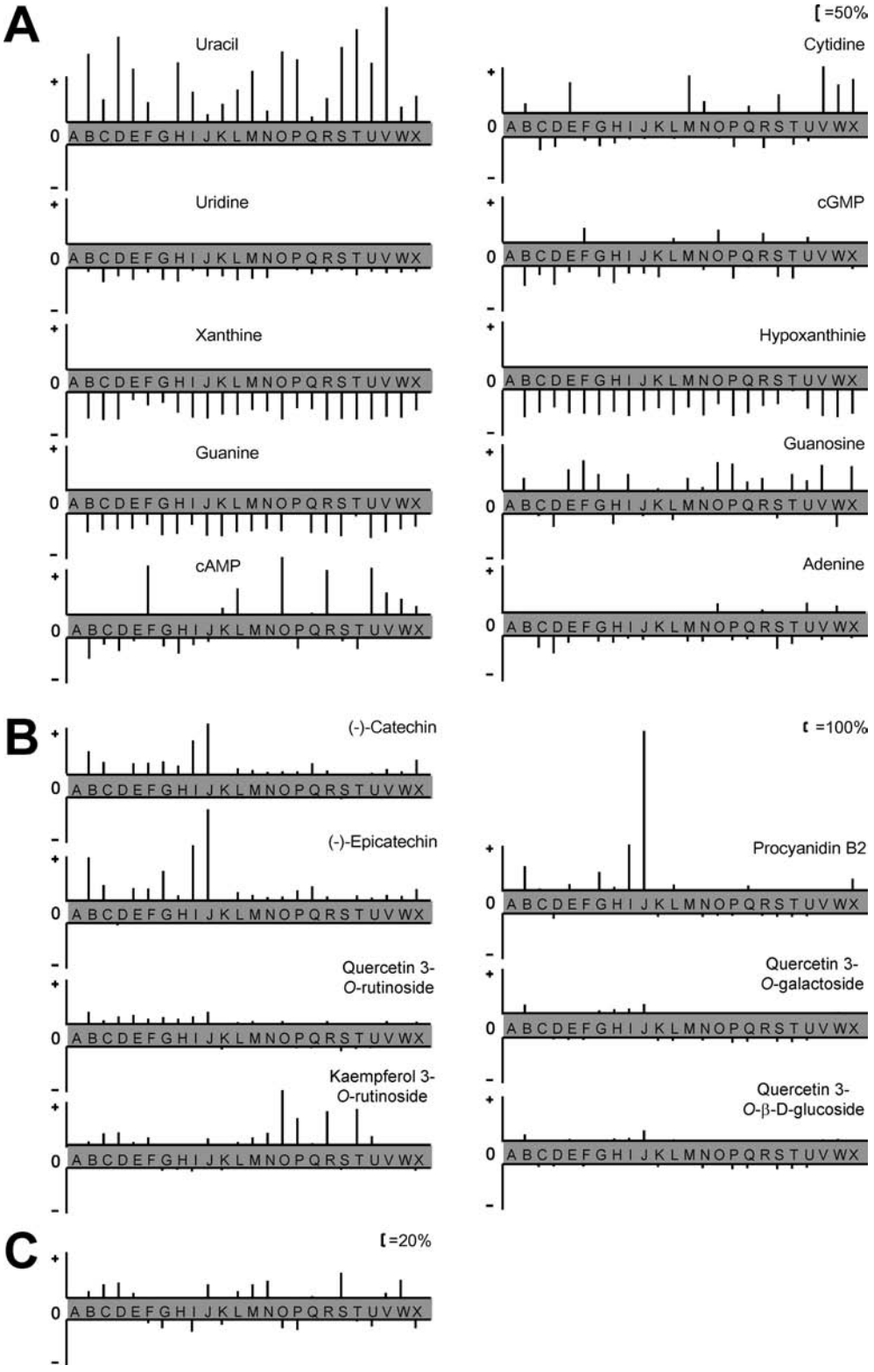

Figure 5. Comparison of the amounts of ingredients in different Chinese date. The chemical composition of dates from different cultivars was determined as in Figure 3. The notations of A to X are corresponding to different geographical sources as listed in Figure 1. (A) shows uracil, cytidine, uridine, cGMP, xanthine, hypoxanthine, guanine, guanosine, cAMP, and adenine. (B) shows (−)-catechin, procyanidin B2, (−)-epicatechin, quercetin 3-*O*-galactoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-*β*-D-glucoside, and kaempferol 3-*O*-rutinoside. (C) shows total polysaccharides. Values are expressed in % of difference by comparing with sample A (Jinsixiaozao), and they are in mean ± SD (n = 3), the SD value is less than 5%, which is not shown for clarify. Bars show the percentage of variation. The content of marker chemical markers in sample A (Jinsixiaozao) was 3.79 (1), 36.93 (2), 194.51 (3), 108.10 (4), 66.50 (5), 25.10 (6), 12.24 (7), 28.17 (8), 196.24 (9), 58.29 (11), 4.02 (12), 7.48 (13), 3.07 (14), 0.24 (15), 25.50 (16), 0.68 (17), and 0.70 (18) μg/g of dried fruit weight, corresponding to the order. The amount of total polysaccharide in sample A (Jinsixiaozao) was 34 mg/g of dried fruit weight.

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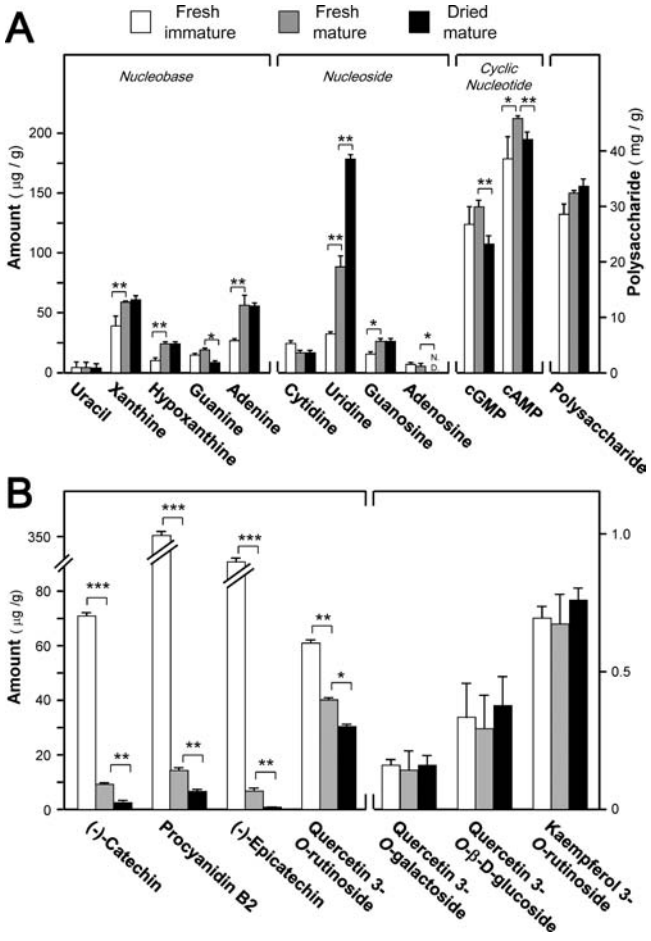

Figure 6. Comparison of the amounts of ingredients in dates from different developmental stages. The amounts of different marker chemicals were determined in the water extracts of dates (sample A: Jinsixiaozao) from different developmental stages, that is, fresh immature, fresh mature and dried mature. The methods were carried out as in Figure 3. (A) shows necleobase, nucleoside, cyclic nucleotide and total polysaccharide. (B) shows flavonoids. Values are expressed in μg/g or mg/g of dried fruit weight, and they are in mean ± SD, where $n = 6$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

