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Chemical Constituent Analysis of *Ranunculus sceleratus* L. Using Ultra-High-Performance Liquid Chromatography Coupled with Quadrupole-Orbitrap High-Resolution Mass Spectrometry

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Abstract: *Ranunculus sceleratus* L.(RS) has shown various pharmacological effects in traditional Chinese medicine. In our previous study, the positive therapeutic effect on α-naphthylisothiocyanate induced intrahepatic cholestasis in rats was obtained using TianJiu treatment with fresh RS. However, the chemical profile of RS has not been clearly clarified, which impedes the research progress on the therapeutic effect of RS. Herein, an ultra-high performance liquid chromatography coupled with quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) method was developed to rapidly separate and identify multiple constituents in the 80% methanol extract of RS. A total of sixty-nine compounds (19 flavonoids, 22 organic acids, 6 coumarins, 4 lignans, 14 nitrogenous compounds, and 4 anthraquinones) were successfully characterized. A total of 12 of these compounds were unambiguously identified by standard samples. Their mass spectrometric fragmentation pathways were investigated. It is worth noting that flavonoids and lignans were identified for the first time in RS. In this study, we successfully provide the first comprehensive report on identifying major chemical constituents in RS by UHPLC-Q-Orbitrap HRMS. The obtained results enrich the RS chemical profile, paving the way for further phytochemical study, quality control, and pharmacological investigation of RS.

Keywords: *Ranunculus sceleratus* L. (RS); UHPLC-Q-Orbitrap HRMS; fragmentation pathway; chemical constituent

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1. Introduction

Ranunculus sceleratus L. (RS), an annual herbaceous plant belonging to the *Ranunculus* L. family, has been listed among the top herbs in the Shennong Traditional Herbal Scriptures, written in the Western Han Dynasty. Compendium of Materia Medica notes that fresh RS can be pasted onto the acupuncture point of Cunkou overnight, (hyperemia and blistering in the skin), to cure jaundice induced by malaria. TianJiu treatment with fresh RS patches exhibited a positive therapeutic effect on α-naphthylisothiocyanate-intrahepatic cholestasis in rats by pasting on the acupuncture points of Dazhui, Ganshu (both sides), and Jizhong in our previous study [1]. The protoanemonin of fresh RS is commonly used as a robust blistering agent. It has been reported that protoanemonin is poisonous, but toxins will be destroyed when fresh RS is heated or dried. Moreover, the anti-inflammatory activity of the extract of RS administered orally at a dose of 100 mg·kg^{-1} was obtained in Wistar rats by inhibiting the induced hind paw edema [2]. A dosage between 3 and 9 g is recorded

in Zhonghuabencao when taken orally as a decoct soup to clear heat, reduce swelling, disperse the knot, and relieve pain. However, little is known about the chemical basis of the therapeutic effect of RS. Over the past few years, several chemical constituents have been isolated from RS, such as emodin, scoparone, isoscopoletin, protocatechualdehyde, protocatechuic acid, hexadecanoic acid, β-sitosterol, stigmast-4-ene-3,6-dione, stigmasterol, 1-docosene, and stigmast-5-en-3-ol [3,4]. In our previous study, 31 volatile compounds were identified in fresh RS by GC-MS analysis. The main chemical constituents were lactones and phenolic acids, including protoanemonin, 2,5-furandione, 2-propanedioic acid, and phenacetic acid [1]. Considering the positive therapeutic application of RS, it is of great importance to establish a rapid and reliable method for chemical profiling RS.

Ultra-high performance liquid chromatography coupled with quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS), with mass accuracy and high sensitivity for precursor and product ions, exhibits high resolution. Conventional separation and identification processes are time and plant-material-consuming. In contrast, the UHPLC-Q-Orbitrap HRMS method has shown high efficiency in the separation, identification, and analysis of multiple chemical constituents of traditional Chinese medicines (TCMs) [5–7]. Therefore, this method can tentatively identify components without reference standards according to exact MS data. The UHPLC-Q-Orbitrap MS method combined the inclusion list, and data-dependent acquisition was established to achieve a comprehensive characterization of the TCM formula Kai-Xin-San, and 211 compounds were identified by comparison with reference standards, literature data, and databases [8]. A total of 18 phenolic acids were identified in *Lycium ruthenicum* Murray by comparing the retention time and the exact m/z in authentic standards using UPLC-Q-Orbitrap MS [9].

To the best of our knowledge, the comprehensive identification of multiple chemical constituents in RS has not been reported. A series of studies were carried out for the rapid characterization of the chemical profile in RS. First, we established a chemical database for the compounds of *Ranunculus* L. family plants and collected as many standards as possible. Second, samples were analyzed by UHPLC-Q-Orbitrap HRMS technology for full-spectrum scanning, and the spectrum information was obtained by Xcalibur 4.1 software. Finally, after characterizing the diagnostic ions and fragmentation rules for the standards, the fragment information in the mass spectrum was compared with the literature to confirm the chemical constituents of RS. In the present study, 19 flavonoids, 22 organic acids, 6 coumarins, 4 lignans, 14 nitrogenous compounds, and 4 anthraquinones from RS were identified by UHPLC-Q-Orbitrap HRMS, providing in-depth knowledge of its chemical constituents and offering valuable information regarding its quality control and further pharmacological study.

2. Results and Discussion

2.1. Optimization of UHPLC-Q-Orbitrap HRMS Conditions

The composition of the UHPLC mobile phase should be systemically optimized to achieve the best chromatographic and mass spectrometric properties for separation and analysis. To assess the resulting peak shape and signal strength, different mobile systems that consisted of methanol−0.1% formic acid aqueous solution or acetonitrile−0.1% formic acid aqueous solution were investigated. In the result, acetonitrile−0.1% formic acid aqueous solution was chosen as the UHPLC mobile phase for gradient elution. In terms of sample processing, 60%, 80%, and 100% methanol (v/v) were examined to obtain the optimal RS extract that contains the more chemical constituents of RS. It was found that 80% methanol showed the best extraction efficiency based on the number of peaks and was selected as the extraction solvent for RS. An 80% methanol extract of RS was then analyzed in the positive and negative ion modes. Most chemical constituents showed higher responses in negative ionization mode than in positive ionization mode. In addition, to improve the sensitivity and accuracy, the ion source voltage and capillary temperature were optimized. The optimal conditions are described in Section 3.3.

