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Phenolic Antioxidants (Hydrolyzable Tannins, Flavonols, and Anthocyanins) Identified by LC-ESI-MS and MALDI-QIT-TOF MS from *Rosa chinensis* Flowers

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Rosa chinensis (Yuejihua) is a well-known ornamental plant, and its flowers are commonly used in traditional Chinese medicine. Methanolic crude extracts of dried *R. chinensis* flowers were used for simultaneous determination of phenolic constituents by liquid chromatography−mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF MS). A total of 36 known and unknown phenolics were identified as hydrolyzable tannins, flavonols, and anthocyanins, mainly including gallotannins (mono-, di-, or trigalloylglucopyranosides), ellagitannins, quercetin, quercetin/kaempferol mono- and diglycosides, and cyanidin/pelargonidin diglycosides. MALDI-QIT-TOF MS was applied not only to verify most phenolics isolated and identified by LC-MS but also to tentatively identify two ellagitannins (rugosins B and C) not isolated and unidentified by LC-MS. This study is the first to demonstrate the rapid and successful use of MALDI-QIT-TOF MS and LC-MS to directly and simultaneously identify phenolics in the crude extracts of *R. chinensis* flowers without any purification. The antioxidant activity of the crude extracts from *R. chinensis* flowers was also measured with three assay methods. The results showed that the phenolic antioxidants from *R. chinensis* flowers exhibited very strong radical scavenging effect and antioxidant power. High levels of flavonols and hydrolyzable tannins might be important bioactive principles in the dried *R. chinensis* flowers.

KEYWORDS: ***Rosa chinensis*; rose; phenolics; antioxidants; hydrolyzable tannins; flavonoids; flavonols; anthocyanins; LC-ESI-MS; MALDI-QIT-TOF MS**

INTRODUCTION

Roses are one of the most important groups of ornamental plants and have been referred to as the queen of flowers. Over 150 rose species and more than 20000 cultivars have been registered, most of which belong to the lineage of Chinese roses (*1*−*3*). *Rosa chinensis* Jacq. (Yuejihua) has been considered to be the important ancestor of modern roses (*4*). *R. chinensis* is a small shrub with beautiful purplish red or pale purplish red or pink flowers and is widely distributed in most parts of China, especially in Jiangsu, Shandong, and Hebei provinces. It is not only a well-known ornamental plant but also a common traditional Chinese medicinal herb. Chinese rose flowers are traditionally used for treating catamenia disorder, trauma, and blood disorders and also for hemostasia and controlling pain and diarrhoea (*5*, *6*).

Plants in the genus *Rosa* of the family Rosaceae are known to contain different types of secondary metabolites, such as phenolic acids (e.g., gallic acid, chlorogenic acid), flavonoids (e.g., flavonols, anthocyanins), fragrant components (essential oils, e.g., monoterpenes, sesquiterpenes), and hydrolyzable and condensed tannins (e.g., rugosins, procyanidins) (*7*−*10*). *Rosa rugosa* flower extracts were reported to contain gallic acid and its derivatives and increased the activities of antioxidant enzymes and reduced lipid peroxidation (*11*, *12*). *Rosa persica* flower extracts contained polyphenolic antioxidant constituents with strong free radical scavenging activity (*13*). *Rosa damascena* flower extracts demonstrated antioxidant and antibacterial activities (*14*). However, so far there have been few studies of the phenolic constituents and antioxidant activity of *R. chinensis* flowers.

High-performance liquid chromatography (HPLC) and HPLC coupled with mass spectrometry (LC-MS) have been extensively used for the isolation and characterization of phenolic compounds (*15*). Fast atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) have been powerful supporting techniques for detailed structural elucidation of phenolic compounds. Mikanagi et al. (*8*), Sumere et al. (*16*), and Velioglu and Mazza (*17*) applied HPLC techniques to identify anthocyanins and other flavonoids (e.g., flavonols) in

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different *Rosa* species and genotypes. Mikanagi et al. (*10*) identified the anthocyanins in flowers of 44 genotypes in the genus *Rosa* using HPLC, FAB-MS, and NMR.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was originally developed for large biomolecules (e.g., proteins, lipids, nucleic acids, carbohydrates) (*18*, *19*) and, recently, also has been used for the analysis of small molecules (e.g., flavonoids) (*20*, *21*). MALDI-TOF MS has several advantages, including ease of sample preparation, rapid generation of spectra, wide applicability combined with a good tolerance toward contaminants, and the ability for the simultaneous determination of masses in complex samples of low and high molecular weight compounds. Recently, a new MALDI MS, that is, matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometer (MALDI-QIT-TOF MS), has been developed. It is a hybrid mass spectrometer equipped with a MALDI source and a quadrupole ion trap (QIT), followed by a time-of-flight analyzer (TOF). This system combines the advantages of conventional MALDI-like rapid analysis time to achieve high throughput with the ability to perform high-efficiency ion trapping and MS/MS and MS^n analysis and with high sensitivity and resolution by using filtered noise and collision-induced dissociation (CID) methods.

MALDI-TOF MS has been recently used to characterize the flavonoids from green tea, onion bulbs, red wine, and fruit juice (*22*, *23*) and the condensed tannins from some plant leaves and tree barks (*24*, *25*). There have been few studies on the analysis of hydrolyzable tannins using MALDI-TOF MS, and information on flavonoids and tannins determined with MALDI-QIT-TOF MS is also scarce. Systematic and simultaneous identification of many phenolic antioxidants from *R. chinensis* flowers by modern analytical techniques and tools (LC-MS and MALDI-QIT-TOF MS) is currently lacking.

In the present study, we used LC-MS to directly analyze the methanolic crude extracts of dried *R. chinensis* flowers and to simultaneously determine and identify more than 30 antioxidant phenolics, including hydrolyzable tannins, flavonols, and anthocyanins. In particular, the new MALDI-QIT-TOF MS technique was developed to successfully verify the results of LC-MS and to identify certain hydrolyzable tannins not isolated and unidentified by LC-MS. The antioxidant activity of *R. chinensis* flower extracts was also evaluated using three assay methods.

MATERIALS AND METHODS

Materials and Reagents. Dried Chinese rose flowers (*R. chinensis*) for medicinal use were from a well-known market for Chinese herbal medicines in Qichun, Hubei, China. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), $FeCl_3 \cdot 3H_2O$, and potassium persulfate were purchased from Sigma/Aldrich (St. Louis, MO); Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was from Fluka Chemie (Buchs, Switzerland); and HPLC grade organic reagents and formic acid were from BDH (Dorset, U.K.). Authentic standards of common phenolic compounds, such as quercetin, kaempferol, and their glycosides (e.g., quercetin 3-*O*-rhamnoside, and kaempferol 3-*O*-glucoside) and gallic acid were from Sigma/Aldrich. Four common vegetables and fruits (broccoli, spring onion, Washington red apple, and tomato) were purchased from a local market in Hong Kong, and immediately freeze-dried, and used as controls for comparison of antioxidant activity.