to reveal the variations among different dates, Jinsixiaozao (A), one of tested samples with the smallest size and yet the most common source, was employed to compare with others (Figure 5). The amounts of nucleotides and its derivatives were simply compared, and they all showed significant variation (Figure 5A). The distinctions having the best content were identified: Uracil from Hamidazao (V); uridine, xanthine and guanine from Jinsixiaozao (A); cAMP from Goutouzao (O); cytidine from Hamidazao (V); cGMP from Yuanzao (F); hypoxanthinie from Jinsixiaozao (A); guanosine from Goutouzao (O); adenine from Hupingzao (U). For flavonoids, the distinctions having the best content were identified (Figure 5B): Kaempferol 3-O-rutinoside from Goutouzao (O); the rest of six flavonoids including (−)-catechin, procyanidin B2, (−)-epicatechin, quercetin 3-O-rutinoside quercetin 3-O-galactoside, and quercetin 3-O-β-D-glucoside from Fupingdazao (J). In addition, the amount of total polysaccharides in dates was the highest in Yuanhongdazao (S) (Figure 5C).

The chemical properties of mature and immature dates were compared. The contents of seven tested chemical markers were higher in mature dates than that of immature ones, which included xanthine, hypoxanthine, adenine, uridine, guanosine, cGMP, and cAMP (Figure 6A).

In considering the influence of drying procedure on dates, the amounts of tested chemicals in fresh and dried mature dates were further compared. After the drying process, the chemical instability was observed in 5 tested chemicals, for example, guanine, uridine and adenosine, cGMP, and cAMP (Figure 6A). These chemicals showed significant decreased after the drying processing, except the amount of uridine was increased. The amount of polysaccharide in the date did not significantly be affected by both maturity and drying processes (Figure 6A). The amounts of 5 flavonoids, including (−)-catechin, procyanidin B2, (−)-epicathin and quercetin 3-*O*-rutinnoside, were much higher in immature dates than that of mature one (Figure 6B). After drying, the contents of (−)-catechin, procyanidin B2, (−)-epicathin, and quercetin 3-*O*-rutinnoside in Chinese dates decreased significantly (Figure 6B).

In order to evaluate the resemblance and variation of dates at different stages, a hierarchical clustering analysis (HCA) was applied to classify among different dates. The results indicated that nucleotide and flavonoid chemicals might be one of the key markers for good discrimination of immature and mature date, as well as a parameter showing the influence of drying factor (SI, Figure S3).

Antioxidative Effects of Date. PC12 cell is a popular study model in analyzing the neuroprotective effect against oxidation and other insults.[19,21,22] Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly correlated to the living cell number. Cell viability as determined by MTT reduction, or by morphology, was markedly decreased after PC12 cell cultures were exposed to tBHP in a dose-dependent manner (Figure 7A). At 150 μM tBHP, about 20−30% cells survived. Different extracts of Chinese dates were tested for antioxidative effect. The water extract possessed the best protective effect in against tBHP-induced cell death in comparison with the ethanol extracts (Figure 7B): this result was in line to the chemical solubility of dates as shown in Figure 4. Moreover, the protective effect of water extract (0.5 to 3.0 mg/mL) was in a dose-dependent manner (Figure 7C). The treatment of vitamin C served as a control.

By determining the formation of ROS in tBHP-treated cultured PC12 cells, the neuroprotective effect of date extract was analyzed. Application of tBHP in cultured PC12 cells induced the formation of ROS in a dose-dependent manner (Figure 7D). Date extracts were applied onto the cultures before the addition of tBHP (at 150 μM), and then the cultures were subjected to the determination of intracellular ROS formation. When compared to the control, the date extract showed an inhibitory effect of tBHP-induced ROS formation in a dose-dependent manner (Figure 7D).

Due to the presence of ARE located on the promoter of defense genes,[23,24] the activation of Nrf2-ARE signaling by date could be one of the molecular mechanisms to account for the date-induced expressions of defensive genes in PC12 cells. To reveal the transcriptional activity of ARE, a luciferase-reporter construct (pARE-Luc), containing four AREs derived from the promoter of defense genes and tagged upstream of a luciferase gene (Figure 8A), was transfected into cultured PC12 cells. The authentication of pARE-Luc was confirmed by its activation in exposing to tBHQ treatment, which was frequently used to investigate the expressions of ARE-driven genes.[25,26] The activation of pARE-Luc by tBHQ treatment was in a dose-