2.2. Identification of Chemical Constituents in RS

Under the optimal UHPLC-Q-Orbitrap HRMS conditions, the base peak chromatograms obtained in positive and negative ionization modes for the 80% methanol extract of RS are shown in Figure 1. As shown in Table 1, a total of 69 compounds (19 flavonoids, 22 organic acids, 6 coumarins, 4 lignans, 14 nitrogenous compounds, and 4 anthraquinones) in the 80% methanol extract of RS were either unambiguously identified (12 compounds) or tentatively characterized (57 compounds). Reference standards of isoscopoletin, scopoletin, and scoparone were detected in positive ionization mode (Figure S1), and reference standards of aesculetin, quercetin, protocatechuic acid, salicylic acid, ferulic acid, luteolin, caffeic acid, emodin, and oleanic acid were detected in negative ionization mode (Figure S2). Next, the names and chemical structures of the other 57 compounds were preliminarily inferred by comparing MS ion fragmentation information with relevant literature. Altogether, the chemical structures of identified 69 compounds are summarized in Figure S3.

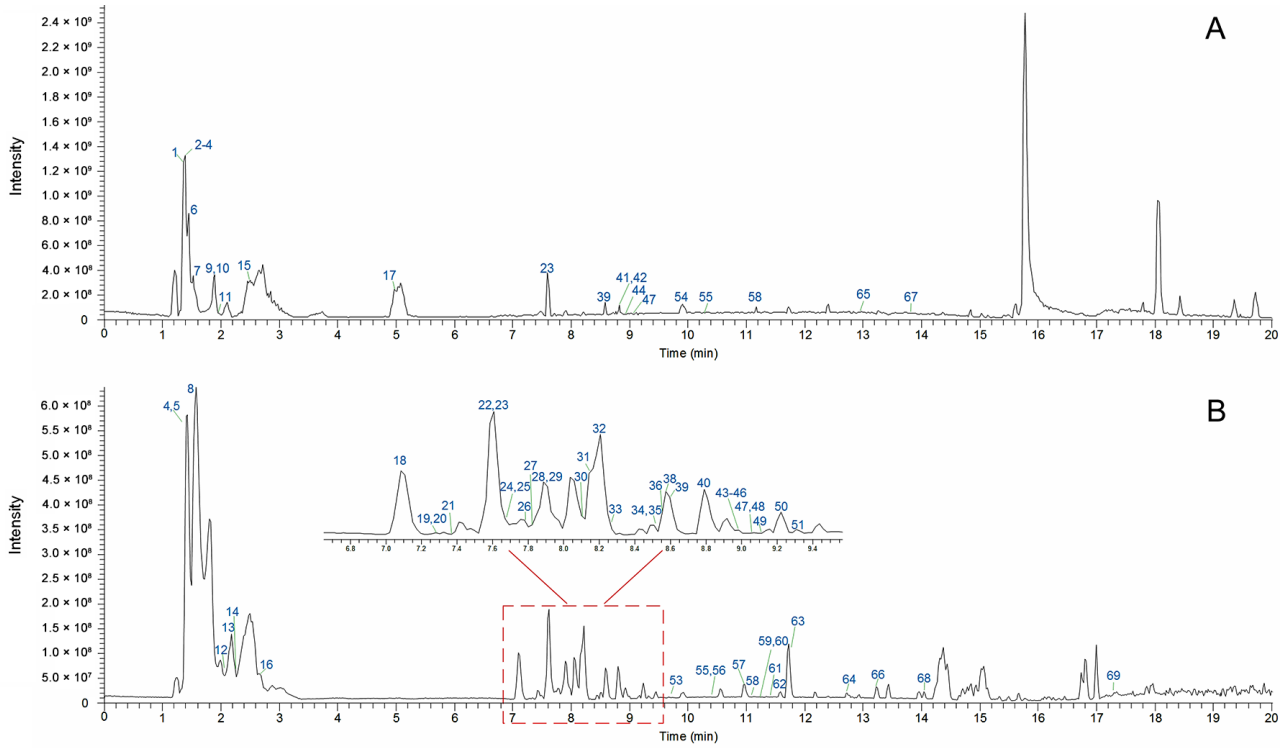

Figure 1. Base peak chromatograms of RS in positive ionization mode (**A**) and negative ionization mode (**B**).

Table 1. 69 compounds identified in the 80% methanol extract of RS and their UHPLC-Q-Orbitrap HRMS data.

