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POLYPHENOLS IN CHINESE KUSHUI ROSE *(ROSA SERTATA × ROSA RUGOSA)* LEAVES

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Polyphenols in Chinese Kushui rose (*Rosa sertata* × *Rosa rugosa*) leaves were first extracted and analysed in this study. Among four fractions (ethyl ether, ethyl acetate, *n*-butanol, and water layer) of crude extracts, the ethyl acetate fraction showed the highest $ABTS^{\bullet+}$ scavenging activity, and the *n*-butanol fraction exhibited the maximum components in composition. On-line HPLC-$ABTS^{\bullet+}$ analysis indicated that there were more than 30 antioxidant compounds from Chinese Kushui rose leaves. The identified polyphenols could be classified into quercetin derivatives, gallic acid derivatives, and proanthocyanidins. Gallic acid was the most antioxidative compound.

Keywords: Chinese Kushui rose leaves, polyphenols, $ABTS^{\bullet+}$ scavenging activity, HPLC-DAD-MS/MS

Roses and their products (essential oils, petals, and extracts) are widely used in food, cosmetic, household cleaning, and medical industries. Rose petals are used as raw materials for products of tea, wine, juice, and jams. Petals and roots of roses have also been utilized in traditional medicine for the treatment of diarrhoea, injuries, gastroenteritis, hepatitis, dysmenorrhea, and blood circulation disorders, as well as pain management and haemostasis maintenance (Ochir et al., 2010). The extract from rose petals showed excellent reducing capacity and free radical scavenging activity (Ge & Ma, 2013), the main antioxidant components of rose petals were gallic acid derivatives (Ng et al., 2004).

Most of reports on rose products were from the species of *Rosa sericea*, *Rosa rugosa*, and *Rosa damascena* Mill. Chinese Kushui rose (*Rosa sertata* × *Rosa rugosa*), mainly planted in Gansu Province of the People's Republic of China, is very famous for its aromatic profile and high content (≥40%) of citronellol in its essential oil (Zhang et al., 2003). As various tissues of a plant should have similar phytochemical composition, it implies that Chinese Kushui rose leaves should be good sources of flavonoids. The purpose of the current study was to analyse total phenol contents in the extract of Chinese Kushui rose leaves, and evaluate its active components by on-line HPLC-$ABTS^{\bullet+}$ assay and HPLC-DAD-MS/MS method.

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1. Materials and methods

1.1. Plant material and chemicals

The dried Chinese Kushui rose (*Rosa sertata* × *Rosa rugosa*) leaves (moisture, 5.8±0.5%) were kindly supplied by Gansu Oriental Tianrun Rose Industry Co. Ltd. (Lanzhou, China).

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu's reagent (2N), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (+)-catechin hydrate, and rutin were purchased from Sigma-Aldrich (Shanghai, China).

1.2. Preparation of the crude extracts and fractions

The rose leaves powder (1 g, d<0.85 mm) was extracted with 30 ml ethanol (50%, v/v) for 120 min (2×60 min) at 70 °C in a shaking bath (Model DSHZ-300, Suzhou, China). The extracts were then filtered and centrifuged at 2000 × *g* for 10 min, then the supernatant was evaporated at 50 °C and –0.08 MPa to make the soluble solid content up to 8%. The concentrates were further fractionized into four portions by ethyl ether, ethyl acetate, *n*-butanol, and water. Each of the four corresponding fractions was evaporated to dryness at 50 °C and –0.08 MPa, and then dissolved in methanol and stored at –18 °C until used.

1.3. Determination of total phenolic content (TPC)

The TPC was measured using aluminium chloride colorimetric assay, following the method described by Gong and co-workers (2012), and expressed in rutin equivalent.

1.4. Evaluation of radical scavenging capacity

The $ABTS^{\bullet+}$ scavenging capacity of Chinese Kushui rose leaves extracts was determined with the procedure described by Gong and co-workers (2012) and expressed in Trolox equivalent.

On-line HPLC-$ABTS^{\bullet+}$ detection was following the method of He and co-workers (2012) with a slight modification. The differences were in the mobile phase (2% (v/v) formic acid (solvent A) and acetonitrile (solvent B)) and its gradient elution. The solvent A was set from 100% to 90% from 0 to 15 min, and kept at 90% till 20 min, decreased to 85% at 30 min, then kept at 85% till 40 min, decreased to 80% at 50 min, kept at 80% till 55 min, and decreased to 70% at 60 min.