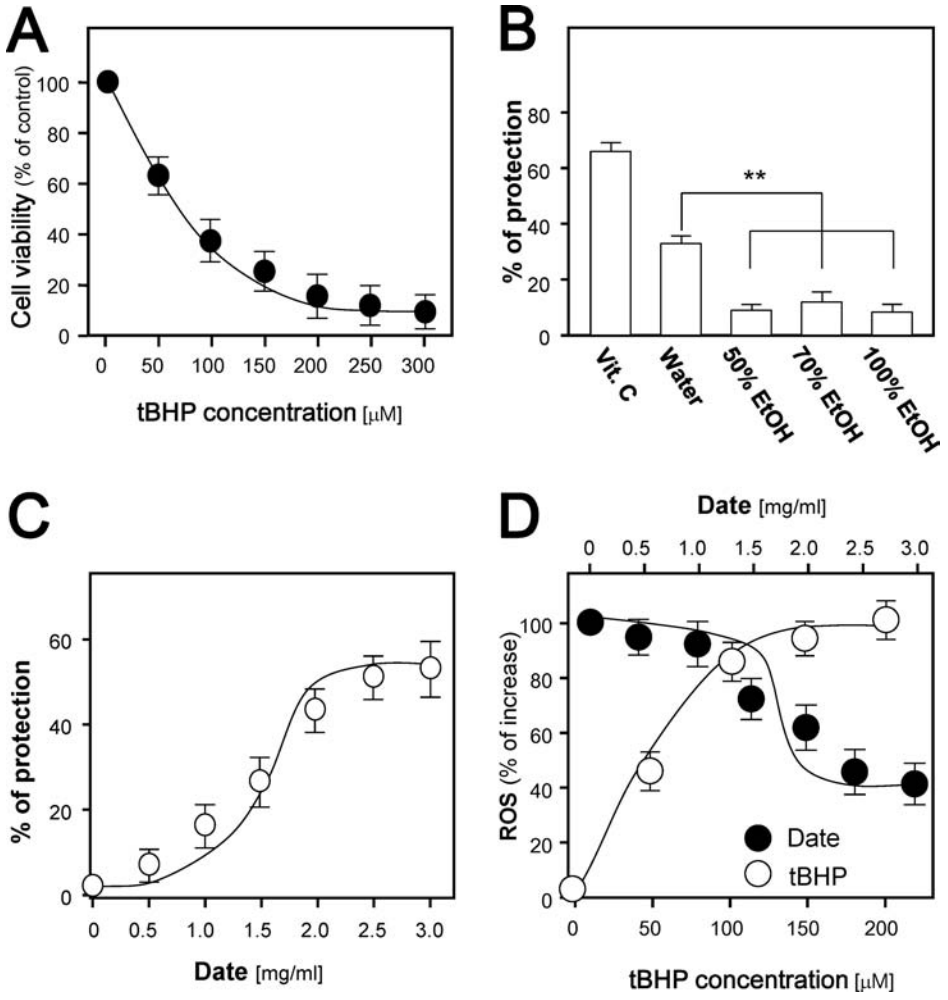

Figure 7. Chinese date extracts protect tBHP-induced PC12 cell cytotoxicity. (**A**): Cultured PC12 cells (2×10^4 cells/well) were exposed to tBHP at various concentrations (0−300 μM) for 3 h, and cell viability was determined by MTT assay. Cell viability was expressed as % of control (cells without tBHP). (**B**): Chinese date was extracted by water of ethanol (50, 70, and 100%). Water extract (1.5 mg/mL) and ethanol extracts (0.15 mg/mL) were pretreated the cells for 24 h before addition of tBHP (150 μM) for cytotoxicity test as in (A). Vitamin C (1 mM) served as positive control. Cell protection was expressed as % of protection relative to control (with tBHP alone). (**C**): Dose-dependent response was performed by pretreating the cultures with the water extract of Chinese date (0−3.0 mg/mL) before addition of tBHP (150 μM) for cytotoxicity test as in (A). (**D**): Cultured PC12 cells were exposed to tBHP (0−200 μM) for 1 h. The level of intracellular ROS was measured. Cultured PC12 cells were pretreated with date water extract (0−3.0 mg/mL) and then exposed to tBHP (100 μM) for 1 h. tBHQ (200 nM), an antioxidant, was used as positive control of having 40% of ROS inhibition. The results were in % of inhibitory effect against ROS formation relative to the control (with tBHP alone). Data were expressed as mean ± SD, n = 5, each with triplicate samples. *$p < 0.05$; **$p < 0.01$.