NO.	Retention Time (min)	Ionization Mode	Molecular Formula	Theoretical (*m*/*z*)	Measured (*m*/*z*)	Error (ppm)	Identified Compounds	MS/MS Fragments (*m*/*z*)	Ref.
1	1.37	$[M + H]^+$	$C_4H_8N_2O_3$	133.0607	133.0604	−2.25	Asparagine	133.0604, 115.0391, 97.0286, 87.0558	[10]
2	1.40	$[M + H]^+$	$C_5H_9NO_4$	148.0604	148.0598	−4.05	Glutamic acid	148.0598, 102.0550, 84.0447	[11]
3	1.40	$[M + H]^+$	$C_4H_9NO_3$	120.0655	120.0655	0.00	Threonine	120.0655, 102.0553, 74.0607, 56.0503	[11]
4	1.40	$[M + H]^+$	$C_5H_7NO_3$	130.0498	130.0499	0.77	Pyroglutamic acid	130.0499, 102.0551, 84.0449	[11]
	1.40	$[M - H]^-$	$C_5H_7NO_3$	128.0342	128.0340	−1.56	Pyroglutamic acid	128.0339, 85.0279, 68.1163	
5	1.42	$[M - H]^-$	$C_6H_{12}O_7$	195.0499	195.0501	1.03	Gluconic acid	195.0501, 129.018, 75.0072	[12]
6	1.47	$[M + H]^+$	$C_5H_9NO_2$	116.0706	116.0704	−1.72	Proline	116.0704, 70.0656	[11]
7	1.51	$[M + H]^+$	$C_4H_5N_3O$	112.0505	112.0507	1.78	Cytosine	112.0507, 95.0244, 69.0455	[13]
8	1.57	$[M - H]^-$	$C_4H_6O_5$	133.0131	133.0129	−1.50	Malic acid	133.0129, 115.0023, 71.0124	[14]
9	1.91	$[M + H]^+$	$C_5H_5N_5O$	152.0567	152.0565	−1.32	Guanine	152.0565, 135.0300, 110.0351	[13]
10	1.91	$[M + H]^+$	$C_6H_5NO_2$	124.0393	124.0394	0.81	Nicotinic acid	124.0394, 106.0291, 96.0448, 80.0501	[15]
11	1.99	$[M + H]^+$	$C_6H_6N_2O$	123.0553	123.0554	0.81	Nicotinamide	123.0554, 80.0501	[16]
12	2.13	$[M - H]^-$	$C_6H_6O_6$	173.0080	173.0082	1.16	Aconitic acid	173.0082, 129.0179, 111.0072, 85.0279	[14]
13	2.17	$[M - H]^-$	$C_5H_4O_3$	111.0076	111.0073	−2.70	Furoic acid	111.0073, 83.0122, 67.0174	[17]
14	2.24	$[M - H]^-$	$C_6H_8O_7$	191.0186	191.0187	0.52	Citric acid	191.0187, 111.0073, 87.0072	[14]
15	2.62	$[M + H]^+$	$C_4H_4N_2O_2$	113.0345	113.0347	1.77	Uracil	113.0347, 96.0084, 70.0294	[11]
16	2.64	$[M - H]^-$	$C_4H_6O_4$	117.0182	117.0179	−2.56	Succinic acid	117.0179, 99.0073, 73.0280	[15]
17	5.05	$[M + H]^+$	$C_9H_{11}NO_2$	166.0862	166.0861	−0.60	Phenylalanine	166.0861, 120.0809, 103.0545	[11]
18	7.09	$[M - H]^-$	$C_{13}H_{16}O_9$	315.0711	315.0722	3.49	Protocatechuic acid-O-glucose	315.0722, 153.0545, 109.0280	[18]
19	7.28	$[M - H]^-$	$C_{14}H_{18}O_9$	329.0867	329.0858	−2.73	Vanillic acid-O-glucopyranoside	329.0858, 167.033, 123.0437	[19]
20	7.28	$[M - H]^-$	$C_{14}H_{20}O_8$	315.1074	315.1092	5.71	Hydroxytyrosol-1-glucopyranoside	315.1092, 153.0545	[19]
21	7.37	$[M-H]^-$	$C_7H_6O_4$	153.0182	153.0181	−0.65	Protocatechuic acid	153.0181, 123.0437, 109.0280, 91.0176	Standard
22	7.61	$[M-H]^-$	$C_{15}H_{18}O_9$	341.0867	341.0873	1.76	Caffeic acid-O-glucopyranoside	341.0873, 179.0339, 135.0439	[19]
23	7.61	$[M + H]^+$	$C_{11}H_{12}N_2O_2$	205.0972	205.0970	−0.98	Tryptophan	205.0970, 188.0703, 159.0914, 146.0598	[11]
	7.61	$[M - H]^-$	$C_{11}H_{12}N_2O_2$	203.0815	203.0818	1.48	Tryptophan	203.0818, 186.0551, 159.091, 142.0649	
24	7.65	$[M - H]^-$	$C_{15}H_{16}O_9$	339.0711	339.0718	2.06	Esculin	339.0718,177.0183	[20]
25	7.67	$[M - H]^-$	$C_{13}H_{16}O_8$	299.0761	299.0777	5.35	Salicylic acid-O-glucopyranoside	299.0777, 137.0231, 93.0331	[19]
26	7.78	$[M - H]^-$	$C_{27}H_{30}O_{16}$	609.1450	609.1464	2.30	Kaempferol-3,7-di-O-glucoside	609.1464, 447.0930, 285.0402, 255.0295, 151.0023	[21]
27	7.82	$[M - H]^-$	$C_7H_6O_3$	137.0233	137.0231	−1.46	Protocatechualdehyde	137.0231, 109.0279, 108.0201, 93.0330	[22]
28	7.89	$[M - H]^-$	$C_{16}H_{18}O_9$	353.0867	353.0865	−0.57	Scopolin	353.0865, 191.0553, 179.0341, 173.0450, 135.0439	[23]

Table 1. *Cont.*

NO.	Retention Time (min)	Ionization Mode	Molecular Formula	Theoretical (*m*/*z*)	Measured (*m*/*z*)	Error (ppm)	Identified Compounds	MS/MS Fragments (*m*/*z*)	Ref.
29	7.91	$[M-H]^-$	$C_{15}H_{18}O_8$	325.0918	325.0928	3.08	*p*-Coumaric acid-O-glucopyranoside	325.0928, 163.0388, 119.0487,	[19]
30	8.12	$[M-H]^-$	$C_{27}H_{30}O_{16}$	609.1450	609.1453	0.49	Kaempferol-O-sophoroside	609.1453, 429.0818, 284.0323, 255.0295, 227.0343	[24]
31	8.16	$[M-H]^-$	$C_9H_6O_4$	177.0182	177.0182	0.00	Aesculetin	177.0182, 149.0231, 133.0280, 121.0280	Standard
32	8.21	$[M-H]^-$	$C_9H_8O_4$	179.0339	179.0342	1.68	Caffeic acid	179.0342, 135.0437, 90.9966	Standard
33	8.25	$[M-H]^-$	$C_{26}H_{28}O_{16}$	595.1294	595.1301	1.18	Quercetin-O-(pentosyl)hexoside isomer	595.1301, 300.0274, 271.0248, 255.0294, 151.0022, 135.0433	[25]
34	8.51	$[M-H]^-$	$C_{11}H_{13}NO_3$	206.0811	206.0813	0.97	*n*-Acetyl-L-phenylalanine	206.0813, 164.0705, 147.0438, 118.9914, 91.0536	[26]
35	8.53	$[M-H]^-$	$C_{21}H_{20}O_{11}$	447.0922	447.0929	1.57	Isoorientin	447.0929, 429.0821, 357.0613, 327.0508, 285.0420, 133.0281	[27]
36	8.55	$[M-H]^-$	$C_8H_{14}O_4$	173.0808	173.0808	0.00	Suberic acid	173.0808, 129.0906, 111.0800	[28]
37	8.59	$[M+H]^+$	$C_{10}H_8O_4$	193.0495	193.0490	−2.95	Isoscopoletin	193.0490, 178.0255, 133.0280	Standard
38	8.60	$[M-H]^-$	$C_{26}H_{32}O_{12}$	535.1810	535.1818	1.49	1-Hydroxylpinoresinol 4′-O-glucopyranoside	535.1818, 373.1284, 343.1182	[19]
39	8.62	$[M+H]^+$	$C_{21}H_{20}O_{12}$	465.1027	465.1024	−0.65	Hyperoside	465.1024, 303.0494, 257.0439, 229.0493, 153.0181	[29]
	8.62	$[M-H]^-$	$C_{21}H_{20}O_{12}$	463.0871	463.0882	2.38	Hyperoside	463.0882, 301.0350, 271.0244, 178.9974, 151.0023	[29]
40	8.77	$[M-H]^-$	$C_9H_8O_3$	163.0389	163.0390	0.61	Coumaric acid	163.0390, 119.0488	[19]
41	8.88	$[M+H]^+$	$C_{16}H_{12}O_7$	317.0656	317.0649	−2.21	Isorhamnetin	317.0649, 302.0416, 285.0388, 274.0467, 153.0180	[30]
42	8.91	$[M+H]^+$	$C_{15}H_{10}O_6$	287.0550	287.0545	−1.74	Kaempferol	287.0545, 269.0437, 231.0651, 213.0541, 153.0180, 121.0285	[29]
43	8.92	$[M-H]^-$	$C_{22}H_{22}O_{12}$	477.1028	477.1034	1.26	Isorhamnetin-3-O-glucoside isomer	477.1034, 314.0430, 285.0402, 271.0247, 243.0295	[22]
44	8.94	$[M+H]^+$	$C_{10}H_8O_4$	193.0495	193.0493	−1.04	Scopoletin	193.0493, 178.0258, 175.1479, 150.0309, 133.0283, 109.0857	Standard
	8.94	$[M-H]^-$	$C_{10}H_8O_4$	191.0339	191.0340	0.52	Scopoletin	191.0340, 176.0105, 146.973,111.0074, 102.9474	
45	8.96	$[M-H]^-$	$C_{21}H_{20}O_{11}$	447.0933	447.0930	−0.67	Luteoloside	447.0930, 285.0420, 255.0296, 241.0500, 217.05.01, 227.0343, 199.0395, 151.0023	[19]
46	8.98	$[M-H]^-$	$C_{10}H_{10}O_4$	193.0495	193.0495	0.00	Ferulic acid	193.0495, 178.0261, 149.0595,134.0360, 121.0282	Standard
47	9.03	$[M+H]^+$	$C_{21}H_{18}O_{11}$	447.0922	447.0924	0.45	Apigenin-7-O-glucuronide	447.0924, 271.0595, 231.1145, 199.2475, 153.0180, 119.0490	[28]