1.5. Identification of polyphenols

The identification of polyphenols was carried out by comparing the characteristics of DAD spectra, retention time of standard compounds, and MS data to those reported in the literature. The mass detector of the HPLC-MS system was a G6310 ion-trap mass spectrometer (Agilent Technologies Inc., Santa Clara, USA) equipped with an electrospray ionization (ESI) system and controlled by Agilent LC/MSD trap software. Nitrogen was used as nebulizing gas at a pressure of 30 psi with a flow rate of 9.0 l min^{-1}. The nebulizer temperature was 325 °C and capillary voltage was maintained at 4000 V. The compounds were measured at the scan range of the mass spectra from m/z 100 u to m/z 1500 u. MS was operated in both positive and negative ion modes.

1.6. Statistical analysis

The whole experiment and all analyses were carried out in triplicate. The data were subjected to analysis of variance (ANOVA) using the Origin Pro 9.0 software (Originlab Corporation, Northampton, USA). Significant differences ($P<0.05$) of means were determined by the Tukey's test.

2. Results and discussion

2.1. ABTS$^{\bullet+}$ scavenging activity of the extracts from Chinese Kushui rose leaves

Table 1 shows four fractions of the crude extract from Chinese Kushui rose leaves. TPC of ethyl acetate layer (39%) was the highest, followed by *n*-butanol layer (37%), ethyl ether layer (16%), and water layer (8%). The reason for that could be the different compositions and polarities of their components (Gong et al., 2012; He et al., 2012). The Trolox equivalent ABTS$^{\bullet+}$ scavenging capacity and TPC values of the extracts could be at linear correlations (R^2=0.925) (He et al., 2012).

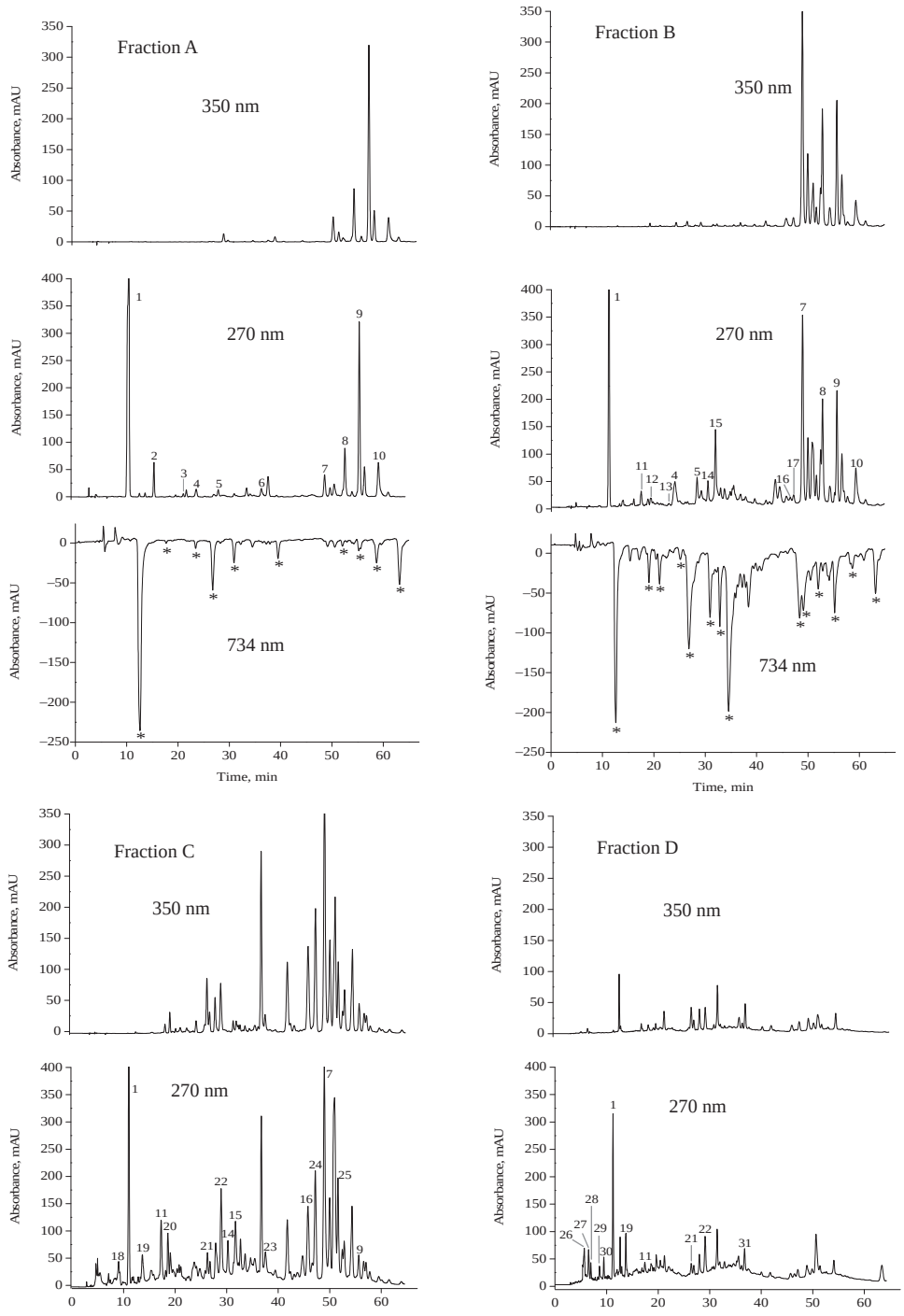
Table 1. Total phenolic content (TPC) and ABTS$^{\bullet+}$ scavenging capacities of different fractions of the extract from Chinese Kushui rose leaves

