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[Xiaoqiong Xu](#)^{*}, [Xican Li](#)^{*}, Shaoman Chen , Yongbai Liang , Chuanyang Zhang , Yuhua Huang

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Article

Simultaneous Qualitative and Quantitative Analyses of 41 Constituents in *Uvaria macrophylla* Leaves Screen Antioxidant Quality-Markers Using Database-Affinity UHPLC-Q-Orbitrap-MS/MS

Xiaoqiong Xu ^{1,*}, Xican Li ^{2,*}, Shaoman Chen ², Yongbai Liang ², Chuanyang Zhang ² and Yuhan Huang ²

¹ College of Pharmacy, Gansu Medical University, Pingliang, 744000, China

² School of Chinese Herbal Medicine, Guangzhou University of Chinese Medicine, Guangzhou, 510006, China

* Correspondence: author: xq198498@163.com (X.L.); lixican@126.com (X.X.)

Abstract: No study has focused on *Uvaria macrophylla* leaves with various traditional efficiencies. This paper therefore applied a database-affinity ultra-high-performance liquid chromatography with quadrupole orbitrap tandem mass spectrometry (UHPLC-Q-Orbitrap-MS/MS) strategy, to analyze the lyophilized aqueous extract of *U. macrophylla* leaves. Through database comparison and MS fragment elucidation, the study has putatively identified 41 constituents belonging to flavonoid, phenolic acid, steroid, saccharide natural product classifications. Significantly, 4 groups of isomers (liquiritigenin *vs* isoliquiritigenin *vs* pinocembrin; oroxylin A *vs* wogonin *vs* galangin 3-methyl ether; isoquercitrin *vs* hyperoside; protocatechuic acid *vs* 2,5-dihydroxybenzoic acid) have been successfully distinguished from each other. All of 41 constituents were then subjected to a quantitative analysis based on linear regression equation established by the above UHPLC-Q-Orbitrap-MS/MS strategy, and an ABTS^{•+}-scavenging antioxidant assay. Finally, the chemical content was multiplied by the corresponding ABTS^{•+}-scavenging percentage to calculate the antioxidant contribution. It was shown that the chemical contents of 41 constituents varied from 0.003 ± 0.000 to 14.418 ± 1.041 mg/g, and gallic acid showed the highest antioxidant contribution. Gallic acid is considered as a suitable antioxidant quality-marker (Q-marker) of *U. macrophylla* leaves. These findings have scientific implications for resource development and quality-control of *U. macrophylla* leaves.

Keywords: *Uvaria littoralis* leaves; UHPLC-Exactive-Q-Orbitrap-MS/MS; quantitation; isomer differentiation

1. Introduction

Uvaria macrophylla Roxburgh (Figure 1A) is a shrub in the annonaceae family and also nominated as *Uvaria littoralis*. It is mainly distributed in low-altitude hilly regions of Eastern Asia, such as Chinese Hainan, Guangdong, and Guangxi provinces [1]. The plant mainly contains polyoxy-substituted, annonaceous acetogenins, flavonoids, alkaloids, and other constituents. In traditional Chinese medicine (TCM), its leaves (Figure 1B) are used [2] for the treatment of cancer, anemia, and inflammation [3,4].

Possibly owing to their traditional effects, three plant species, *U. macrophylla*, *U. microcarpa*, and *U. kurzii*, have attracted interests from pharmacists and chemists. However, a substantial proportion of studies focused on *U. microcarpa*. For example, in 2009, Yang has systematically studied the chemical constituents of different parts of *U. macrocarpa* [4,5]. Liu explored the chemical constituents of *U. microcarpa* leaves [2]. Lv obtained 19 chemical constituents from another species *U. kurzii*, by means of conventional phytochemical approaches [3].

By contrast with *U. microcarpa*, *U. macrophylla* has received less attention from chemists. To our knowledge, only Wang's team has focused on the constituents in recent two decades [6–8]. However, Wang's work only involved in the root and stem of *U. macrophylla*. Therefore, there is a limitations

regarding chemical studies of *U. macrophylla*, that is, no study has focused on the *U. macrophylla* leaves, which have also been documented to the resource of *Ziyupan* [9].



Figure 1. *Uvaria macrophylla* Roxb plant (aboveground, A) and its leaves (B). The inserted images are enlarged views (40 ×). All photos were taken by Xican Li in Guangzhou.

This study thereby attempted to analyze *U. macrophylla* leaves using a novel chemical strategy, i.e., database-affinity ultra-high-performance liquid chromatography with quadrupole orbitrap tandem mass spectrometry (UHPLC-Q-Orbitrap-MS/MS) strategy. The strategy has been proven to be highly reliable and effective, for it could completely elucidate MS fragments and strictly discriminate different isomers [10–12]. Meanwhile, this novel strategy was able to fulfill the quantification for these constituents [13]. Such simultaneous qualitative and quantitative analyses would bring about an in-depth understanding of *U. macrophylla* leaves, from the angle of chemistry. The in-depth chemical understanding is believed to lead to three scientific implications, i.e., clarification of substance basis of traditional herbal medicine *Ziyupan*, promotion of resource development of *U. macrophylla* plant, and establishment of quality-control method for *U. macrophylla* leaves.

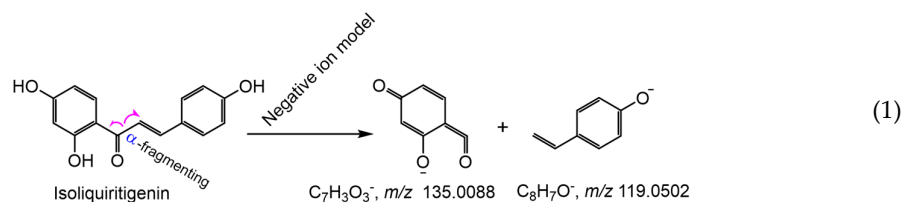
2. Results and Discussion

Sample solutions of *U. macrophylla* leaves were analyzed for constituent identification using UHPLC-Q-Orbitrap-MS/MS strategy. Constituent identification however was accomplished by comparison with authentic standards in the database and subsequent MS/MS fragment elucidation. The identification results were intuitively shown in the total ion current (TIC) diagram (Figure 2); while the structures of all identified constituents were detailed in Figure 3. The main information for constituent identification was listed in Table 1, including R.T. values, molecular ion peak, MS/MS fragments, observed m/z value, theoretical m/z value, and error between observed m/z value and theoretical m/z value.