Table 1. *Cont.*

NO.	Retention Time (min)	Ionization Mode	Molecular Formula	Theoretical (*m*/*z*)	Measured (*m*/*z*)	Error (ppm)	Identified Compounds	MS/MS Fragments (*m*/*z*)	Ref.
	9.03	$[M-H]^-$	$C_{21}H_{18}O_{11}$	445.0765	445.0752	−2.92	Apigenin-7-O-glucuronide	445.0752, 269.0452, 175.0233, 113.0229	
48	9.06	$[M-H]^-$	$C_{21}H_{18}O_{12}$	461.0714	461.0727	2.82	Luteolin-7-O-glucuronide	461.0727, 285.0402, 151.0022, 133.0279	[31]
49	9.10	$[M-H]^-$	$C_{26}H_{32}O_{11}$	519.1861	519.1873	2.31	Matairesinoside	519.1972, 357.1340, 342.1098, 313.1464, 221.0804, 161.0595	[19]
50	9.22	$[M-H]^-$	$C_9H_{16}O_4$	187.0964	187.0966	1.07	Azelaic acid	187.0966, 125.0958, 97.0644	[32]
51	9.31	$[M-H]^-$	$C_9H_{10}O_3$	165.0546	165.0545	−0.61	*p*-Hydroxybenzene propanoic acid	165.0545, 147.0439, 136.9310, 119.0488, 72.9916	[33]
52	9.33	$[M-H]^-$	$C_{20}H_{22}O_7$	373.1282	373.1289	1.88	1-Hydroxylpinoresinol	373.1289, 343.1176, 313.1081, 298.0844, 147.0439, 123.0074, 109.0277	[19]
53	9.68	$[M-H]^-$	$C_7H_6O_3$	137.0233	137.0232	−0.73	Salicylic acid	137.0232, 93.0331	Standard
54	9.82	$[M+H]^+$	$C_{11}H_{10}O_4$	207.0652	207.0649	−1.45	Scoparone	207.0649, 191.0337, 163.0388, 151.0752, 121.0648, 105.0703	Standard
55	10.28	$[M+H]^+$	$C_{15}H_{10}O_6$	287.0550	287.0545	−1.74	Luteolin	287.0545, 153.0180, 131.0439	Standard
	10.28	$[M-H]^-$	$C_{15}H_{10}O_6$	285.0393	285.0403	3.51	Luteolin	285.0403, 175.0387, 133.0281,121.0279, 107.0125, 83.0124	
56	10.32	$[M-H]^-$	$C_{15}H_{10}O_7$	301.0343	301.0355	3.99	Quercetin	301.0355, 229.0504, 201.0565, 178.9975, 151.0024, 121.0281	Standard
57	10.83	$[M-H]^-$	$C_{20}H_{22}O_6$	357.1332	357.1341	2.52	Matairesinol	357.1341, 342.1097, 313.0370, 283.0078,	[19]
58	11.06	$[M+H]^+$	$C_{15}H_{10}O_5$	271.0601	271.0595	−2.21	Apigenin	271.0595, 153.0181, 119.0492	[26]
	11.06	$[M-H]^-$	$C_{15}H_{10}O_5$	269.0444	269.0453	3.35	Apigenin	269.0453, 251.0590, 227.0341, 181.0644, 151.0025, 117.0332	
59	11.16	$[M-H]^-$	$C_{16}H_{12}O_6$	299.0550	299.0560	3.34	Chrysoeriol	299.0560, 284.0326, 256.0372, 255.0293, 227.0345	[31]
60	11.19	$[M-H]^-$	$C_{15}H_{10}O_6$	285.0394	285.0403	3.16	7-Hydroxy-emodin	285.0403, 257.0449, 211.0380	[34]
61	11.41	$[M-H]^-$	$C_{16}H_{12}O_6$	299.0550	299.0561	3.68	Diosmetin	299.0561, 284.0325, 256.0378, 255.0296, 227.0344	[31]
62	11.51	$[M-H]^-$	$C_{16}H_{12}O_7$	315.0499	315.0508	2.86	Rhamnetin	315.0508, 300.0271, 151.0024,107.0123	[35]
63	11.67	$[M-H]^-$	$C_{16}H_{12}O_5$	283.0601	283.0611	3.53	1-O-Methyl-emodin	283.0611, 268.0376, 239.0346, 211.0395	[34]
64	12.75	$[M-H]^-$	$C_{15}H_{12}O_4$	255.0651	255.0659	3.14	Pinocembrin	255.0659, 213.0549, 171.0441, 151.0024	[36]
65	12.95	$[M+H]^+$	$C_{17}H_{14}O_6$	315.0863	315.0857	−1.90	7,3′-Dihydroxy-8,4′-dimethoxyisoflavone/isomer	315.0857, 300.0623	[37]
66	13.21	$[M-H]^-$	$C_{16}H_{12}O_5$	283.0601	283.0610	3.18	Physcion	283.0610, 268.0377, 239.0346, 211.0393	[34]