Fractions	TPC (mg RE/g)	ABTS$^{\bullet+}$ (mmol TE/g)
Crude extract	74.12±1.26	1.23±0.12^{d}
Ethyl ether layer (Fa)	12.73±0.17	0.26±0.01^{b}
Ethyl acetate layer (Fb)	29.64±0.83	0.48±0.01^{c}
n-Butanol layer (Fc)	29.85±0.94	0.51±0.02^{c}
Water layer (Fd)	6.12±0.02	0.09±0.01^{a}

RE: rutin equivalent; TE: Trolox equivalent
Values are expressed as means ± S.D. (n=3); the numbers in each column marked with different superscript letters were significantly ($P<0.05$) different.

The HPLC profiles of components and ABTS$^{\bullet+}$ scavenging antioxidants in the four fractions of the extract are significantly different (Fig. 1). To compare the free radical scavenging capacity of each component, negative absorbance lower than –100 mAU was chosen as a standard in comparison. It must be noted that ABTS radical scavenging property is only an additional physico-chemical characteristic of polyphenols. On-line HPLC-ABTS$^{\bullet+}$ analysis is used to support the identification of polyphenols and screen them quickly. The intensity of this property cannot be directly associated with better in vivo bioefficacy of identified polyphenols.

In ethyl ether fraction (Fraction A, Fa), compound 1 exhibited higher ABTS$^{\bullet+}$ scavenging activity. In ethyl acetate fraction (Fb), compounds 1, 4, 15 had higher ABTS$^{\bullet+}$ scavenging capacity. In *n*-butanol fraction (Fc), compounds 1, 11, 14, 15, 20, 22, 25 showed higher ABTS$^{\bullet+}$ scavenging activity. In water fraction (Fd), compounds 1 and 26 exhibited higher antioxidant activity. Interestingly, compound 4 was the main radical scavenger in Fb, but not the main scavenger in Fa. This could be ascribed to the different concentrations of compound 4 in Fa and Fb. Compound 1 was the most significant contributor in all four fractions.


Fraction A
350 nm
Fraction B
350 nm
270 nm
270 nm
734 nm
734 nm
Time, min
Time, min
Fraction C
350 nm
Fraction D
350 nm
270 nm
270 nm
Absorbance, mAU

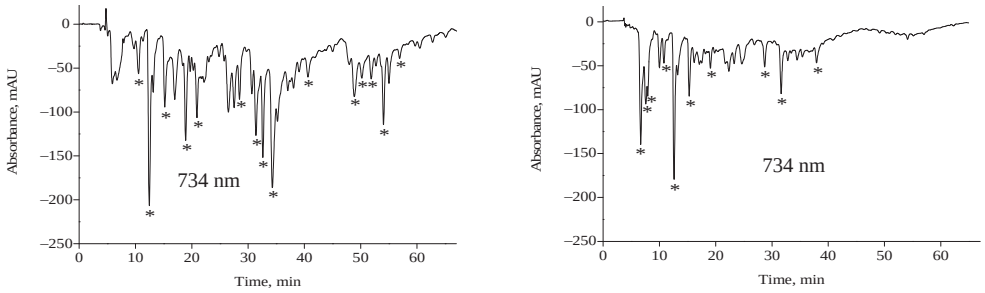


Fig. 1. HPLC chromatograms of Chinese Kushui rose leaves extract (Fraction A: ethyl ether layer fraction; Fraction B: ethyl acetate layer fraction; Fraction C: *n*-butanol layer fraction; Fraction D: water layer fraction; Absorbance at 270, 350, and 734 nm were detected for gallic acid derivatives, flavonoids, and $ABTS^{\bullet+}$ scavenging compounds; Peak numbers refer to those in Table 2.)