In negative ion mode, the aqueous extract of *U. macrophylla* leaves was shown to enrich 36 constituents, most of which were flavonoids and phenolic acids. In positive ion mode, 5 constituents were identified as D-fructose (17), cholesteryl acetate (34), (+)-4-cholesten-3-one (35), betulin (36), and L-(-)-proline (41). All 41 constituents have been elucidated for their MS/MS spectra in Suppls. 1–41. For example, the MS elucidation spectra of tiliroside 11 were shown in Figure 4.

Significantly, the constituent identification has successfully discriminated 4 groups of isomers. The discrimination of isomers group *i* (1, 2, and 3) is representative of the process. As seen in Figure 5, when formula $C_{15}H_{11}O_4$ was extracted by Xcalibur in negative ion mode, the chromatograph was observed to show three peaks with m/z 255. Nevertheless, their MS/MS profiles were quite different from each other because the three isomers (1, 2, and 3) formed a group of hybridized isomers rather than simple positional isomers (or functional group isomers or steric isomers). Thus, they displayed

quite different fragmenting pathways, according to our previous study [14]. Isoliquiritigenin **2**, for example, underwent α -fragmentation to give two strong MS/MS peaks, m/z 135 and 119 (Eq. 1). Another two constituents liquiritigenin **1** and pinocembrin **3** did not give rise to these MS/MS peaks. Through comparison of these characteristic MS/MS peaks, as well as the different R.T. values in the database, the members in isomers group *i* were readily distinguished from each other. The identification processes and fragmentation process was detailed in Suppls. 1-3.



Similarly, three members (**4**, **5**, and **6**) in isomers group *ii* constructed positional isomerism and were differentiated by their different R.T. values (Figure 2A, and (Suppls. 4-6). Isomers group *iii* (**7** and **8**, Figure 3) however included two hybridized isomers. Isomers group *IV* (**23** and **24**, Figure 3) were positional isomerism too. Nonetheless, all these members have been completely differentiated by means of comparisons of their different R.T. values and MS/MS profiles. Their MS/MS fragments have been elucidated in Suppls. 4-8, 23-24. The success in differentiation of these isomers has implied that the database-aided UHPLC-Q-Orbitrap-MS/MS was a reliable strategy.

Using the reliable database-aided UHPLC-Q-Orbitrap-MS/MS strategy, the study has putatively identified 41 constituents in *U. macrophylla* leaves. Subsequently, the contents of all 41 identified constituents (**1-41**) were also quantitatively analyzed using the database-aided UHPLC-Q-Orbitrap-MS/MS strategy. As seen in Table 1, their chemical contents varied from 0.003 ± 0.000 to 14.418 ± 1.041 mg/g. One steroid cholesteryl acetate (**34**) displayed the highest chemical content while another steroid (+)-4-cholesten-3-one (**35**) exhibited the lowest chemical content.

From the angle of pharmacology, these constituents showed various beneficial effects, such as anti-tumor (e.g., pinocembrin), anti-inflammatory effect (e.g., wogonin), neuroprotection (e.g., gallic acid), cardiovascular protection (e.g., citric acid). Especially, five constituents are actually nutrients which can improve anemia [15], including sucrose, 2,5-dihydroxybenzoic acid, 4-hydroxybenzaldehyde, and L-(-)-proline. Nine constituents have been reported to be natural antioxidants [15], such as gallic acid and ellagic acid (Table 1). These beneficial effects are relevant to the aforementioned traditional efficiencies of *Ziyupan*. Nevertheless, there is not a certain bioactivity to effectively characterize the traditional efficiencies of *Ziyupan*. Thereby, the study had to comparatively evaluate their relative antioxidant abilities, because antioxidation plays an indispensable role in these traditional efficiencies and bioactivities [16–18].

To guarantee the comparability, all 41 constituents were individually analyzed by ABTS^{•+}-scavenging antioxidant assay at the same dose ($3 \mu\text{L} \times 0.5 \mu\text{g}/\mu\text{L}$). As seen in Figure 6A, these constituents showed different antioxidant levels in ABTS^{•+}-scavenging assay. Some constituents (e.g., **34-41**) exhibited negligible ABTS^{•+}-scavenging percentages; while 6 constituents (e.g., **18**, **22**, **24**, **29**, **32**, and **33**) exhibited 100% ABTS^{•+}-scavenging percentage. However, the six showed different chemical contents, as indicated in Table 1. Therefore, the study defined a new formula (Eq. 2), to calculate the antioxidant contribution and then to characterize the role of one antioxidant constituent in whole *U. macrophylla* leaves.

$$\text{Antioxidant contribution} = \text{relative contribution level} \times \text{chemical content} \quad (2)$$

In line with the calculation, gallic acid (**29**) possessed the highest antioxidant contribution value, which was also much higher than the sum of antioxidant contribution values of other constituents (Figure 6B). Therefore, gallic acid (**29**) was considered as the most antioxidant contributor in *U. macrophylla* leaves.

Herein the study used a novel database-aided UHPLC-Q-Orbitrap-MS/MS strategy, to simultaneously qualitatively and quantitatively analyze 41 constituents in *U. macrophylla* leaves. These constituents covered several natural product classifications, including flavonoid, phenolic acid,

steroid, and saccharide. Particularly, the qualitative analysis has successfully discriminated 10 isomeric constituents, to accomplish a putative identification of all 41 constituents. Thereafter, all 41 constituents were quantitatively analyzed using the above database-aided UHPLC-Q-Orbitrap-MS/MS strategy. Finally, these constituents were evaluated for the relative antioxidant level using ABTS^{•+}-scavenging assay, to calculate their individual antioxidant contribution. One phenolic acid gallic acid showed substantial chemical content and extremely high antioxidant contribution towards *U. macrophylla* leaves. Therefore, it was recommended as the antioxidant quality-marker (Q-marker) of *U. macrophylla* leaves. Apparently, all these in-depth chemical understandings have not only facilitated the resource development and quality-control of *U. macrophylla* leaves, but also helped to clarify the substance basis of traditional herbal medicine *Ziyupan*.

Table 1. The main experimental data and documental bioactivity of 41 constituents (1-41) in *U. macrophylla* leaves.