Table 1. *Cont.*

NO.	Retention Time (min)	Ionization Mode	Molecular Formula	Theoretical (*m*/*z*)	Measured (*m*/*z*)	Error (ppm)	Identified Compounds	MS/MS Fragments (*m*/*z*)	Ref.
67	13.77	$[M + H]^+$	$C_{18}H_{39}NO_3$	318.3002	318.2996	−1.89	2-Amino-1,3,4-octadecanetriol	318.2996, 300.2891, 282.2787, 264.2681, 60.0452	[38]
68	14.02	$[M - H]^-$	$C_{15}H_{10}O_5$	269.0444	269.0455	4.09	Emodin	269.0455, 241.0504, 225.0552, 210.0320	Standard
69	17.25	$[M - H]^-$	$C_{30}H_{48}O_3$	455.3531	455.3528	−0.66	Oleanic acid	455.3528, 240.9500, 206.1664, 82.4031	Standard

2.2.1. Flavonoids

Flavonoids refer to a class of naturally occurring bioactive compounds in herbal medicine. The principal mass spectrometric fragmentation mechanisms for flavonoids are loss of neutral fragments, such as H_2O, CH_3, CO, CO_2, and the cleavage of retro-Diels-Alder (RDA) at glycosyl bonds. The product ion spectrum of compound **39** shown in Figure 2A is representative. In ESI^-, compound **39** produced the deprotonated molecular ion at m/z 463.0882 $[M - H]^-$, which could form the fragment ion m/z 301.0350 $[M - H - C_6H_{10}O_5]^-$ by losing one glucose sugar group ($C_6H_{10}O_5$). After undertaking the RDA reaction, a series of product ions could be produced, such as m/z 151.0023 and 107.0123. By comparing their molecular formulas and fragmentation patterns with those reported in the literature [29], compound **39** was tentatively identified as hyperoside, reported here for the first time in RS. Similarly, the $[M - H]^-$ ion of compound **47** was shown at m/z 445.0752, and was tentatively identified as apigenin-7-O-glucuronide. Characteristic fragment ions m/z 269.0452 were produced by successive loss of glucuronide [28]. Glucuronide residues at m/z 175.0233 and m/z 113.0229 could always be observed in glycosides. The mass spectra and proposed major fragmentations with structures are shown in Figure 2B. Except for luteolin (**55**) and quercetin (**56**), confirmed by reference standards, all other flavonoids were identified similarly. The other flavonoids identified according to their molecular mass, formulas, MS/MS fragments, and related literature studies, including kaempferol-3,7-di-O-glucoside (**26**), kaempferol-O-sophoroside (**30**), quercetin-O-(pentosyl) hexoside isomer (**33**), isoorientin (**35**), isorhamnetin (**41**), kaempferol (**42**), isorhamnetin-3-O-glucoside isomer (**43**), luteoloside (**45**), luteolin-7-O-glucuronide (**48**), apigenin (**58**), chrysoeriol (**59**), diosmetin (**61**), rhamnetin (**62**), pinocembrin (**64**), 7,3′-dihydroxy-8,4′-dimethoxyisoflavone/isomer (**65**). Flavonoids were identified for the first time in RS, and pharmacological studies have demonstrated that flavonoids have good efficacy in treating cholestatic liver disease [39].

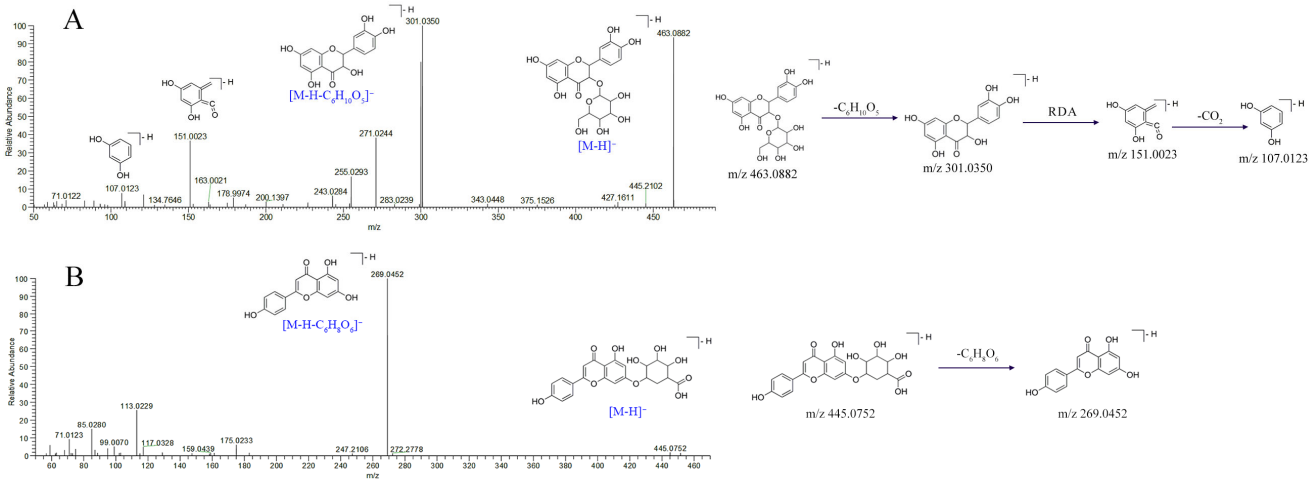

Figure 2. Characteristic of MS/MS spectra and possible fragmentation pathways of hyperoside (**A**) and apigenin-7-O-glucuronide (**B**).