2.2. Identification of polyphenols in the extract from Chinese Kushui rose leaves

The chromatographic HPLC-ESI/MS and UV data for the identification of major free radical scavengers, showed in Figure 1, are shown in Table 2.

2.2.1. Quercetin derivatives. The presence of quercetin-3-rutinoside (rutin, 16, 17, 31) was revealed in the extract from Chinese Kushui rose leaves. The ESI-MS spectrum displayed an intense parent ion at m/z 609, a fragment at m/z 301 matched with quercetins derivatives. The fragment at m/z 463 was due to a loss of rhamnose moiety from the parent ion. Moreover, by comparison with the UV-Vis spectra data reported in the literature and retention time of the standard compound, compounds 16, 17, and 24 were identified as epimers of quercetin-rhamnosylgalactoside (Tiberti et al., 2007; He et al., 2010).

Compounds 7 and 31 gave a $[M-H]^-$ ion at m/z 463, and their λ_{max} was about at 356 nm. Furthermore, the deprotonated molecular ion $[M-H]^-$ yielded a fragment at m/z 301 on MS^2 spectra, therefore, they could be identified as isomers of quercetin hexoside (Seeram et al., 2006; Kajdžanoska et al., 2010).

Two compounds (8, 9) in the ether fraction were detected with m/z 433 in the MS^1 analysis, and a fragment at m/z 301 was detected in the MS^2 analysis. According to the DAD spectra data (λ_{max}=354 nm), the fragmentations of both constituents were well in accordance with quercetin pentoside (Carocho et al., 2014).

Compound 10 gave a $[M-H]^-$ ion at m/z 585, and its λ_{max} was about at 360 nm. Furthermore, the deprotonated molecular ion $[M-H]^-$ yielded fragments at m/z 301 and 433 on MS^2 spectra. It could be identified as quercetin-O-(O-galloyl)-pentoside (Saldanha et al., 2013).

Compound 22 was assigned to quercetin-dihexoside according to its pseudomolecular ion ($[M-H]^-$ at *m/z* 625) and fragmentation ions at *m/z* 463 $[M-162-H]^-$ and 301 $[M-162-162-H]^-$. Such fragmentation pattern and its UV spectrum (λ_{max}=352 nm) confirmed the presence of quercetin-dihexoside (William et al., 2003).

Compound 23 was identified as an O-glycoside of quercetin according to the UV spectrum (λ_{max}=256, 364 nm) and a major MS^2 ion at m/z 300 (M-H-448). This analysis also showed that compound 23 contained one additional deoxyhexose sugar unit (MS^2 ion at m/z 591 $[M-H-164]^-$). The loss of the deoxyhexose residue (146 amu for deoxyhexose) yielded a MS^2 ion at m/z 609 (Simirgiotis et al., 2009).

Table 2. Polyphenols in the extract of Chinese Kushui rose (*Rosa sertata* × *Rosa rugosa*) leaves