No.	RT min	Name	Molecular ion	Observed m/z value	Theoretical m/z value	Error (δ ppm)	Content (mg/g) n=3	Characteristic MS/MS fragment peak m/z	Bioactivity
1	10.56	liquiritigenin	C ₁₅ H ₁₁ O ₄ ⁻	255.0661	255.0663	0.7843	0.577±0.013	255.0659, 119.0492, 91.0178	anti-inflammatory [19]
2	11.50	isoliquiritigenin	C ₁₅ H ₁₁ O ₄ ⁻	255.0661	255.0663	0.7843	0.012±0.000	255.0661, 119.0492, 91.0178	antitumor [20]
3	12.20	pinocembrin	C ₁₅ H ₁₁ O ₄ ⁻	255.0662	255.0663	0.3921	0.072±0.000	255.0661, 213.0552, 151.0029	antitumor [21]
4	12.29	oroxylin A	C ₁₆ H ₁₁ O ₅ ⁻	283.0612	283.0612	0	0.048±0.002	283.0611, 268.0377	antioxidant [22]
5	12.35	wogonin	C ₁₆ H ₁₁ O ₅ ⁻	283.0611	283.0612	0.3533	0.026±0.001	283.0611, 163.0027, 268.0374 109.9998	anti-inflammatory [23]
6	12.52	galangin 3-methyl ether	C ₁₆ H ₁₁ O ₅ ⁻	283.0612	283.0612	0	0.012±0.000	239.0346, 211.0395, 167.0494	antibiotic [24]
7	9.54	isoquercitrin	C ₂₁ H ₁₉ O ₁₂ ⁻	463.0873	463.0882	1.9438	0.017±0.002	463.0874, 300.0273, 271.0247 255.0298	anti-inflammatory [25]
8	9.43	hyperoside	C ₂₁ H ₁₉ O ₁₂ ⁻	463.0875	463.0882	1.5118	0.025±0.002	463.0874, 300.0273, 271.0247	anti-inflammatory [26]
9	9.57	quercetin 3-O-β-D-glucuronide	C ₂₁ H ₁₇ O ₁₃ ⁻	477.0674	477.0675	0.2096	0.138±0.005	301.0353, 151.0028, 109.0284	antimicrobial [27]
10	9.93	phloridzin	C ₂₁ H ₂₃ O ₁₀ ⁻	435.1280	435.1297	3.9080	0.034±0.001	273.0770, 167.0341, 123.0442, 119.0492	antioxidant [28,29]
11	10.96	tiliroside	C ₃₀ H ₂₅ O ₁₃ ⁻	593.1298	593.1301	0.5059	0.542±0.007	285.0397, 255.0292, 227.0341	antioxidant [30]
12	11.36	kaempferol	C ₁₅ H ₉ O ₆ ⁻	285.0405	285.0405	0	0.033±0.000	285.0405, 117.0335, 93.0334	anti-inflammatory [31]
13	11.93	7-hydroxyflavone	C ₁₅ H ₉ O ₃ ⁻	237.0553	237.0557	1.6877	0.027±0.000	237.0553, 208.0524, 91.0178	antibacterial [32]
14	12.51	chrysin	C ₁₅ H ₉ O ₄ ⁻	253.0504	253.0506	0.7905	0.267±0.002	251.0500, 209.0598, 143.0491	antitumor [33]

15	12.67	galangin	C₁₅H₉O 5⁻	269.0 456	269.04 55	0.3 717	0.247± 0.001	269.0456, 169.0650	anti-inflammatory [34]
16	9.86	myricetin	C₁₅H₉O 8⁻	317.0 301	317.03 03	0.6 309	0.036± 0.005	317.0301, 151.0028, 137.0234, 109.0284	antioxidant [35]
17	13.82	D-fructose	C₆H₁₃O 6⁺	181.0 715	181.07 07	4.4 198	0.247± 0.000	163.0385, 149.0229, 65.0393	antioxidant [36]
18	9.46	1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose	C₄₁H₃₁O₂₆⁻	939.1 146	939.11 09	3.9 403	0.547± 0.700	169.0134, 125.0234, 107.0127, 95.0126	antioxidant [37]
19	0.53	sucrose	C₁₂H₂₁O₁₁⁻	341.1 086	341.10 89	0.8 797	0.986± 0.048	341.1086, 179.0553, 161.0448, 89.0233	sweetening agent [38]
20	0.55	quinic acid	C₇H₁₁O 6⁻	191.0 555	191.05 61	3.1 413	0.377± 0.003	191.0555, 93.0335, 85.0283	antioxidant [39]
21	8.18	salicylic acid	C₇H₅O₃⁻	137.0 234	137.02 44	7.2 992	0.056± 0.001	137.0234, 93.0333	anti-inflammatory [40,41]
22	3.06	methyl gallate	C₈H₇O₅⁻	183.0 291	183.02 99	4.3 715	0.007± 0.000	183.0291, 124.0156, 78.0099	anti-inflammatory [42]
23	1.6	protocatechuic acid	C₇H₅O₄⁻	153.0 186	153.01 93	4.5 751	0.026± 0.006	153.0186, 109.0285, 108.0206	antimicrobial [43]
24	2	2,5-dihydroxybenzoic acid	C₇H₅O₄⁻	153.0 186	153.01 93	4.5 751	0.015± 0.003	153.0182, 109.0284, 108.0206	improvements in vascular function [44]
25	3.22	4-hydroxybenzaldehyde	C₇H₅O₂⁻	121.0 285	121.02 95	8.2 644	0.006± 0.001	121.0285, 92.0257	improvements in vascular function [45,46]
26	4.6	caffeic acid	C₉H₇O₄⁻	179.0 339	179.03 50	6.1 452	0.012± 0.000	179.0345, 136.0474, 135.0441, 133.0282	antibacterial [47]
27	7.11	cis-4-Hydroxycinnamic acid	C₉H₇O₃⁻	163.0 392	163.04 01	5.5 214	0.094± 0.005	119.0492	anti-SARS [48]
28	8.71	ferulic acid	C₁₀H₉O 4⁻	193.0 496	193.05 06	5.1 813	0.258± 0.301	193.0136, 178.0262, 134.0364	antibacterial [49,50]
29	0.86	gallic acid	C₇H₅O₅⁻	169.0 133	169.01 42	5.3 254	4.800± 0.103	169.0133, 125.0234	antioxidant [51]
30	9.5	ellagic acid	C₁₄H₅O 8⁻	300.9 989	300.99 90	0.3 333	0.485± 0.000	300.9990, 145.0286, 117.0335	antioxidant [52]
31	0.52	citric acid	C₆H₇O₇⁻	191.0 189	191.01 97	4.1 884	0.757± 0.007	111.0078, 87.0076, 85.0248	improvements in vascular function [53]
32	7.98	gallocatechin gallate	C₂₂H₁₇O₁₁⁻	457.0 736	457.07 76	8.7 527	0.274± 0.004	169.0133, 125.0234	antiviral [54]