dependent manner (Figure 8A). The application of date extract increased the luciferase activity in a dose-dependent manner (Figure 8B).

The variations of antioxidative effect among different dates were compared. The fresh immature date performed better effect on antioxidation activity against tBHP-induced cytotoxicity as compared to mature dates (Figure 9A). In contrast, the inhibition of ROS formation and the activation of transcriptional activity of ARE were better in mature dates, in particular the dried one (Figure 9A). In different geographical sources of dates, the antioxidative effects showed a great variation, and the date from Yuanzao (F) had better activities in cell protection and pARE-Luc assays (Figure 9B).

■ DISCUSSION

The cultivation history of dates has over few thousand years in China, and today China has about 700 cultivars, located in various geographical regions. The HPLC fingerprints of 24 cultivars of Chinese dates were rather similar indicating that they might have similar chemical compositions. The active ingredients of dates have been proposed,[15,16] including nucleotide, flavonoid, and polysaccharide. The profiles of nucleotide, as well as its derivatives, and flavonoids in Chinese dates have been quantified by the current study. In line to the previous study,[27] the contents of these chemicals were very different among different sources of dates. The chemical variation could be accounted by many factors, for example, genetic variation, plant origin, and climate or geography (soil or minerals). The cyclic nucleotides, cAMP, and cGMP, showed a high concentration in date extract, which was in accord to the study of Liu and Wang.[28] From our results, the dates from Yuanzzo (F), Goutouzao (O), Zanhuangdazao (R), and Hupingzao (U) contained much higher amounts of these cyclic nucleotides. In parallel, numerous flavonoids were isolated and identified from *Ziziphus* species,[4,29,30] and here we measured the amounts of seven flavonoids in Chinese dates.

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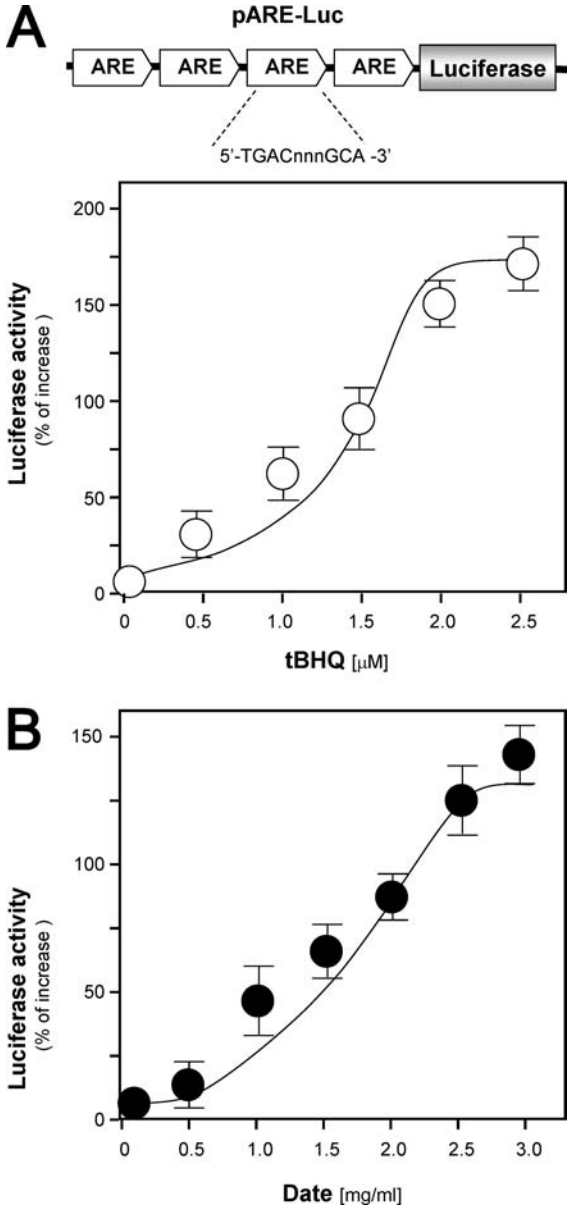