Preparation of Crude Extracts from *R. chinensis* Flowers. Dried *R. chinensis* flowers and freeze-dried vegetables and fruits were ground into a fine powder (710 μm) and passed through a sieve (24-mesh). Two grams of the powdered sample was extracted with 50 mL of 80% methanol at room temperature (~23 °C) for 24 h in a shaking water bath. The crude extract was filtered by a Millipore filter with a 0.2-μm nylon membrane under vacuum at 23 °C. The filtrate was then separated into two parts; one part was stored at 4 °C within 24 h for analysis of antioxidant activity, and the other part was stored at 4 °C for analysis by LC-MS and MALDI-QIT-TOF MS.

Sample Preparation for LC-MS and MALDI-QIT-TOF MS Analysis. The sample for LC-MS analysis was prepared by diluting the filtrate to half of the original concentration. The sample used for MALDI-QIT-TOF MS analysis was prepared by 10 times dilution of the filtrate plus an addition of 0.5% formic acid and directly used for MALDI-QIT-TOF MS analysis without using any matrices. One microliter of the sample solution was dropped onto a sample plate and then allowed to air-dry at ambient temperature before the sample plate was loaded into the Amixa-QIT instrument.

LC-MS. The LC-MS-2010A system applied in this study consisted of an LC-10ADvp binary pump, an SIL-10Avp autosampler, a photodiode array detector (PDA), a central controller, and a single-quadrupole MS detector with electrospray ionization (ESI) interface (Shimadzu). The system was equipped with a 250 × 2.0 mm ODS-VP C_{18}, 5-μm column (Nomura Chemical Co. Ltd., Seto, Japan). LC conditions were as follows: solvent A, 0.1% formic acid, and solvent B, MeOH with 0.1% formic acid. A gradient elution used was 0−5 min, 5% B; 5−90 min, 5−45% B; 90−100 min, 45% B; 100−101 min, 45−5% B. Flow rate was 0.2 mL/min, and injection volume was 10 μL. Detection was at 280 nm. The LC eluate was introduced directly into the ESI interface without flow splitting. The ESI voltage was 4.5 kV in positive ion mode and 3.5 kV in negative ion mode. A nebulizing gas of 1.5 L/min and a drying gas of 10 L/min were applied for ionization using nitrogen in both cases.

MALDI-QIT-TOF MS. MALDI MS analysis was performed on an Axima MALDI-QIT-TOF MS instrument [Shimadzu (Asia Pacific) Pte. Ltd., Singapore]. All spectrometric data were processed and analyzed using Lachlan software (Kratos Analytical Ltd., Manchester, U.K.). This instrument uses a three-dimensional ion trap with a time-of-flight mass measurement stage. MALDI was produced using pulsed laser light (337 nm, 3-ns pulse width) generated by a nitrogen laser with a maximum pulse rate of 10 Hz. Each profile resulted from the accumulation of data from two laser shots. A small bias voltage (6−30 V) was applied to the sample plate. The ions were extracted by a negative potential (−10 kV), following ionization. Upon trapping, the ions were cooled using helium. The pressure in the trap was held at 6×10^{-3} Pa. For CID, argon was used as the collision gas. In the MS modes, ions were extracted by applying a potential between the two end-caps and pulsed into the TOF system with an accelerating voltage of 10 kV. The detector was a microchannel plate, and acquisition was made using an 8-bit transient recorder. The instrument was operated in the positive ion mass mode (ranges of 50−300 and 300−800 Da). Mass spectra from a sum of 1000 laser shots were recorded using a laser power of 70 arbitrary units (range of laser power 0−180, where 0 represents minimum and 180 maximum transmission). External mass calibration was performed daily using fullerite clusters.

Total Phenolics. Total phenolic content was estimated following the Folin−Ciocalteu colorimetric method (*26*). Briefly, the appropriate dilutions of Chinese rose flower extracts were oxidized with 0.5 N Folin−Ciocalteu reagent, and then the reaction was neutralized with saturated sodium carbonate (75 g/L). The absorbance was read at 760 nm by a Spectronic Genesys 5 spectrophotometer (Milton Roy, New York) after incubation for 2 h at room temperature (~23 °C). The results were expressed as milligrams of gallic acid equivalent (mg of GAE) per 100 g of dry weight (DW).

Radical Cation $ABTS^{\bullet+}$ Scavenging Activity Assay. The total antioxidant capacity of *R. chinensis* flower extracts was measured with a Spectronic Genesys 5 spectrophotometer according to the improved $ABTS^{\bullet+}$ method (*27*) as modified by Cai et al. (*26*). $ABTS^{\bullet+}$ cation solution (3.9 mL, absorbance of 0.70 ± 0.005) was added to 0.1 mL of Chinese rose flower extracts and mixed thoroughly. The reaction mixture was kept at room temperature for 6 min, and the absorbance was immediately recorded at 734 nm. Trolox standard solution in 80% ethanol was prepared and assayed under the same conditions. The results

were expressed in terms of Trolox equivalent antioxidant capacity [TEAC, mM Trolox equiv per 100 g of DW].

Radical DPPH Scavenging Activity Assay. The traditional DPPH method (*28*) was modified for use in this study. DPPH$^{\bullet}$ solution (60 μM) prepared in 80% ethanol (3.9 mL; absorbance of 0.68 ± 0.005 at 515 nm) was added to 0.1 mL of Chinese rose flower extracts. The reaction for scavenging DPPH$^{\bullet}$ radicals was carried out at 23 °C in the dark for 120 min, and then the absorbance of the reactive mixture was recorded at 515 nm. The results were expressed using the parameters of EC_{50} = concentration (micrograms per milliliter) of the extracts required for a 50% decrease in absorbance of DPPH$^{\bullet}$ radicals. The detailed procedure was described in our previous study (*29*).

Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was performed as previously described by Benzie and Strain (*30*) and Faria et al. (*31*) with some modifications. Briefly, FRAP reagent (10 volumes of 300 mM acetate buffer, pH 3.6, + 1 volume of 10 mM TPTZ in 40 mM HCl + 1 volume of 20 mM $FeCl_3 \cdot 3H_2O$) was diluted to 1:3 with methanol. The test was conducted at 37 °C. Working FRAP reagent (3 mL) was mixed with 0.1 mL of diluted samples (extracts). This mixture was thoroughly shaken and incubated at 37 °C for 8 min. The absorbance was recorded at 593 nm using the spectrophotometer. A standard curve was established with Trolox. The results were expressed as micromolar Trolox equivalents (TE) per gram on dried basis (μM TE/g of DW).

RESULTS AND DISCUSSION

In previous studies, the crude extracts of methanol or other solvents treated and partially or fully purified by paper chromatography (PC), thin-layer chromatography (TLC), and Sephadex LH-20, Dianion HP-20, and other preparative columns were used for analysis by HPLC and HPLC/LC-MS (*8*, *10*, *13*, *17*). Previous MALDI-TOF MS analyses of flavonoids and condensed tannins also required the samples to be purified by preparative HPLC or Sep-Pak C_{18} cartridge or other methods (*21*−*24*). In the present study, methanolic crude extracts of the dried *R. chinensis* flowers were not purified and directly used for simultaneous determination of phenolic compounds by LC-MS under our chromatographic conditions and also directly used for MALDI-QIT-TOF MS analyses without using any matrices.

Identification of Phenolic Compounds by LC-MS. Because the availability of reference standards is limited and the simultaneous identification of a large number of compounds on the basis of only chromatographic behaviors and UV spectra is difficult or impossible, HPLC coupled to MS can be extremely helpful for peak assignment and rapid and further characterization and elucidation of many individual constituents. In this study, LC-MS was employed to simultaneously identify many phenolic constituents separated directly from crude extracts of the dried *R. chinensis* flowers. Our LC-MS analysis results showed that the major phenolic constituents in the dried *R. chinensis* flowers were hydrolyzable tannins, flavonols, and anthocyanins, consisting of over 30 known and unknown compounds. The results were similar to those for other *Rosa* plant flowers reported in previous studies (*8*, *10*, *13*, *17*). However, previous researchers did not carry out simultaneous separation and identification of many flavonoids and hydrolyzable tannins from the crude extracts of the genus *Rosa* plants by HPLC or HPLC/LC-MS. We simultaneously separated and identified a great number of flavonoids and tannins from the crude extracts of *R. chinensis* flowers using the same chromatographic conditions.

The general structures and substitution patterns of hydrolyzable tannins, flavonols, and anthocyanins identified in the dried *R. chinensis* flowers are shown (**Figure 1**). **Figure 2** displays LC-UV/MS chromatographs of methanolic crude extracts from the dried *R. chinensis* flowers, and **Table 1** lists retention times,

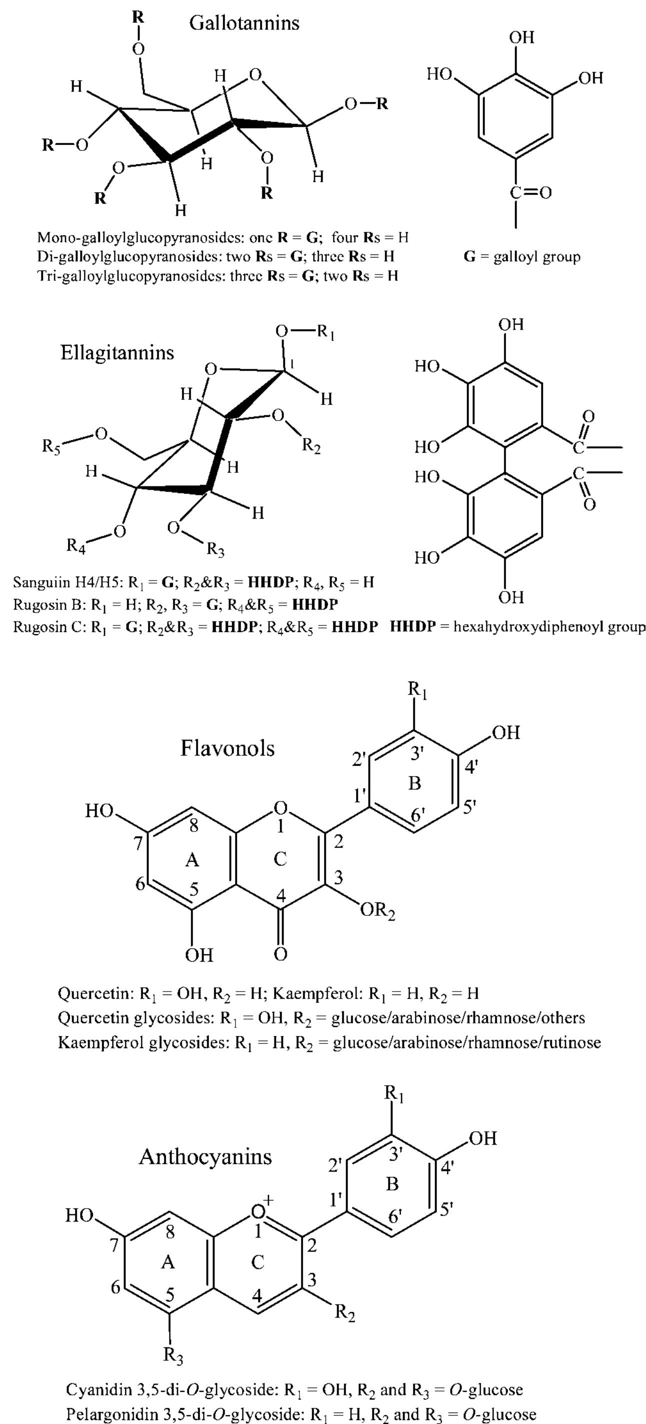

Figure 1. General structures and substitution patterns of hydrolyzable tannins, flavonols, and anthocyanins identified in the dried *R. chinensis* flowers. Sanguiins H4/H5 are the HHDP-galloylglucopyranoside isomers at C_1.

UV spectra, characteristic ions, and molecular masses from LC-MS analysis. Although the *R. chinensis* flower extracts contained quite complex constituents, most phenolic constituents were separated well under our chromatographic conditions (**Figure 2A**), and both positive (**Figure 2B**) and negative (**Figure 2C**) ESI-MS (full scan modes) were sensitive for the determination of most peaks isolated by HPLC. The molecular masses of the phenolic constituents isolated in the dried *R. chinensis* flowers were determined from the $[M + H]^+$, $[M + Na]^+$, and $[M - H_2O]^+$ ions in positive ESI and from the prominent $[M - H]^-$ ions in negative ESI (**Table 1**).