33	8.68	epicatechin gallate	C ₂₂ H ₁₇	441.0	441.08	0.9	0.020±	289.0715, 169.0134,	antiox
			O ₁₀ ⁺	831	27	070	0.000	125.0234, 109.0285	ident [55]
34	16.03	cholesteryl acetate	C ₂₉ H ₄₉	429.3	429.37	4.8	14.418	429.3706, 165.0912,	antitu
			O ₂ ⁺	706	27	951	±1.041	91.0545, 81.0705,	mor [56]
35	16.67	(+) -4-cholesten-3- one	C ₂₇ H ₄₅	385.3	385.34	1.5	0.003±	385.3454, 109.0649,	antitu
			O ⁺	459	65	584	0.000	97.0650, 91.0545	mor [57]
36	15.38	betulin	C ₃₀ H ₅₁	443.3	443.38	1.3	0.130±	443.3497, 105.0700,	antitu
			O ₂ ⁺	878	84	544	0.005	91.0547, 81.0705	mor [58]
37	15.18	oleanolic acid	C ₃₀ H ₄₇	455.3	455.35	0	0.026±	455.3531	antitu
			O ₃ ⁺	531	31	0	0.004		mor [59]
38	16.22	ethyl stearate	C ₂₀ H ₃₉	311.2	311.29	0	0.209±	311.1682, 183.0114,	antiox
			O ₂ ⁺	956	56	0	0.013	119.0492	ident [60]
39	15.62	oleic acid	C ₁₈ H ₃₃	281.2	281.24	0.3	0.046±	281.2487	antioxid
			O ₂ ⁺	487	86	558	0.000		ant [61]
40	15.8	stearic acid	C ₁₈ H ₃₅	283.2	283.26	0	0.197±	283.2643, 92.1626	antioxid
			O ₂ ⁺	643	43	0	0.002		ant [62]
41	0.54	L-(-)-proline	C ₅ H ₁₀ N	116.0	116.07	1.7	0.940±	116.0706, 70.0657	immune
			O ₂ ⁺	708	06	241	0.045		modulation [63]

Note: *m/z* values below 50 were also found by the Xcalibur 4.1 software package, despite the scan mode range of *m/z* 100-1500. *m/z* values in square brackets are the molecular ion peaks. Nominal level (NL) values were obtained by the Xcalibur 4.1 software package. Five compounds (17, 34, 35, 36, and 41) annotated with “+” under *Molecular ion* were identified in positive ion mode. All other compounds were identified in negative ion mode.

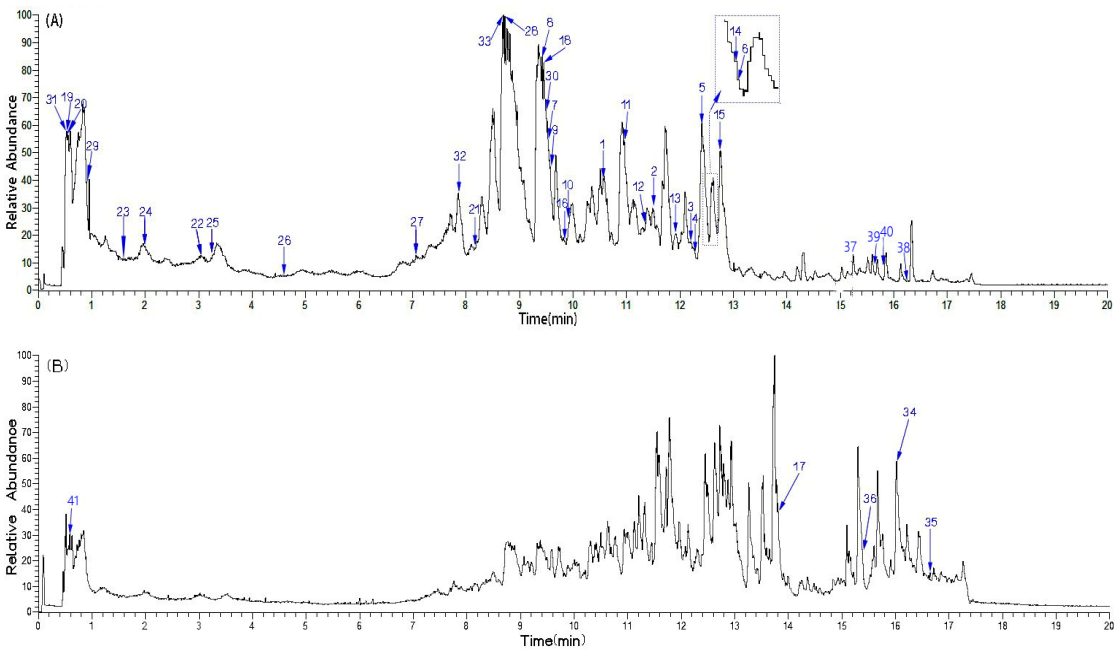


Figure 2. Total ion current (TIC) chromatograms of *U. macrophylla* leaves in the UHPLC-Q-Orbitrap-MS/MS analysis: (A) negative ion mode; (B) positive ion mode.