Peak No.	R.T. (min)	λ (nm)	M.W.	Negative ion mode (m/z)		Positive ion mode (m/z)		Compound	I.D.
				$[M-H]^-$	MS/MS ions	$[M+H]^+$	MS/MS ions		
Fraction A									
1	11.05	238/272	170	**169**	169, 125	–	–	gallic acid	$MS^{1, 2}$ + RT
2	15.80	260/292		–	–	–	–	unknown	
3	21.52	272	578	**577**	425, 407, 451	–	–	catechin-catechin	MS^3 + RT
4	23.72	240/278	290	**289**	289, 245, 205, 179, 125	–	–	catechin	MS^3 + RT
5	27.74	244/300/322;	636	**635**	635, 465, 483	–	–	trigalloyl glucose	MS^4
6	36.12	262		**275**	275, 258, 229, 247	–	–	unknown	
7	48.38	234/254/358	464	**463**	463, 301, 151, 179, 342	**465**	303	quercetin hexoside	MS^5
8	52.50	256/354	434	**433**	301, 151, 179, 342, 414	–	–	quercetin pentoside	MS^5
9	55.26	256/352	434	**433**	301, 151, 179, 342, 414	**435**	435, 322, 336, 209, 226	quercetin pentoside	MS^5
10	58.83	268/360	586	**585**	301, 585, 433	**587**	285, 303, 267, 417, 205	quercetin-O-(O-galloyl)-pentoside	MS^6
Fraction B									
11	17.36	252/280	484	**483**	483, 169, 331, 313	–	–	digalloyl glucose derivatives	MS^7
12	19.27	244/276	634	**633**	632, 301, 481, 463, 275, 257	–	–	galloyl-HHDP-glucose	$MS^{8, 9}$
13	22.55	282	578	**577**	577, 425, 289, 407	**579**	247, 291, 427, 301, 289	epicatechin-epicatechin	MS^3 + RT
14	29.91	270	786	**785**	633, 301	–	–	digalloyl-HHDP-hexoside	MS^{10}
15	31.40	274	786	**785**	785, 301, 483, 419, 633	–	–	digalloyl-HHDP-hexoside	MS^{10}
16	45.43	258/354/358	610	**609**	609, 301	**611**	303, 449, 465	quercetin-rhamnosylgalactoside	MS^{11}
17	46.68	254/354/358	610	**609**	609, 301	**611**	303, 465, 449	quercetin-3-rhamnosylglucoside	MS^{11}

Table 2. continued

Peak No.	R.T. (min)	λ (nm)	M.W.	Negative ion mode (m/z)		Positive ion mode (m/z)		Compound	I.D.
				$[M-H]^-$	MS/MS ions	$[M+H]^+$	MS/MS ions		
Fraction C									
18	9.56	240/276	332	**331**	331, 125, 169, 193, 211	–	–	galloyl-glucose	MS[4]
19	13.96	236/276	484	**483**	483, 169, 193, 313, 331	–	–	digalloyl glucose derivatives	MS[7]
20	18.19	240/278	484	**483**	483, 169, 193, 271, 313, 331	–	–	digalloyl glucose derivatives	MS[7]
21	26.25	244/324	354	**353**	191, 179	–	–	5-O-caffeoylquinic acid	MS[12] + RT
22	28.58	278/352	626	**625**	625, 301, 463	**627**	303, 465	quercetin-dihexoside	MS[13]
23	37.71	256/354	756	**755**	300, 591, 609	–	–	Q-3-O-(2'rhamnosyl)-rutinoside	MS[14]
24	46.67	258/354	610	**609**	609, 301	**611**	303, 465, 449, 146, 162	quercetin-rhamnosylgalactoside	MS[11]
25	50.80	264/356		**761**	761, 301, 459, 609	**763**	–	unknown	
Fraction D									
26	5.76	242/276	482	**481**	481, 301, 421, 275, 257, 319, 437	**465**	277, 303, 321, 447, 259, 385, 483,	HHDP-hexoside	MS[8, 9]
27	6.60	242/276	482	**481**	481, 301, 421, 275, 249	**465**	303, 235, 277, 218, 250	HHDP-hexoside	MS[8, 9]
28	7.04	244/276	482	**481**	481, 301, 275, 257, 249	**465**	465, 483, 321, 277, 303	HHDP-hexoside	MS[8, 9]
29	8.74	242/274	332	**331**	331, 125, 169, 193, 211, 271, 313	–	–	galloyl-glucose	MS[4]
30	9.59	240/282		**337**	337, 249, 293, 161, 205	**339**	339, 321, 293, 275, 311	unknown	
31	35.97	256/335	464	**463**	463, 301, 259, 343, 445, 619, 648	–	–	quercetin hexoside	MS[15, 16]

I.D.: identification methods; MS+RT: mass spectrum agrees with those of authentic compounds analysed in our laboratory. MS: mass spectrum agrees with those of spectrum in the literature. Compounds identified with references are indicated by superscript numbers. 1: Prakash et al., 2007; 2: Lee et al., 2005; 3: Sun et al., 2007; 4: Barros et al., 2013; 5: Carocho et al., 2014; 6: Saldanha et al., 2013; 7: Romeo et al., 2015; 8: Regueiro et al., 2014; 9: Mena et al., 2012; 10: Fischer et al., 2011; 11: Tiberti et al., 2007; 12: He et al., 2010; 13: William et al., 2003; 14: Simirgiotis et al., 2009; 15: Kajdžanoska et al., 2010; 16: Seeram et al., 2006.