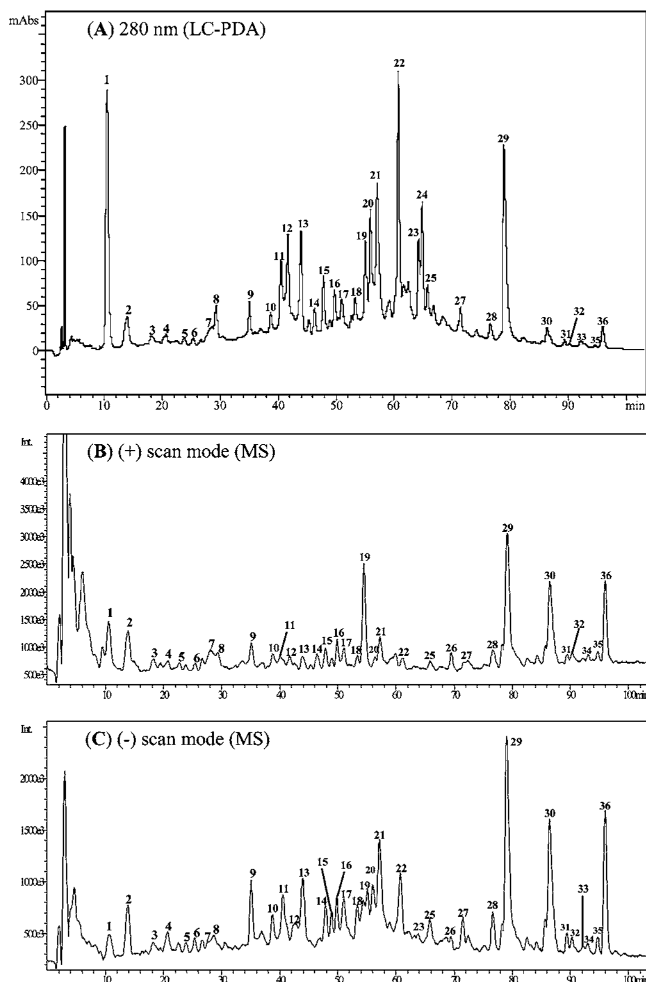

Figure 2. LC-MS chromatograms of methanolic crude extracts from *R. chinensis* flowers: (**A**) photodiode array detection (PDA) at 280 nm; (B) positive ESI-MS in full scan mode; (C) negative ESI-MS in full scan mode. For peak assignments, see **Table 1**. Conditions are described in the text.

Hydrolyzable tannins, including gallotannins and ellagitannins, are derivatives of 1,2,3,4,5,6-penta-*O*-galloyl-β-D-glucopyranose (*32*). Because they contain many hydroxyl groups (**Figure 1**), gallotannins and ellagitannins exhibit very strong polarity and shorter retention time under reversed-phase chromatographic conditions (*33*). They have strong absorption in the UV region at ~260−270 nm. The peaks 1−6, 9, and 10 exhibited typical UV spectroscopic features and chromatographic behaviors of hydrolyzable tannins. Through determination of the negative and positive ions by ESI-MS and by comparison with the literature (*7*, *32*−*34*), the molecular masses of peaks 1−5 and 10 showed that they were a series of gallotannin molecules (mono-, di-, or trigalloylglucopyranosides). Peaks 3−5 were identified as three di-*O*-galloyl-β-D-glucopyranosides (isomers), and peak 10 was identified as a tri-*O*-galloyl-β-D-glucopyranoside. Peak 2 seemed to be a glucopyranoside with one galloyl unit, but its *m/z* values of negative and positive ions observed did not wholly agree with the calculated molecular mass (332) of mono-*O*-galloyl-β-D-glucopyranoside. In **Figure 2A** (HPLC profile), peak 1 was the largest, accounting for 12.2% of total peak area, but not fully isolated by HPLC. The coupled MS analysis showed that peak 1 contained several signals which were mono-*O*-galloyl-β-D-glucopyranoside (MW 332), di-*O*-galloyl-β-D-glucopyranosides (MW 484), other unknown galloylglucopyranosides (MW 284 and 582), and gallic acid (MW 170) (**Table 1**; **Figure 2B**,**C**). In a previous study (*35*), a large amount of gallic acid was also isolated from the flowers of *R. rugosa*, which is a close relative of *R. chinensis*. In addition, the molecular masses of negative ion (*m/z* 633 for $[M - H]^-$) and positive ions (*m/z* 635 for $[M + H]^+$ and *m/z* 657 for $[M + Na]^+$) observed by ESI-MS and their UV spectroscopic characteristics showed that peaks 6 and 9 were monomeric ellagitannins (MW 634), that is, hexahydroxydiphenoyl (HHDP)-galloylglucopyranoside isomers, such as sanguiin H4 or sanguiin H5 depending on the location of the galloyl group (*34*). However, other monomeric ellagitannins (rugosins B and C) were not isolated by HPLC under our chromatographic conditions. Rugosins B and C could be directly identified by MALDI-QIT-TOF MS.

Gallotannins and ellagitannins have been frequently found in the leaves, roots, and flowers of the genus *Rosa* (*7*, *9*). Condensed tannins (catechin derivatives or oligomeric and polymeric proanthocyanidins) were also detected in the roots and leaves of *R. rugosa* and *R. persica*, but fewer were detected in the flowers of the genus *Rosa* (*9*, *13*). Khanbabaee and Van Tee (*32*) reported that gallotannins and ellagitannins have many complex galloyl derivatives; complex tannins are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin. Peaks 11−13, 15, 16, and 19 also exhibited UV maxima around 260−270 nm, similar to the identified gallotannins/ellagitannins and most condensed tannins, but had longer retention times than the identified gallotannins/ellagitannins. These unidentified peaks (unknown tannins) might be galloyl derivatives of gallotannins/ellagitannins or complex tannins consisting of combinations of hydrolyzable and condensed tannins. Peak 20 did not exhibit the typical UV spectrum, and its mass was measured as 552 but could not yet be identified. For peak 24 (**Figure 1A**), the corresponding mass signal peak and molecular ions were not observed in either positive or negative scan mode (**Figure 1B**,**C**). Characterization of the unidentified and unknown peaks requires other spectroscopic and structural techniques, such as NMR.