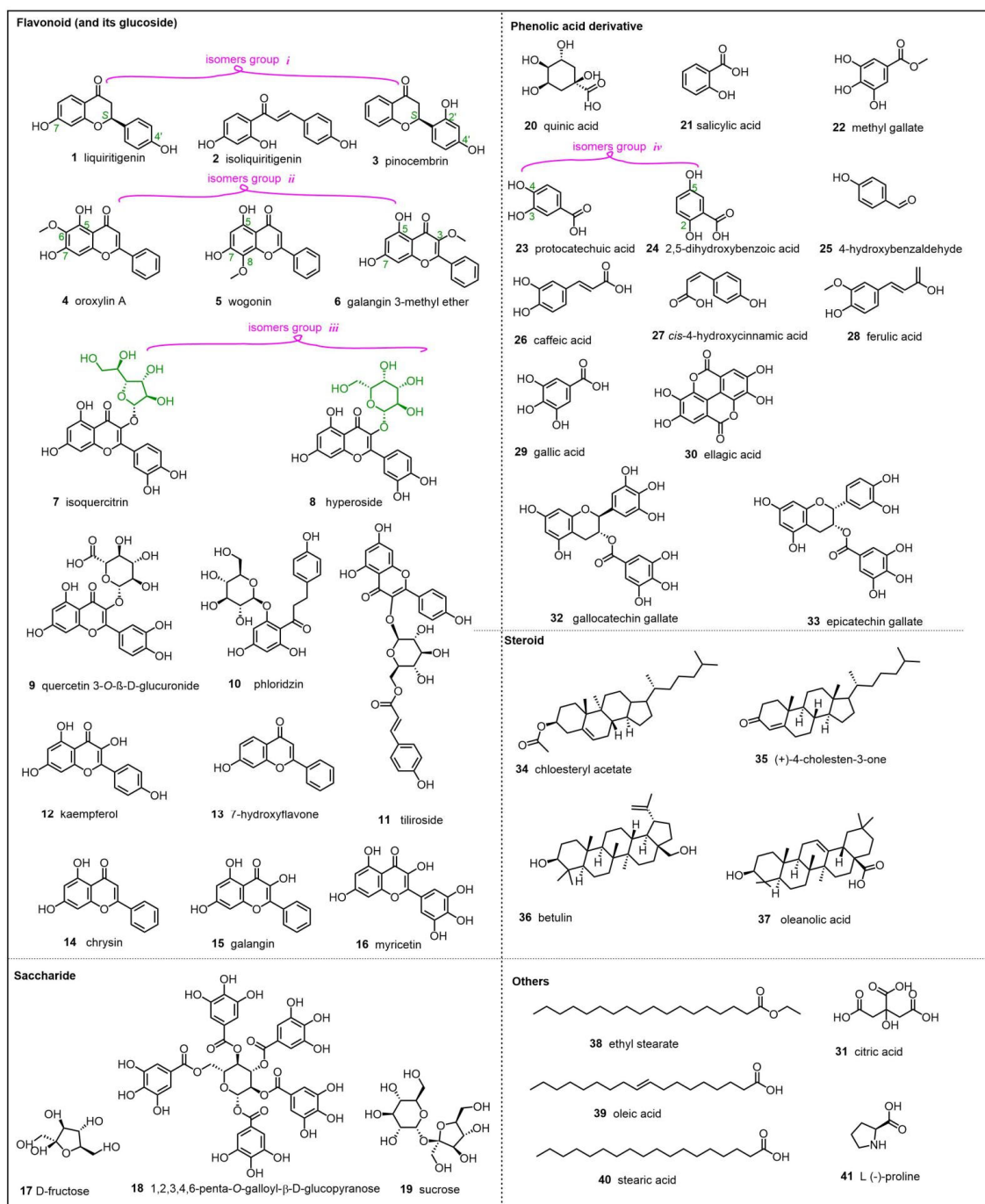


Figure 3. Structures and configurations of 41 identified constituents (1-41).

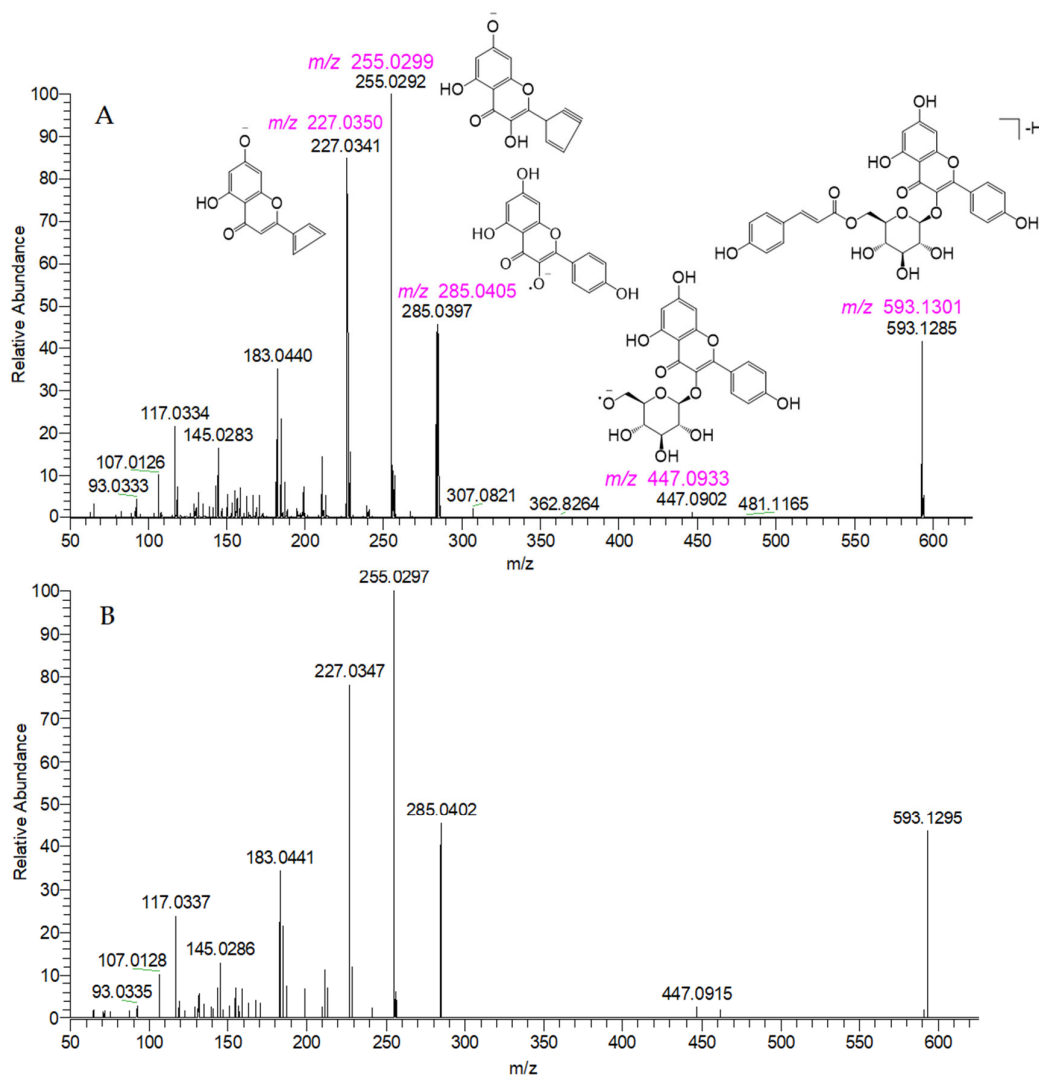


Figure 4. The typical MS fragments of tiliroside: (A) for an authentic standard of tiliroside; and (B) for tiliroside in *U. macrophylla* leaves sample solution (R.T. 10.96 min). The m/z values in purple are calculated based on the relative atomic masses of C (12.0000), H (1.007825), and O (15.994915).