Figure 8. Date extract stimulates the ARE-mediated transcriptional activity (**A**): A luciferase-reporter containing four AREs and a downstream luciferase-reporter gene, namely as pARE-Luc, was used as a study tool (upper panel). Cultured PC12 cells, transfected with pARE-Luc, were treated with tBHQ (0−2.5 μM) for 24 h. The cell lysates were subjected to luciferase assay to measure the activity driven by ARE (lower panel). (**B**): The extracts of Chinese date (0−3.0 mg/mL) were applied onto pARE-Luc-expressed PC12 cells for 24 h. The cell lysates were subjected to luciferase assay. Values are expressed as the percentage of increase to basal reading (untreated culture), and they are in mean ± SD, where n = 4, each with triplicate samples.

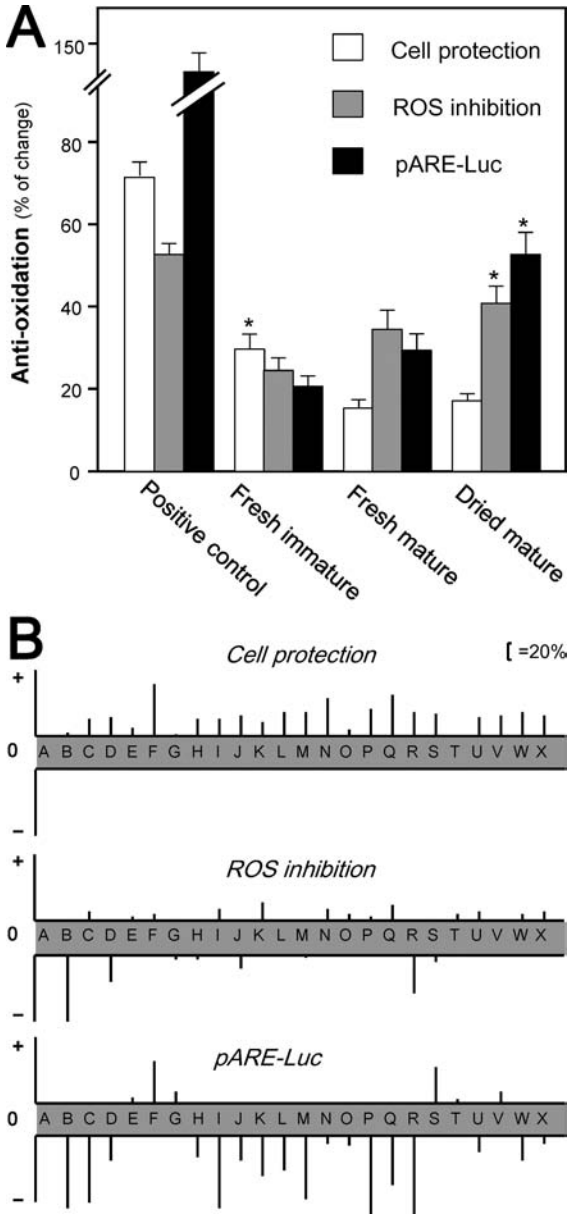

Figure 9. Comparison of antioxidation activity on Chinese dates from different stages and different sources. Antioxidation activities, including cell protection, ROS inhibition and pARE-Luc assays, as described in Figures 7 and 8, were employed here. (**A**): The antioxidative properties of different stage of date extract at 1.5 mg/mL (sample A: Jinsixiaozao) were measured. Vitamin C (1 mM), tBHQ (200 nM), and tBHQ (2 μM) were served as positive control for the cell protection, ROS inhibition and pARE-Luc assay, respectively. The cell protection values are expressed as % of protection relative to control (with tBHP alone), the ROS inhibition values are in % of inhibitory effect against ROS formation relative to the control (with tBHP alone), the pARE-Luc values are expressed as the percentage of increase to basal reading (untreated culture), and they are in mean ± SD, where n = 4, each with triplicate samples. Statistical comparison was made with the lowest effect; *p < 0.05. (**B**): The antioxidative properties of dates extracts from different sources were measured. The geographical sources from A to X were listed as in Figure 1. Values are expressed in % of difference by comparing with sample A (Jinsixiaozao), and they are in mean ± SD (n = 3), the SD value is less than 5%, which is not shown for clarify. Bar shows the percentage of variation.

The date from Fupingdazao (J) contained significant higher levels of various flavonoids. In addition, our finding suggested that the water extract contained higher amount of cyclic nucleotide, as well as its derivatives, and flavonoids than that of the ethanol extract, which therefore supported the water extract possessed better effect in cell protection. We speculated that these chemicals might be the active ingredients contributing to its cell protection. In addition, the antioxidative properties of these compounds have been reported.[3,4,38]

Apart from dried mature dates being commonly consumed in our daily life, the immature Chinese dates were also eaten freshly as fruits. Indeed, the maturity of date could affect the chemical composition, as supported by our results here as well as others.