By comparison with hydrolyzable tannins, flavonols usually have weaker polarity and much longer retention time under reversed-phase chromatographic conditions. Flavonols normally exhibit additional strong absorption (λ_{max}) in the UV region at ~350−370 nm, quite different from the UV spectra and chromatographic behaviors of hydrolyzable tannins and other flavonoids (e.g., anthocyanins, flavanols, flavanones). Peaks 17, 18, 21−23, and 25−36 exhibited the typical UV spectra of flavonols (**Figure 2A**; **Table 1**). Negative and positive ESI-MS scan modes gave the molecular masses of these peaks. By comparison with the spectroscopic characteristics and retention times of relevant standards and literature data, the flavonols in the dried *R. chinensis* flowers were identified as quercetin, and quercetin or kaempferol monoglycosides, such as quercetin or kaempferol glucosides, rhamnosides, arabinosides, and their diglycosides (e.g., rutinoside). The tentative identities of the flavonols are listed in **Table 1**. Peaks 18, 21, 26, 31, and 33 were preliminarily identified as flavonol (quercetin and kaempferol) mono- or diglycosides on the basis of their UV spectra and typical ion fragments, but their structures could not be completely elucidated. Peak 22 was identified as quercetin diglycoside (MW 596), which might be quercetin 3-glucosylarabinoside or quercetin 3-galactosylarabinoside. Peaks 26, 31, and 35 were also flavonol diglycosides. Because the flavonol diglycosides have two sugar moieties, the negative and/or positive ions observed were >595. Additionally, **Figure 2A** shows that peaks 22 (quercetin diglycoside) and 29 (quercetin)

Table 1. LC-MS Analysis (UV Spectra, Characteristic Ions, and Molecular Masses) of Phenolic Compounds in Methanolic Crude Extracts from Dried Flowers of *R. chinensis*

peak	retention time (min)	tentative names of phenolic compounds	UV–vis spectra (HPLC-DAD) λ_{max} (nm)	calcd MW	mass of LC-MS obsd adduct ions (*m*/*z*)		
					negative ion mode	positive ion mode	
					$[M-H]^-$	$[M+H]^+$	$[M+Na]^+$
1[a]	10.50	gallic acid and different galloyl-glucopyranosides	215, 270	170, 284, 332, 484, 582	169, 283, 331, 483, 581		307, 355, 507
2	13.96	mono-*O*-galloyl-β-D-glucopyranoside?	220, 273	332, 344	343	345	367
3[a]	18.15	di-*O*-galloyl-β-D-glucopyranoside	263	484	483		507
4[a]	20.68	di-*O*-galloyl-β-D-glucopyranoside	260	484	483		507
5[a]	23.83	di-*O*-galloyl-β-D-glucopyranoside	259	484	483		507
6[b]	25.37	HHDP-galloylglucopyranoside	219, 257	634	633	635	657
7	28.42	cyanidin 3,5-di-*O*-glycoside	273, 522	610		611	
8	29.32	cyanidin di-*O*-glycoside (isomer of 7)	273, 522	610		611	
9[b]	35.04	HHDP-galloylglucopyranoside	221, 268	634	633	635	657
10	38.72	tri-*O*-galloyl-β-D-glucopyranoside	276	636	635		659
11	40.50	unknown tannins?	217, 270	477	476		
12	41.65	unknown tannins?	217, 271	466		467	
13	43.92	unknown tannins (isomer of 11)?	216, 270	477	476		
14	46.27	pelargonidin 3,5-di-*O*-glycoside	271, 510	594			617
15	47.82	unknown tannins?	221, 257	644	643		667
16	49.72	unknown tannins?	219, 265	504		505	
17	50.98	unknown flavonol	271, 361	292	291	293	
18[c]	53.26	flavonol glycoside	254, 370	470	469		493
19	55.08	unknown tannins?	217, 274	469	468		
20	55.93	unknown	217, 257sh	552	551		
21[c]	57.08	flavonol glycoside	255, 365	470	469	471	
22[c]	60.72	quercetin diglycoside	216, 275, 365	596	595		
23	64.23	quercetin 3-*O*-glucoside	216, 273, 363	464	463		
24[d]	64.81	unknown	216, 276		no mass peak		
25	65.78	quercetin 3-*O*-rhamnoside (quercitrin)	216, 274, 370	448	447	448	
26	69.54	flavonol diglycoside	270, 357	640	639		663
27[e]	71.44	quercetin rhamnoside (isomer of 25)	216, 276, 360	448	469		
28	76.58	quercetin 3-*O*-arabinoside	253, 361	434	433	435	457
29	78.92	quercetin	253, 367	302	301	303	
30	86.38	kaempferol 3-*O*-glucoside	255, 350	448	447		471
31	89.38	flavonol diglycoside	266, 352	600	599		623
32	90.27	kaempferol 3-*O*-arabinoside	265, 352	418	417		441
33	92.19	flavonol glycoside	255, 363	426	425	427	
34	93.00	kaempferol arabinoside (isomer of 32)	265, 356	418	417		441
35	94.67	kaempferol 3-rutinoside (diglycoside)	263, 346	594	593		617
36	95.97	kaempferol 3-*O*-rhamnoside	264, 343	432	431		455

[a] The $[M-H_2O]^+$ positive ion was also observed in positive scan mode. [b] Referred to as sanguiin H4/H5 (isomers). HHDP, hexahydroxydiphenoyl group. [c] The $[M-CO_2]^-$ negative ion was also detected in negative scan mode. [d] There was no mass peak, and no molecular ions were observed. [e] *m*/*z* 469 was the mass of the $[M-2H+Na]^-$ negative ion.

were two of the three largest peaks, accounting for 9.6 and 10.2% of total peak area, indicating that these were the predominant phenolic constituents in the dried *R. chinensis* flowers. Mikanagi et al. (*8*) detected a total of 19 known flavonols and 7 unidentified flavonols in the fresh flowers from 10 sections of the subgenus *Rosa*. We determined a total of 11 known flavonols and 6 unidentified flavonols in the dried *R. chinensis* flowers, but did not find the flavonols 3-glucuronide and 3-sophoroside observed in a previous study (*5*).