2.2.2. Organic Acids

Organic acids widely occur in natural plants, especially in herbs. In negative ionization mode, organic acids exhibited deprotonated molecular peaks and easily lost CO_2 and CO to generate corresponding fragment ion peaks. Moreover, the losses of small molecules (like H_2O) or radicals (like CH_3) sometimes occurred. For instance, protocatechuic acid (compound **21**) showed a molecular formula of $C_7H_6O_4$ and a deprotonated molecule $[M - H]^-$ peak at m/z 153.0181. The deprotonated molecule lost a CO_2 moiety to form a fragment ion $[M - H - CO_2]^-$ at m/z 109.0280. Then, it was dehydrated to form the $[M - H - CO_2 - H_2O]^-$ fragment ion of m/z 91.0176. The fragmentation pathways of compound **21** are shown in Figure 3A. Compound **21** was unambiguously identified as protocatechuic acid

by comparing its MS/MS fragmentation pattern and retention time of reference standard. Likewise, compounds **32**, **46**, **53**, and **69** were identified as caffeic acid, ferulic acid, salicylic acid, and oleanic acid by reference standards. The deprotonated molecule $[M-H]^-$ of compound **25** peak was at m/z 299.0777, and its chemical structure and fragmentation pathway are shown in Figure 3B. Compound **25** was identified as salicylic acid-O-glucopyranoside according to the literature [19]. The other compounds were identified according to their molecular mass, formulas, MS/MS fragments, and related literature studies, including gluconic acid (**5**), malic acid (**8**), aconitic acid (**12**), furoic acid (**13**), citric acid (**14**), succinic acid (**16**), protocatechuic acid-O-glucose (**18**), vanillic acid-O-glucopyranoside (**19**), hydroxytyrosol-1-glucopyranoside (**20**), caffeic acid-O-glucopyranoside (**22**), protocatechualdehyde (**27**), *p*-coumaric acid-O-glucopyranoside (**29**), suberic acid (**36**), coumaric acid (**40**), azelaic acid (**50**), and *p*-hydroxybenzene propanoic acid (**51**).

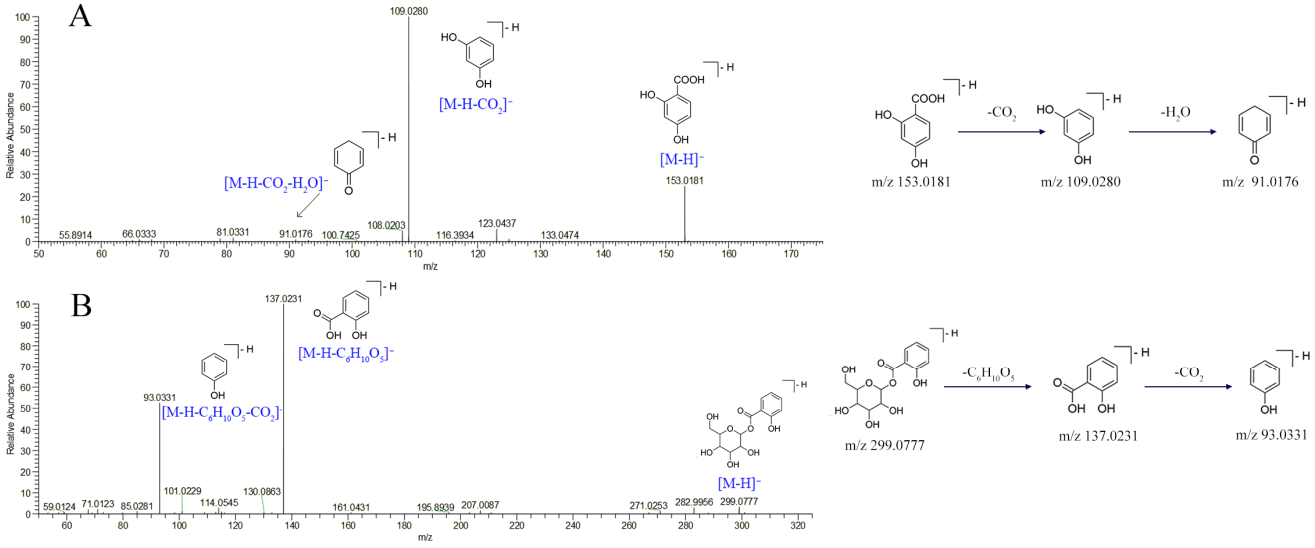

Figure 3. Characteristic of MS/MS spectra and possible fragmentation pathways of protocatechuic acid (**A**) and salicylic acid-O-glucopyranoside (**B**).

2.2.3. Coumarins and Lignans

Six coumarins were identified from the 80% methanol extract of RS, including esculin (**24**), scopolin (**28**), aesculetin (**31**), isoscopoletin (**37**), scopoletin (**44**), and scoparone (**54**). Their fragmentation patterns in mass spectrometry were investigated, and neutral losses of CO and CO_2 could be commonly observed. A typical coumarin, scopoletin (**44**), was taken as an example to investigate the MS/MS fragmentation pattern of coumarin in RS. The protonated molecular ion of compound **44** was m/z 193.0493 $[M + H]^+$ in positive ESI mode. Scopoletin produced a fragment ion $[M + H - CH_3]^+$ at m/z 178.0258 by demethylation, which further lost one CO moiety, and the fragment ions of $[M + H - CH_3 - CO]^+$ at m/z 150.0309 were generated. Scopoletin also directly lost one CH_4 and CO_2 neutral moiety to generate a product ion $[M + H - CH_4 - CO_2]^+$ at m/z 133.0283, indicating the C-6 methoxy substituents and lactone structures. The possible fragmentation pathway for scopoletin is proposed in Figure 4A. The esculin (**24**) and scopolin (**28**) were identified according to related literature studies [20,23]. Compounds **31**, **37**, **44,** and **54** were confirmed by comparison with the available reference standards.