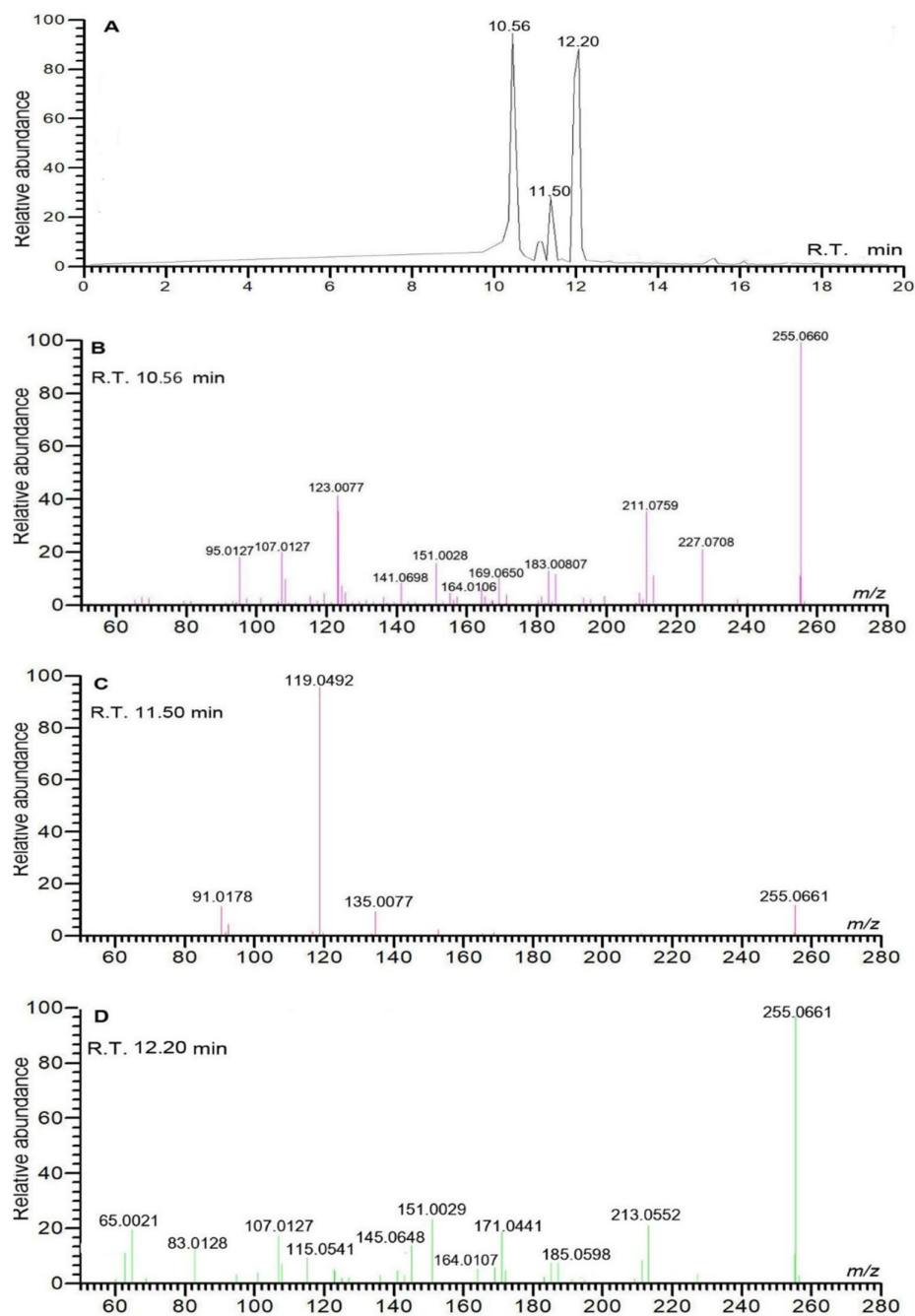


Figure 5. The discrimination of three isomers (liquiritigenin, isoliquiritigenin, and pinocembrin): (A) chromatogram for $C_{15}H_{11}O_4$ - extracted by Xcalibur in negative mode; (B) MS/MS of peak at 10.56 min; (C) MS/MS of peak at 11.50 min; and (D) MS/MS of peak at 12.20 min.