Anthocyanins occur widely in plant flowers. The genus *Rosa* plant flowers contain a great amount of anthocyanins responsible for their beautiful and bright purple-red color. Mikanagi et al. (*10*) identified a total of 15 anthocyanins from the flowers of the genus *Rosa*, mainly including monoglycosides or diglycosides of cyanidin, pelargonidin, and peonidin. In this study, the tested *R. chinensis* flowers were the dried sample from a common traditional Chinese medicine drugstore. The dried *R. chinensis* flowers contained fewer anthocyanins than fresh flowers because the anthocyanins are easily degraded during drying and storage of the flowers, but we still separated and identified three anthocyanins from the dried *R. chinensis* flowers. The anthocyanins were easier to identify than hydrolyzable tannins and flavonols. Anthocyanins exhibit strong absorption in the visible region of ~520 nm, whereas hydrolyzable tannins and flavonols do not have any absorption in the visible region. Furthermore, the polarity (retention time) of the anthocyanins is mostly stronger (shorter) than that of the flavonols, but slightly weaker (longer) than that of most hydrolyzable tannins. Peaks 6, 7, and 14 were readily identified as anthocyanins according to their maximum visible absorbance wavelength (λ_{max}) and chromatographic behaviors. Peaks 6 and 7 exhibited the same $[M+H]^+$ ions at *m*/*z* 611, and peak 14 showed an $[M+Na]^+$ ion at *m*/*z* 617 (**Table 1**). According to our MS results and by comparison with literature data (*8*, *10*), peaks 6 and 7 were cyanidin 3,5-di-*O*-glycoside (MW 610) and its isomer (MW 610) and peak 14 was pelargonidin 3,5-di-*O*-glycoside (MW 594).

Verification and Identification of Phenolic Compounds by MALDI-QIT-TOF MS. MALDI-QIT-TOF MS analysis is usually used for rapid and accurate identification of molecular weights. The technique was applied in this study not only to confirm the identification of almost all phenolic compounds in the crude extracts of the dried *R. chinensis* flowers by LC-MS but also to identify the hydrolyzable tannins unisolated and unidentified by LC-MS. **Table 2** shows that MALDI-QIT-TOF MS verified and identified 31 of all 36 known and unknown

Table 2. MALDI-QIT-TOF MS Analysis of Phenolic Compounds in Methanolic Crude Extracts from Dried Flowers of *R. chinensis*

phenolic compound	calcd mass	mass of MALDI-QIT-TOF MS obsd adduct ions[a] (*m*/*z*)
	36 Known/Unknown Phenolics Isolated/identified by LC-MS[b]	
(1) gallic acid and different galloyl-glucopyranosides	170, 284, 332, 484, 582	[M + K]+ (209), [M + 2K – H]+ (247), [M + H]+ (285), [M + H]+ (333), [M + K]+ (523), [M + K]+ (621)
(2) mono-*O*-galloyl-β-D-glucopyranoside	332	[M + H]+ (333)
(3) di-*O*-galloyl-β-D-glucopyranoside	484	[M + K]+ (523)
(4) di-*O*-galloyl-β-D-glucopyranoside	484	[M + K]+ (523)
(5) di-*O*-galloyl-β-D-glucopyranoside	484	[M + K]+ (523)
(6) HHDP-galloylglucopyranoside	634	[M + Na]+ (657)
(7) cyanidin 3,5-di-*O*-glycoside	610	[M + H]+ (611), [M + 2Na – H]+ (655)
(8) cyanidin di-*O*-glycoside (isomer of 7)	610	[M + H]+ (611), [M + 2Na – H]+ (655)
(9) HHDP-galloylglucopyranoside	634	[M + Na]+ (657)
(10) tri-*O*-galloyl-β-D-glucopyranoside	636	[M + 2Na – H]+ (681)
(11) unknown tannins?	477	[M + Na]+ (500), [M + 2Na – H]+ (522), [M + 2K – H]+ (554)
(12) unknown tannins?	466	no adduct ions observed
(13) unknown tannins (isomer of 11)?	477	[M + Na]+ (500), [M + 2Na – H]+ (522), [M + 2K – H]+ (554)
(14) pelargonidin 3,5-di-*O*-glycoside	594	[M + 2Na – H]+ (639)
(15) unknown tannins?	644	no adduct ions observed
(16) unknown tannins?	504	no adduct ions observed
(17) unknown flavonol	292	[M + K]+ (331)
(18) flavonol glycoside	470	[M + Na]+ (493)
(19) unknown tannins?	469	[M + H]+ (470), [M + Na]+ (492)
(20) unknown	552	[M + H]+ (553), [M + Na]+ (575)
(21) flavonol glycoside	470	[M + Na]+ (493)
(22) quercetin diglycoside	596	[M + Na + K – H]+ (657)
(23) quercetin 3-*O*-glucoside	464	[M + H]+ (465)
(24) unknown		
(25) quercetin 3-*O*-rhamnoside (quercitrin)	448	[M + 2Na – H]+ (493)
(26) flavonol diglycoside	640	[M + 2K – H]+ (717)
(27) quercetin rhamnoside (isomer of 25)	448	[M + Na]+ (493)
(28) quercetin 3-*O*-arabinoside	434	no adduct ions observed
(29) quercetin[c]	302	[M + Na]+ (325), [M + 2Na – H]+ (347), [M + 2K – H]+ (379)
(30) kaempferol 3-*O*-glucoside	448	[M + 2Na – H]+ (493)
(31) flavonol diglycoside	600	[M + Na]+ (623)
(32) kaempferol 3-*O*-arabinoside[d]	418	[M + 2Na – H]+ (463)
(33) flavonol glycoside	426	[M + Na]+ (449)
(34) kaempferol arabinoside (isomer of 32)	418	[M + 2Na – H]+ (463)
(35) kaempferol 3-rutinoside	594	[M + 2Na – H]+ (639)
(36) kaempferol 3-*O*-rhamnoside[d]	432	[M + Na]+ (455)
	2 Phenolics Unisolated/Unidentified by LC-MS[e]	
(37) rugosin B	954	[M + H]+ (955), [M + Na]+ (977), [M + K]+ (993)
(38) rugosin C	1104	[M + H]+ (1105), [M + 2Na – H]+ (1149)

[a] The observed adduct ions were mostly positive ions. Fewer negative ions were observed. [b] Based on the HPLC peak and retention time order in **Table 1**. Theoretical masses of 36 compounds were also from **Table 1**. [c] Shown in **Figure 3A** (quercetin). [d] Shown in **Figure 3B** (kaempferol 3-*O*-arabinoside and kaempferol 3-*O*-rhamnoside). [e] Rugosins B and C were the ellagitannins unisolated/unidentified by LC-MS, but could be identified by MALDI-QIT-TOF MS. Shown in **Figure 3C** (rugosin B).

phenolics isolated/identified by LC-MS and also identified two hydrolyzable tannins that were not isolated and identified by LC-MS.