2.2.2. Gallic acid derivatives. The aglycone of ellagic acid showed the same $[M-H]^-$ ion at m/z 301 like quercetin. However, ellagic acid produced fragments at m/z 258, 229 and 185 in the MS^2 analysis, whereas quercetin generated fragments at m/z 179 and 151 (Romeo et al., 2015). Furthermore, both compounds significantly differed in their UV-Vis spectra (λ_{max}; ellagic acid: 275 and 367 nm; quercetin: 252, 286, and 372 nm), thus allowing them differentiated unambiguously.

Compounds 11, 19, 20 gave the same $[M-H]^-$ ion at m/z 483 in accordance with a molecular formula $C_{20}H_{20}O_{14}$. Their λ_{max} were about 276 nm. Their deprotonated molecular ions $[M-H]^-$ yielded four fragments at m/z 331, 313, 193, and 169 on MS^2 spectra, which represented different diagnostic fragmentations of galloyl, glucose, digalloyl, and gallic acid, respectively. Moreover, the relative intensities of ions in each spectrum were significantly different at the same MS^2 condition. In this study, there were some conspicuous fragmentation differences amongst the three compounds. The fragmentation behaviours of both constituents were in accordance with those detected by Meyers and co-workers (2006). Identification of compounds 11, 19, 20 was achieved by comparing their ESI-MS^2 spectra and they could be identified as digalloyl glucose derivatives. Compound 5 was identified as trigalloyl glucose, and compounds 18 and 29 were identified as galloyl-glucose (Barros et al., 2013).

In water fraction, compounds 12, 26, 27, and 28 were detected with m/z 481 in the MS^1 analysis. All these compounds produced a fragment at m/z 301, and their λ_{max} were about at 275 nm, which indicated an ellagic acid moiety released. Three compounds were tentatively identified as hexahydroxydiphenoyl-hexoside (HHDP-hexoside) (Mena et al., 2012; Regueiro et al., 2014).

Compounds 14 and 15 were characterized as digalloyl-HHDP-hexoside (pedunculagin II). The DAD spectra data and fragmentation behaviours of both compounds were in accordance with literature data (Fischer et al., 2011). Their $[M-H]^-$ ion were at m/z 785 and typical ellagitannin and gallotannin fragments were released at m/z 483 (digalloyl-hexoside), 301 (ellagic acid), and 633 (galloyl-HHDP-hexoside). Their different retention times were ascribed to their isomeric structures resulting in their different fragmentation patterns.

Compound 1, the most abundant and effective antioxidative component, gave a negative ion at m/z 169 $[M-H]^-$ and fragment ion at m/z 125 $[M-CO_2]^+$, the compound could be recognized as gallic acid by comparison with the published data (Lee et al., 2005; Prakash et al., 2007).

2.2.3. Proanthocyanidins. The mass spectra showed the deprotonated molecule $[M-H]^-$ of catechin at m/z 289 with the characteristic MS^2 ions at m/z 245, 205, 179, 125, and UV maximum at 280 nm. For epicatechin, its mass spectra were same to those of catechin, but the λ_{max} of epicatechin was 270 nm (Dincheva et al., 2013).

Compounds 3 and 13 had $[M-H]^-$ at m/z 577 and main fragmentation with a loss of 152 amu were recognized as proanthocyanidin dimmers (Sun et al., 2007). On considering the λ_{max} of catechin and epicatechin, Compound 3 was recognized as the dimer of catechin-catechin, compound 13 was recognized as the dimer of epicatechin-epicatechin, and compound 4 was recognized as catechin.

2.2.4. Miscellaneous. Compound 21 generated a $[M-H]^-$ ion at m/z 353, and two fragment ions at m/z 191 and 179. Based on its λ_{max} at 325 nm and the retention time of authentic compound, compound 21 was identified as 5-O-caffeoylquinic acid (He et al., 2010).

3. Conclusions

In conclusion, 50% ethanol solution was an effective solvent to obtain crude extract from Chinese Kushui rose leaves. Although the in vitro $ABTS^{\bullet+}$ scavenging capacity of a compound is not equivalent to its in vivo physiological action, on-line HPLC-$ABTS^{\bullet+}$ analysis is still helpful in screening bioactive components in herbal extracts. There were 31 main polyphenols contributing to the $ABTS^{\bullet+}$ scavenging activity of the extract from Chinese Kushui rose leaves, and the compound with the most antioxidant capacity was gallic acid.

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