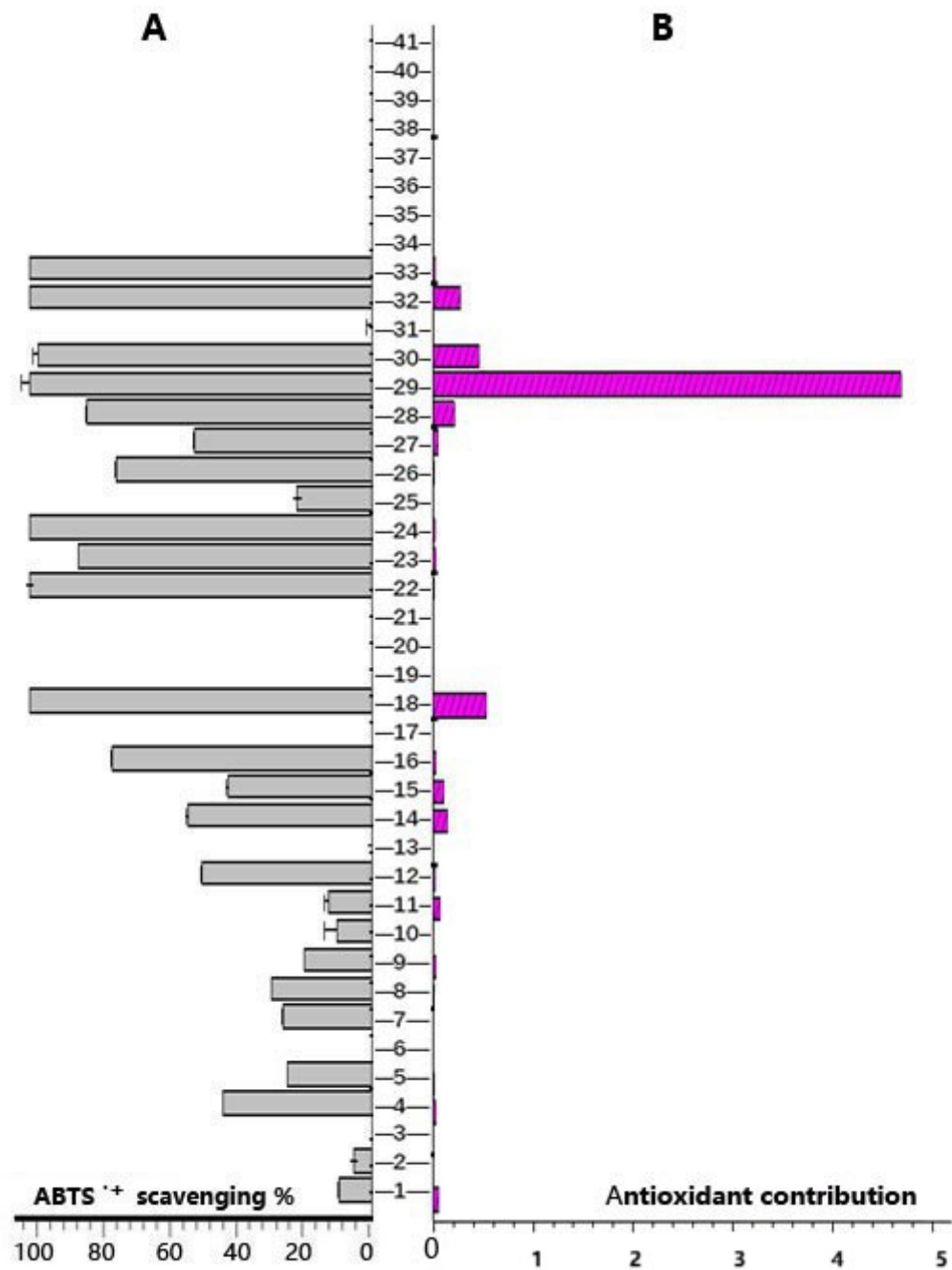


Figure 6. The relative antioxidant levels of 41 components (1-41, A) and corresponding antioxidant contributions (B). The relative antioxidant level was evaluated using ABTS^{•+}-scavenging assay.

3. Materials and Methods

3.1. Plant Materials and Chemicals

The *U. macrophylla* leaves (10 g) were clipped at Yaowang Mountain, Guangzhou University of Chinese Medicine (23.039° N, 113.388° E, altitude 20 m, Guangzhou, China) on July 12, 2023. Samples were identified as genuine by Professor Wang Xifang of Shaanxi University of Chinese Medicine, were dried at low temperature, were placed into a self-sealing bag and labeled as "*Uvaria macrophylla*

Roxburgh", and were refrigerated (2-8 °C). The methanol and water used for the preparation and analysis of the sample solution were of MS purity (Merck Sigma-Aldrich, Shanghai, China).

3.2. Authentic Standards

Liquiritigenin (CAS 578-86-9, C₁₅H₁₂O₄, MW 256.25, 98%), isoliquiritigenin (CAS 961-29-5, C₁₅H₁₂O₄, MW 256.25, 98%), pinocembrin (CAS 480-39-7, C₁₅H₁₂O₄, MW 256.25, 98%), oroxylin A (CAS 480-11-5, C₁₆H₁₂O₅, MW 284.26, 98%), wogonin (CAS 632-85-9, C₁₆H₁₂O₅, MW 284.26, 98%), galangin 3-methyl ether (CAS 6665-74-3, C₁₆H₁₂O₅, MW 284.26, 98%), isoquercitrin (CAS 21637-25-2, C₂₁H₂₀O₁₂, MW 464.38, 98%), and hyperoside (CAS 482-36-0, C₂₁H₂₀O₁₂, MW 464.38, 98%) were obtained from Shanghai Maclin Biochemical Technology Co., Ltd. Quercetin 3-O-β-D-glucuronide (CAS 22688-79-5, C₂₁H₁₈O₁₃, MW 478.36, 98%), phloridzin (CAS 60-81-1, C₂₁H₂₄O₁₀, MW 436.13, 98%), tiliroside (CAS 20316-62-5, C₃₀H₂₆O₁₃, MW 594.52, 98%), kaempferol (CAS 520-18-3, C₁₅H₁₀O₆, MW 286.24, 98%), 7-hydroxyflavone (CAS 6665-86-7, C₁₅H₁₀O₃, MW 238.24, 98%), chrysin (CAS 480-40-0, C₁₅H₁₀O₄, MW 254.24, 99%), galangin (CAS 548-83-4, C₁₅H₁₀O₅, MW 270.24, 98%), myricetin (CAS 529-44-2, C₁₅H₁₀O₈, MW 318.24, 98%), D-fructose (CAS 57-48-7, C₆H₁₂O₆, MW 180.16, 98%), and 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose (CAS 14937-32-7, C₄₁H₃₂O₂₆, MW 940.68, 98%) were obtained from Merck-Sigma Co., Ltd. (Shanghai, China). Sucrose (CAS 57-50-1, C₁₂H₂₂O₁₁, MW 342.3, 99.5%), quinic acid (CAS 77-95-2, C₇H₁₂O₆, MW 192.17, 98%), salicylic acid (CAS 69-72-7, C₇H₆O₃, MW 138.12, 99.5%), methyl gallate (CAS 99-24-1, C₈H₈O₅, MW 184.15, 99%), protocatechuic acid (CAS 99-50-3, C₇H₆O₄, MW 154.12, 99%), 2,5-dihydroxybenzoic acid (CAS 490-79-9, C₇H₆O₄, MW 154.12, 99%), 4-hydroxybenzaldehyde (CAS 123-08-0, C₇H₆O₂, MW 122.12, 98%), and caffeic acid (CAS 331-39-5, C₉H₈O₄, MW 180.16, 99%) were purchased from Sichuan Weikeyi Biological Technology Co., Ltd. (Chengdu, China). Cis-4-Hydroxycinnamic acid (CAS 4501-31-9, C₉H₈O₃, MW 164.16, 99%), ferulic acid (CAS 1135-24-6, C₁₀H₁₀O₄, MW 194.18, 98%), gallic acid (CAS 149-91-7, C₇H₆O₅, MW 170.12, 98%), ellagic acid (CAS 476-66-4, C₁₄H₆O₈, MW 302.19, 98%), citric acid (CAS 77-92-9, C₆H₈O₇, MW 192.12, 99.5%), gallic acid (CAS 149-91-7, C₇H₆O₅, MW 170.12, 98%), epicatechin gallate (CAS 1257-08-5, C₂₂H₁₈O₁₀, MW 442.37, 99%), cholesteryl acetate (CAS 604-35-3, C₂₉H₄₈O₂, MW 428.69, 98%), (+)-4-cholesten-3-one (CAS 601-57-0, C₂₇H₄₄O, MW 384.64, 98%), betulin (CAS 473-98-3, C₃₀H₅₀O₂, MW 442.72, 98%), oleanolic acid (CAS 508-02-1, C₃₀H₄₈O₃, MW 456.7, 98%), ethyl stearate (CAS 111-61-5, C₂₀H₄₀O₂, MW 312.53, 98%), oleic acid (CAS 112-80-1, C₁₈H₃₄O₂, MW 282.46, 98%), stearic acid (CAS 57-11-4, C₁₈H₃₆O₂, MW 284.48, 98%), and L-(-)-proline (CAS 147-85-3, C₅H₉NO₂, MW 115.13, 98%) were obtained from Shanghai Yuanye Biotechnology Co., Ltd.