In MALDI-QIT-TOF MS positive mode, many adduct ions were observed in ESI for the tested phenolics, such as $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, $[M + 2Na - H]^+$, $[M + 2K - H]^+$, and $[M + Na + K - H]^+$. **Table 2** shows that most phenolic compounds were found to have one to three different adduct ions, and their molecular masses calculated from MALDI-QIT-TOF MS observed adduct ions were identical to those calculated from LC-MS observed adduct ions (**Table 1**). **Figure 3A**,**B** shows typical MALDI-QIT-TOF MS positive ion spectra of quercetin, kaempferol 3-*O*-arabinoside, and kaempferol 3-*O*-rhamnoside in the crude extract of the dried *R. chinensis* flowers. The results indicated that MALDI-QIT-TOF MS could generate abundant positive ions and provide more structural information for the identification and confirmation of the hydrolyzable tannins, flavonols, and anthocyanins in the crude extracts of the dried *R. chinensis* flowers. Only two compounds (**12** and **15**) were not observed to have any adduct ions by MALDI-QIT-TOF MS. Because compound **24** did not have a mass peak in LC-MS analysis and its mass was not calculated, we could not find the corresponding adduct ions and verify it by MALDI-QIT-TOF MS.

MALDI-TOF MS was originally used for large biomolecules, such as proteins, lipids, oligosaccharides, and tannins (condensed tannins) (*18*−*20*, *24*). So far, there has been no information about hydrolyzable tannins analyzed by MALDI-TOF MS or MALDI-QIT-TOF MS. In the present study, we used MALDI-QIT-TOF MS not only to confirm and identify the hydrolyzable tannins isolated and identified by LC-MS but also to identify two hydrolyzable tannins that could not be isolated and identified by LC-MS. The two hydrolyzable tannins belonged to ellagitannins, tentatively identified as rugosins B and C. **Figure 3C** displays MALDI-QIT-TOF MS positive ion spectra of rugosin B, and three observed adduct ions were $[M + H]^+$ (*m*/*z* 955), $[M + Na]^+$ (*m*/*z* 977), and $[M + K]^+$ (*m*/*z* 993). Early studies showed that a series of mono- and oligomeric ellagitannins (e.g., rugosins A−G) were isolated and identified from fresh *R. rugosa* flowers using traditional tools and methods (*9*, *36*, *37*). Dried *R. chinensis* flowers were used for analysis in the present study. Because drying and long-term storage of the flowers may easily degrade hydrolyzable tannins, a few ellagitannins were isolated and identified from the dried flowers.

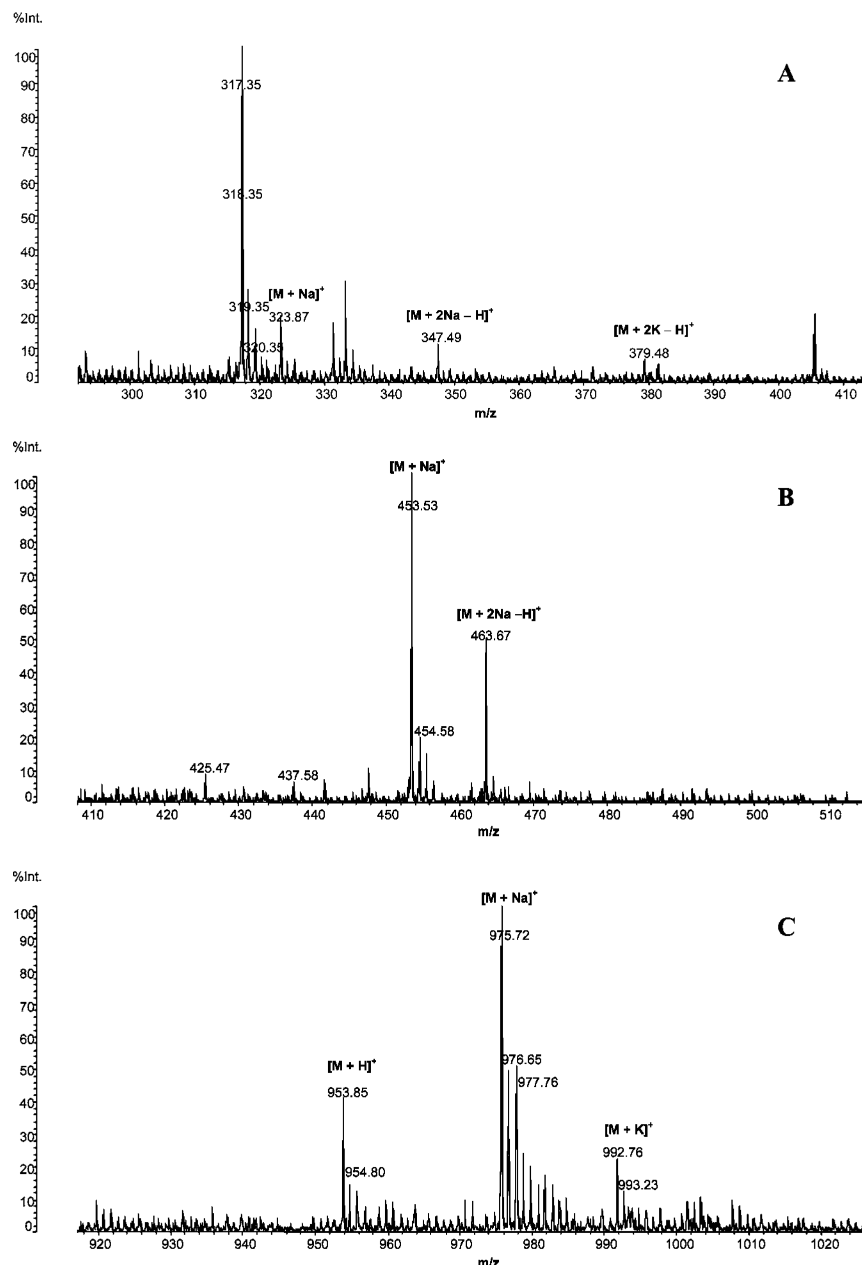

Figure 3. MALDI-QIT-TOF MS positive ion spectra of (**A**) quercetin, (**B**) kaempferol 3-*O*-arabinoside and kaempferol 3-*O*-rhamnoside, and (**C**) ellagitannin (rugosin B) in the crude extract of the dried *R. chinensis* flowers.

In MALDI-QIT-TOF MS negative-ion mode, few signal peaks were observed and much less useful information on molecular ions was acquired. This indicated that MALDI-QIT-TOF MS negative mode data could not provide useful identification information for phenolics from the dried *R. chinensis* flowers. Wang and Sporns (*23*, *38*) also reported that for conventional MALDI-TOF MS, the intensity or abundance and useful information from molecular ions in the negative ion mode was much less than from the positive ion mode.