[31−34] In addition, the influence of drying process on the active compounds of Chinese dates was demonstrated. The amounts of nucleotides and their derivatives were changed due to drying process. One of the chemicals, adenosine was not detected in Chinese dates after drying, which indicated that this chemical could be highly sensitive to drying. Recently, the analyses of phenolic and flavonoid compounds have been used as chemical markers to study the influence of maturity and drying techniques on some edible fruits in the market.[35,36] In their studies, there was a sharp decrease of phenolic and flavonoid compounds during maturity. Our results on Chinese dates, in terms of flavonoid compounds, agreed very well with their findings. In addition, the polysaccharide in Chinese date was also investigated under different developmental stages. It is generally considered that the fruit ripeness could influence the polysaccharide composition.[37] In this study, the increase of total polysaccharide in Chinese date, as revealed here, was small during developmental stage. And the postharvest treatment did not alter its polysaccharide amount. As for dates from different geographical sources, slight variations among their amounts were observed.

Flavonoids could be one of the ingredients in Chinese date contributing to the antioxidative effects, despite the levels of individual flavonoid were not correlated with the activity. We found that immature date with significantly higher amount of flavonoids performed better effect in cell protection, but not in ROS inhibition and pARE-Luc activation. Similarly, the date from Fupingdazao (J) contained the highest amount of flavonoids among dates from different sources but which did not show maximal antioxidative activities. Our finding was in agreement with the report by Choi et al.[4] that the ferric reducing activity power (FRAP) was not significantly correlated with total HPLC-determined flavonoids. In contrast, Zhang et al.[38] reported that FRAP effect was highly correlated with the amount of total flavonoids. We speculated that nucleotides and polysaccharides, as found significantly in date, might also play a role in antioxidative effects: higher amount of which in mature date could explain better activity in both ROS inhibition and pARE-Luc activation. However, this was not observed for dates from different sources. Thus, the interaction among these three types of ingredients within date might occur, which together could account for its antioxidative effect.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Structures of chemical markers analyzed in Chinese date (Figure S1), optimization of extraction cycles in extraction (Figure S2) and HCA analysis of Chinese date at different stages (Figure S3). HPLC method validation results (Tables S1−S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TCM, traditional Chinese medicine; tBHP, *tert*-butyl hydroperoxide; tBHQ, *tert*-butyl hydroquinone; cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; HPLC, high performance liquid chromatography; DAD, diode array detector; MS, mass spectrometry; MRM, multiple reaction monitoring; MTT, thiazolyl blue tetrazolium bromide; DMSO, dimethyl sulfoxide; ROS, reactive oxygen species; DCFH-DA, 2′,7′-Dichlorofluorescin diacetate; ARE, antioxidant response element; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HCA, hierarchical clustering analysis

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