3.3. Preparation of Sample and Authentic Standard Solutions

3.3.1. Preparation of Lyophilized Aqueous Extract and Sample Solutions

The extraction process is depicted in Figure 7. To avoid possible insoluble impurities and solvent effects [64], 10.0 g *U. macrophylla* leaves powder was accurately weighed and placed in a round-bottomed flask with a stopper. After soaking for 5 minutes in 50 mL water, boiling extraction was carried out twice, the first time for 2 h, the second for 1 h. After cooling and filtering, the filtrates were lyophilized according to a previous report [65], to yield 0.3 g brown lyophilized powder. The powder was re-dissolved using 80% methanol at 30 mg/mL concentration. The methanolic solution was filtered using a 0.45 μm organic filter membrane to afford the sample solution which was stored in the refrigerator at 2–8 °C for further analysis.

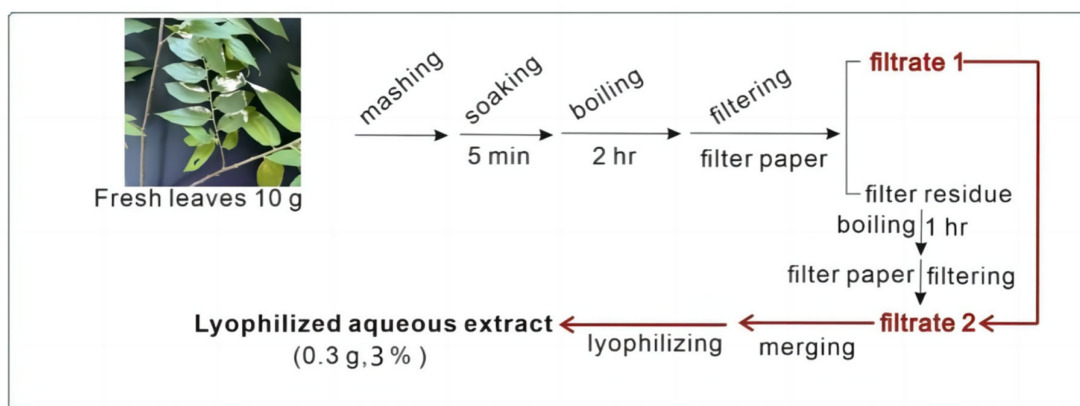


Figure 7 Flow chart for preparation of lyophilized aqueous extract of *U. macrophylla* leaves

3.3.2. Preparation of Authentic Standard Solutions

All authentic standards listed in Section 2.2 were dissolved in methanol at 30 µg/mL concentration and filtered through a 0.45 µm membrane. Filtrates were stored at 2–8 °C for further analysis [66].

3.4. Simultaneous Qualitative and Quantitative Analyses Using Database-Affinity UHPLC-Q-Orbitrap-MS/MS

The main operations of injection, data acquisition, and analysis were controlled by the Xcalibur 4.1 package (Thermo Fisher Scientific Inc., Waltham, MA, USA) comprising TraceFinder, mzVault, and FreeStyle viewing softwares with the UHPLC-Q-Exactive Orbitrap-MS apparatus. Before data acquisition, the background sign was zeroed using blank solvent. The acquired data were then exported to TraceFinder for *m/z* extraction and to afford the corresponding MS spectra [67]. The main parameters were set as follows: mass range 100–1500 Da; mass tolerance 10 ppm; S/N threshold 5; and isotopic pattern fit threshold 90%. The MS spectra and corresponding top 5 secondary spectra were screened using Xcalibur 4.1. By comparing 4 parameters with authentic standards (retention time, molecular ion peak, MS/MS profile, and characteristic fragments), the constituents were preliminarily nominated by TraceFinder and were putatively identified manually [15].

Quantitative analysis of the 41 identified constituents was carried out according to a published method with minor modifications [68]. In brief, linear regression equations were first established using authentic standard solutions. TraceFinder and Xcalibur 4.1 software offered peak area parameters for these authentic standard solutions. *U. macrophylla* leaves sample solutions were subsequently analyzed under the same chromatography and MS spectra conditions. In line with the linear regression equations and the peak areas of the identified constituents, the contents of the *U. macrophylla* leaves constituents were quantified and expressed as mean ± SD (standard deviation).

3.5. Relative Antioxidant Level Evaluation Experiment

Relative antioxidant level was evaluated using ABTS^{••}-scavenging assay [69], with some modifications. ABTS^{••} was produced by mixing 700 µL (NH₄)₂ABTS aqueous solution (7.4 mmol/L) with 700 µL of K₂S₂O₈ aqueous solution (2.6 mmol/L). The mixture was incubated in the dark at room for 12 h. Thereafter, the incubated mixture was diluted with methanol (at a ratio of approximately 1: 15) so that its absorbance at 734 nm was measured to be 0.65 ± 0.01 using a microplate reader (Multiskan FC, Thermo Scientific, Shanghai, China). Then 3 µL sample solution (0.5 µg/µL) was added to 17 µL of methanol and treated with 80 µL of ABTS^{••} reagent to determine the scavenging activity. The absorbance at 734 nm was measured using the above microplate reader after initial mixing. After a 6-minute incubation, the absorbance at 734 nm was measured. The ABTS^{••}-scavenging percentage was calculated as Eq. 3.

$$\text{Scavenging \%} = \frac{A_0 - A}{A_0} \times 100\% \quad (3)$$

where A_0 was the absorbance at 734 nm of the control (reaction system without sample) and A is the absorbance at 734 nm of the reaction mixture with the sample. Methanol was used as the blank group.

3.6. Statistical Analysis

Each quantitative analysis experiment and each ABTS^{•+}-scavenging assay experiment were performed in triplicate. The data are shown as the mean $\pm$ SD from three independent measurements. Correlation coefficients (R values) were calculated by linear analysis using Origin 6.0 professional software (Origin-Lab Corporation, Northampton, MA, USA).

4. Conclusions

U. macrophylla leaves contain at least 41 constituents (including 10 isomers). These constituents belong to several natural product classifications, such as flavonoid, phenolic acid, steroid, and saccharide. They show different chemical contents and antioxidant contributions. Gallic acid has the highest antioxidant contribution and is applicable to act as the antioxidant Q-marker of *U. macrophylla* leaves.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Suppls. S1–S41: UHPLC-Q-Orbitrap MS spectra and identification of 1–41.

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