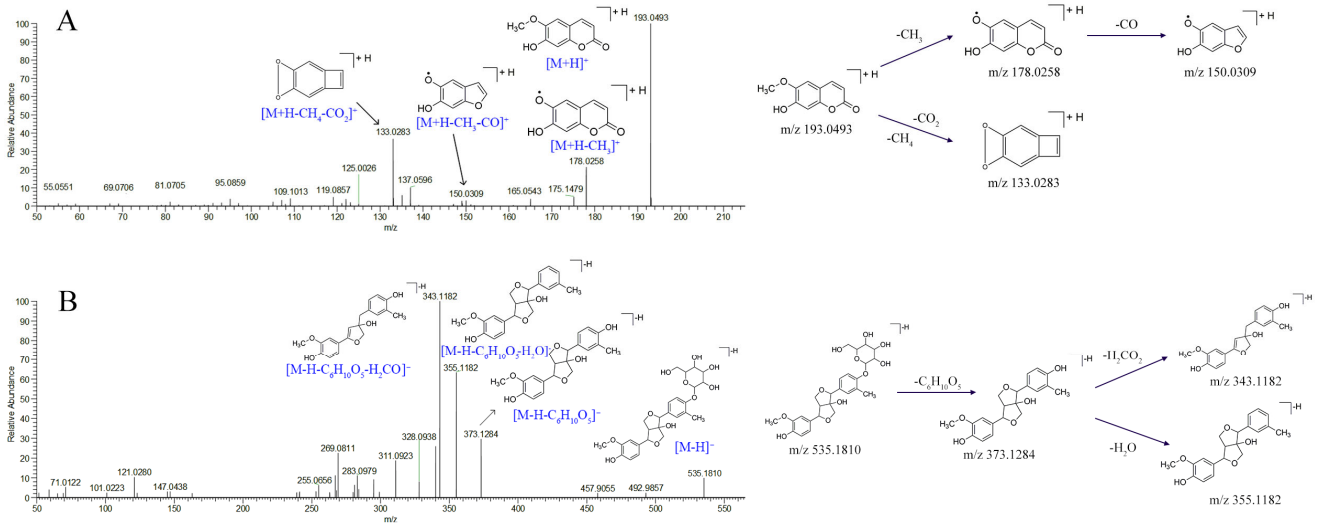

Figure 4. Characteristic of MS/MS spectra and possible fragmentation pathways of scopoletin (**A**) and hydroxylpinoresinol 4′-O-glucopyranoside (**B**).

Lignans are a class of natural compounds synthesized by polymerizing two phenylpropanoid derivatives (C6-C3 monomers). Compounds **38**, **49**, **52**, and **57** were furofuran-type lignans. Taking Compound **38** as an example, the deprotonated molecular ion m/z 535.1772 was detected in the spectrum. Its MS/MS fragment ions at m/z 373.1284 $[M - H - C_6H_{10}O_5]^-$, m/z 355.1182 $[M - H - C_6H_{10}O_5 - H_2O]^-$, and m/z 343.1182 $[M - H - C_6H_{10}O_5 - H_2CO]^-$ were observed in negative ionization mode. Compound **38** was identified as 1-hydroxylpinoresinol 4′-O-glucopyranoside according to the literature [19]. The possible fragmentation mechanism of compound **38** is depicted in Figure 4B. Compounds **49**, **52,** and **57** were tentatively identified as matairesinoside, 1-hydroxypinoresinol, and matairesinol according to their MS/MS fragments and related literature studies [19].

2.2.4. Nitrogenous Compounds

Amino acids, nucleobases, and other nitrogenous compounds respond strongly in positive ionization mode, and most of the second mass spectra are broken in the center of N^+. In the present study, 3 nucleobases, 7 amino acids, and other nitrogenous compounds in RS were characterized. The usual fragmentation pathways, including the losses of NH_3, H_2O, and HCOOH, were observed in these compounds. Compound **6** was identified as proline based on short retention time and specific fragments. It has a pseudomolecular ion of m/z 116.0704, indicative of the molecular formula $C_5H_9NO_2$. The fragment ion at m/z 70.0656 for $[M + H - HCOOH]^+$ agrees with the literature studies due to losing one carboxyl group [11]. The chemical structures and fragmentation pathways are shown in Figure 5. In this way, other amino acids can be successfully characterized according to related literature studies, including asparagine (**1**), glutamic acid (**2**), threonine (**3**), pyroglutamic acid (**4**), phenylalanine (**17**), and tryptophan (**23**). In addition, nucleobases (compounds **7**, **9,** and **15**) and other nitrogenous compounds (compounds **10**, **11**, **34,** and **67**) were also detected and tentatively identified based on databases and the literature.

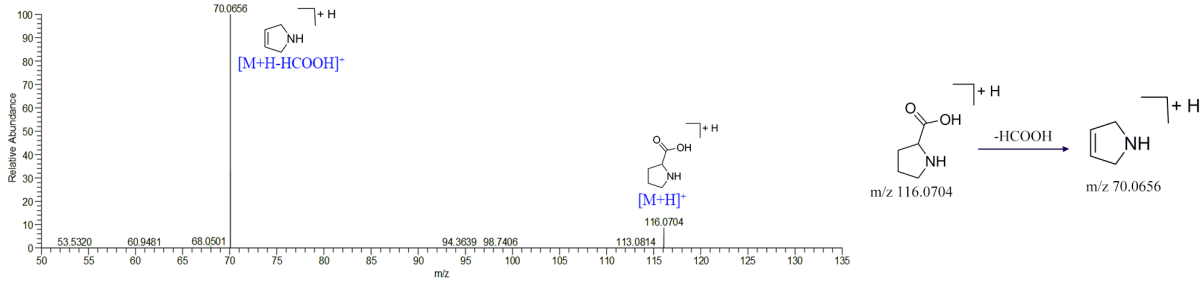

Figure 5. Characteristic of MS/MS spectra and possible fragmentation pathways of proline.

2.2.5. Anthraquinonoids

By comparing the retention time and MS spectrum with authentic standards, compound **68** was identified as emodin. The protonated molecular ion of emodin was m/z 269.0455 $[M - H]^-$ in negative ionization modes. Its MS/MS fragment ions were at m/z 241.0504 $[M - H - CO]^-$ and m/z 225.0552 $[M - H - CO_2]^-$. The chemical structures and fragmentation pathways are shown in Figure 6. In addition, compounds **60**, **63,** and **66** were tentatively identified as 7-hydroxy-emodin, 1-O-methyl-emodin, and physcion according to their MS/MS fragments and related literature studies [34].

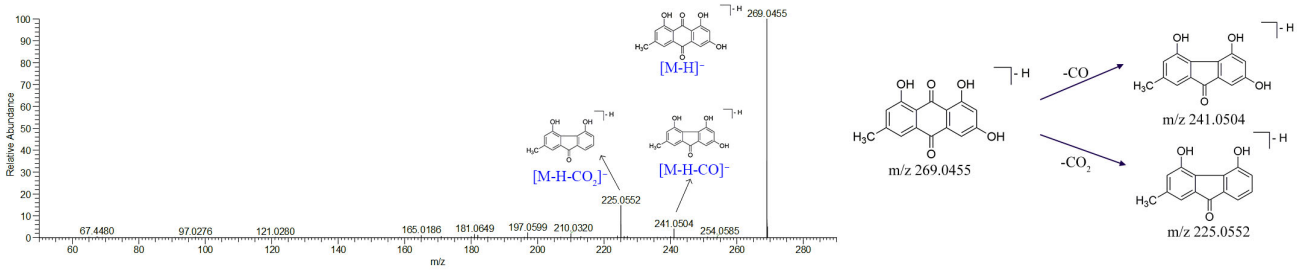

Figure 6. Characteristic of MS/MS spectra and possible fragmentation pathways of emodin.