Proper preparation of the sample is key to obtaining good MALDI-MS spectra (*20*). For the analysis of any compound using MALDI-MS, the selection of matrices, preparation of matrix and sample, signal-to-noise ratio, and spot-to-spot repeatability must normally be considered (*23*). A disadvantage of MALDI for low molecular weight compound analysis is the generation of high matrix background signals and abundant matrix adduct ions, which make the mass identification more difficult. The present study developed a novel testing method of MALDI-QIT-TOF MS; that is, the crude extract sample was dropped into the sample plate without matrix for direct analysis. This matrixless testing method could eliminate complex background peaks and generate relatively simple ionization data. However, without a matrix, the laser beam hits the sample directly, which is normally referred to as laser desorption ionization (LDI). Because LDI may cause decomposition of target compounds, one must be careful to "fire" the sample using as low a laser power as possible. In the present study, we used MALDI-QIT-TOF MS to collect good quality of MS spectra, but the collected MS/MS spectra quality was poor, because of the complexity of the constituents in the crude extract samples. The MS/MS or MS^n test will be carried out with the purified samples in the future.

Additionally, both LC-MS and MALDI-QIT-TOF MS could directly and simultaneously identify a great number of phenolics in the crude extract samples in this study. However, their advantages and disadvantages were different. MALDI-QIT-TOF MS analysis, a more rapid technique, normally took only several minutes per run, whereas LC-MS analysis took >100 min per

Table 3. Antioxidant Activity and Total Phenolic Content of Methanolic Crude Extracts from Dried *R. chinensis* Flowers and Common Vegetables and Fruits[a]

methanolic crude extract	antioxidant activity			total phenolic content
	TEAC[b] (mM/100 g of DW)	DPPH[c] (EC_{50})(μg/mL)	FRAP[d] (μM TE/g of DW)	(g of GAE/100 g of DW)
R. chinensis	235.8 ± 16.7	21.3	46.2 ± 0.01	18.9 ± 0.13
broccoli	4.5 ± 0.01	0.38	0.95 ± 0.01	0.56 ± 0.01
spring onion	2.2 ± 0.00	0.26	0.67 ± 0.00	0.37 ± 0.01
Washington red apple	5.6 ± 0.02	0.44	1.24 ± 0.03	0.68 ± 0.02
tomato	6.4 ± 0.01	0.46	1.22 ± 0.02	0.44 ± 0.00

[a] All measurements were conducted in triplicate. [b] Assayed by the improved $ABTS^{\bullet+}$ method. [c] Concentration required for 50% reduction of 60 μM DPPH radicals. [d] Expressed as micromolar Trolox equivalents (TE) per gram on dried basis (μM TE/g).

run in the present study. This was the biggest advantage of MALDI-QIT-TOF MS. Moreover, MALDI-QIT-TOF MS was more suitable for determination of larger biomolecules (e.g., tannins) and needed very simple sample preparation. Nevertheless, MALDI-QIT-TOF MS had a disadvantage in that it gave only masses of molecular ions and could not distinguish isomers of the identified phenolics. HPLC coupled with MS could differentiate isomers of many phenolics in most cases, because it not only provided molecular masses but also gave retention times and UV−vis data. The isomers had the same molecular masses and similar UV−vis spectra, but normally possessed different retention times, such as peaks 7 versus 8, 25 versus 27, and 32 versus 34 (**Figure 2**; **Table 1**).

Total Phenolics and Antioxidant Activity of Crude Extracts. Table 3 shows that the methanolic crude extracts from the dried *R. chinensis* flowers contained very high level of total phenolics (18.9 g of gallic acid equiv/100 g of DW), which were significantly (∼37-fold) higher than those from four common vegetables and fruits (broccoli, spring onion, Washington red apple, and tomato) (mean = 0.51 g/100 g of DW). As described above, the phenolics in methanolic crude extracts from the dried *R. chinensis* flowers were identified as hydrolyzable tannins, flavonols, and anthocyanins. Hydrolyzable tannins and flavonols accounted for 34.0 and 39.9% of total peak area (**Figure 2A**). Because these identified phenolics, especially gallotannins and ellagitannins, possess many phenolic hydroxyl groups, including *o*-dihydroxy groups (**Figure 1**), they have very strong radical scavenging effect and antioxidant power (*39*). As shown in **Table 3**, the methanolic crude extracts from the dried *R. chinensis* flowers exhibited potent antioxidant activity. Three methods, TEAC ($ABTS^{\bullet+}$), DPPH (EC_{50}), and FRAP, were used for antioxidant activity assays of the methanolic crude extracts from the dried *R. chinensis* flowers. TEAC of methanolic crude extracts from the dried *R. chinensis* flowers was 235.8 mM/100 g DW, significantly (∼50-fold) higher than those of controls consisting of four common vegetables and fruits (mean = 4.7 mM/100 g of DW). Their EC_{50} and FRAP values were 21.3 μg/mL and 46.2 μM TE/100 g of DW, respectively, also markedly higher than those of controls (mean = 0.39 μg/mL and 1.02 μM TE/100 g of DW). This indicated that high levels of phenolic antioxidants might be important bioactive principles in the dried *R. chinensis* flowers used as traditional Chinese medicine. The dried *R. chinensis* flowers are usually used as hemostatic and antidiarrheal agents. High levels of hydrolyzable tannins found in the dried *R. chinensis* flowers should be the important bioactive principles responsible for the related medical effect. In previous studies, the extracts of the flowers from other *Rosa* species (e.g., *R. persica*, *R. damascena*, and *R. rugosa*) closely related to *R. chinensis* also demonstrated good antioxidant activity (*12*−*14*).

In conclusion, a large number of known/unknown phenolics were isolated from the methanolic crude extracts of dried *R. chinensis* flowers and simultaneously characterized by LC-MS and MALDI-QIT-TOF MS, including 16 hydrolyzable tannins (gallotannins and ellagitannins), 17 flavonols, and 3 anthocyanins. MALDI-QIT-TOF MS not only verified 33 of all 36 phenolics isolated/identified by LC-MS but also tentatively identified 2 larger molecules of hydrolyzable tannins (rugosins B and C) not isolated and unidentified by LC-MS. This study marks the first report on direct investigation of phenolic antioxidants in the crude extracts from *R. chinensis* flowers by LC-MS and MALDI-QIT-TOF MS, very valuable techniques for the rapid analysis of hydrolyzable tannins and flavonoids. The assay results of antioxidant activity of the methanolic crude extracts indicated that the phenolic antioxidants from *R. chinensis* flowers exhibited potent antioxidant effects. High levels of phenolic antioxidants might be important bioactive principles in the dried *R. chinensis* flowers used as traditional Chinese medicine.

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