3. Materials and Methods

3.1. Chemicals and Reagents

High-performance liquid chromatography (HPLC)-grade acetonitrile and formic acid were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). Analytical-grade methanol was purchased from Chinasun Specialty Products Co., Ltd. (Jiangsu, China). The aesculetin (Batch No. 5483, 99%), isoscopoletin (Batch No. 3620, 98%), scopoletin (Batch No. 5257, 98%), quercetin (Batch No. 1115, 98%), scoparone (Batch No. 1902, 98%), protocatechuic acid (Batch No. 5809, 99%), salicylic acid (Batch No. 5328, 99%), caffeic acid (Batch No. 2681, 98%), ferulic acid (Batch No. 8042, 99%), and emodin (Batch No. 8171, 98%) were purchased from Shanghai Standard Technology Co., Ltd. (Shanghai, China). Standards of luteolin (Batch No. C11352540, 98%) and oleanic acid (Batch No. M180850633, 98%) were obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Ultrapure water (18.2 MΩ·cm^{-1}) was purified by a Millipore system (Millipore Corp., Burlington, MA, USA).

The batch of *Ranunculus sceleratus* L. was collected in the Xinhe community of Feidong County (Hefei, China) and authenticated by Professor Huasheng Peng (School of Pharmacy, Anhui University of Chinese Medicine). Voucher specimens (Batch No. 20210401) were deposited in the herbarium of the School of Pharmacy, Anhui Medical University (Hefei, China).

3.2. Preparation of Sample and Standard Solutions

An aliquot of 0.1 g fine powder (<65 mesh) of RS samples was accurately weighed and placed in a 50 mL of a conical flask, and then 10 mL of 80% methanol−water (v/v) was added into the conical flask. After sonication for 30 min, the sample solution was

cooled to room temperature. All the standards of aesculetin, isoscopoletin, scopoletin, quercetin, scoparone, protocatechuic acid, salicylic acid, ferulic acid, luteolin, caffeic acid, emodin and oleanic acid were dissolved in 80% methanol−water (*v*/*v*) at a concentration of 10 μg·mL^{-1} to prepare standard solutions. All the solutions were filtered through a 0.22 μm filter membrane (Bandao Corp., Shanghai, China) before analysis.

3.3. UHPLC-Q-Orbitrap HRMS System and Conditions

A UHPLC Dionex Ultimate 3000 (Thermo Scientific, San Jose, CA, USA) equipped with a cooling autosampler and column oven was utilized. The separation was performed on a Shim-pack GISS UHPLC C_{18} column (100 mm × 2.1 mm, 1.9 μm) (Shimadzu, Japan) with a column temperature maintained at 30 °C at a flow rate of 0.2 mL·min^{-1}. Binary mobile solvents consisted of acetonitrile (A) and water containing 0.1% formic acid (B), and the following gradient elution program was used: 0−3 min, 2% A; 3–5 min, 2–30% A; 5–12 min, 30–70% A; 12–14 min, 70–95% A; 14–18 min, 95% A; 18–20 min, 95–2% A. The injection volume was set at 2 μL.

A Q-Exactive plus hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) with heat electrospray ionization (HESI) was employed. The mass conditions were set as follows: capillary temperature, 320 °C; auxiliary gas heater temperature, 200 °C; spray voltage, 4 kV/3.5 kV (positive/negative); Apex trigger, 2–6 s; Loop count, 5; S-lens RF level, 50 V. Full MS/dd-MS^2 scan mode conditions were set as follows: Scan range, 75–1125 *m*/*z*; Full MS resolution, 70,000; dd-MS^2 resolution, 17,500; Maximum injection time (IT), 50 ms; Isolation window, 1.0 *m*/*z*; Normalized collision energy (NCE), 20/40/60 eV; Automatic gain control (AGC) target, 1.0 × 10^5; Dynamic exclusion, 10 s. Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the HCD cell, and as the damping gas in the C-trap.

3.4. Data Processing and Analysis

Tune 2.9 (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the mass spectrometer, and Xcalibur 4.1 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the instrument for data acquisition and analysis. The mass tolerance of MS and MS^2 was within 5 ppm. The chemical formulas for all parent and fragment ions were calculated according to the exact mass, and the parameters are set as follows: C (0–60), H (0–120), O (0-60), and N (0–10).

4. Conclusions

The inherent variety of natural products in TCM has presented a big challenge in separation and detection techniques for the rapid characterization of its chemical profiling. In the present study, the chemical constituents of RS extract were determined by UHPLC-Q-Orbitrap HRMS. A total of 69 compounds, including 19 flavonoids, 22 organic acids, 6 coumarins, 4 lignans, 14 nitrogenous compounds, and 4 anthraquinones, were identified based on the comparison of their accurate masses, fragment ions, literature studies, and standard samples. Isoscopoletin, scopoletin, scoparone, aesculetin, quercetin, protocatechuic, salicylic acid, ferulic acid, luteolin, caffeic acid, emodin and oleanic acid were identified by standard samples. It is worth noting that flavonoids and lignans were identified for the first time in RS. This work can provide an essential chemical basis for quality control and further studies on the pharmacological and clinical application of RS.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27103299/s1. Figure S1: Product ion spectra of standard samples of isoscopoletin (A), scopoletin (B), and scoparone (C) in positive ionization mode. Figure S2: Product ion spectra of standard samples of protocatechuic acid (A), aesculetin (B), caffeic acid (C), ferulic acid (D), salicylic acid (E), luteolin (F), quercetin (G), emodin (H) and oleanic acid (I) in negative ionization mode. Figure S3: Chemical structures of 69 compounds identified in the 80% methanol extract of RS.

Author Contributions: Writing—original draft and formal analysis, S.C.; data curation, M.H.; formal analysis, L.Y. and F.C.; resources, M.L.; methodology, Z.S.; writing—review & editing, W.C.; supervision, Y.Z.; software, S.W.; project administration and writing—review & editing, Q.Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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