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Article

Evaluation of the Antioxidant Potential of *Citrus medica* from Different Geographical Regions and Characterization of Phenolic Constituents by LC−MS

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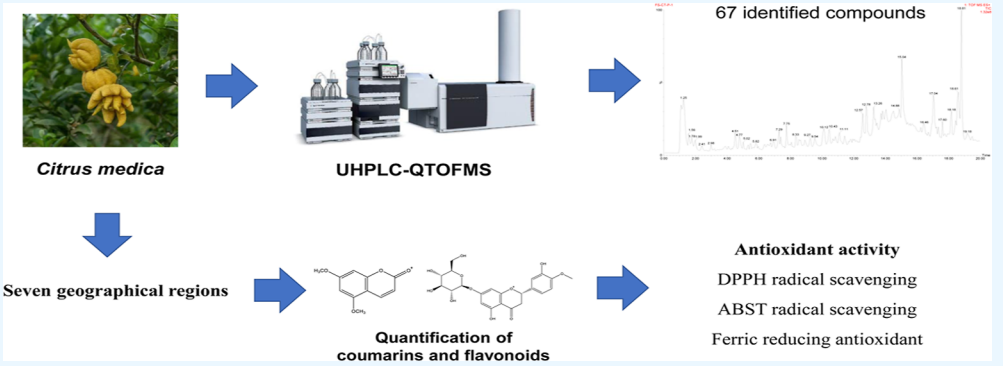

ABSTRACT: The varying antioxidant potential of *Citrus medica* associated with different geographical regions makes the evaluation of *C. medica* for natural antioxidants essential. This work aimed to compare the antioxidant potential of the phenolic constituents from different geographical regions. The chemical compositions were characterized by ultra-high-performance liquid chromatography (UPLC) coupled with mass spectrometry (MS). A total of 67 compounds including 29 coumarin derivatives and 38 flavonoids were tentatively identified by UPLC−quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). To evaluate the quality of *C. medica* from seven different geographical regions, water and 80% methanol fractions were subjected to quantitative analysis. Antioxidant potentials were determined by 2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), iron chelation, and reduction methods. The samples collected from Sichuan province showed the highest content of total phenolic compounds. Combined with antioxidant results, the sample from Sichuan province presented good antioxidant activity. This study also showed that total phenolic compounds significantly contributed to the antioxidant activities (2,2-azinobis(3-ethyl-benzothiazoline-6-sulphonic acid) and radical scavenging activity) of *C. medica* samples ($p < 0.01$). These results provided chemical information and potential antioxidant value for further research, providing ideal evidence for the quality evaluation and exploitation of the source.

1. INTRODUCTION

CitriSarcodactylis Fructus (Foshou in Chinese) has been prominent in treating liver conditions, harmonizing stomach, and expelling phlegm in traditional Chinese medicine system. One of its origins is the fruits of *Citrus medica* L. var. sarcodatylis Swingle.[1] *C. medica* L. var sarcodatylis belonging to Rutaceae family, widely known as the bergamot orange or bergamot, is an evergreen shrub or a small tree originated in India but now is spread across the globe.[2] In addition to its utility in the traditional Chinese medicine system, a wealth of modern pharmacological investigations verified its effectiveness in the treatment of tracheitis, hypertension, respiratory tract infections, angiocardiopathy, and asthma.[3] The major compounds in *C. medica* L. var sarcodatylis include flavonoids; coumarins; alkaloids; volatile oils; polysaccharides;[4] and other active components responsible for various pharmacological activities such as immunostimulatory,[5] nerve regeneration,[6] anti-inflammatory effect,[7] antioxidant activity,[8] anti-biofilm activity,[9] and so forth. Phenolic compounds such as flavonoids, phenolic acids, coumarins, and tannins are common antioxidants, and they can make cells more resistant to oxidative stress. The typical

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phenolic compound in *C. medica* L. var sarcodatylis is flavonoids—used for treating hyperlipidemia and atherosclerosis, scavenging reactive oxygen species, and improving insulin resistance by increasing the glucose consumption[4]—which include hesperidin, scoparone, scopoletin, 5,7-dimethoxycoumarin, and chrysoeriol-7-*O*-glucoside. For example, Pla-Pagà and his colleagues performed a metabolomic analysis suggesting that hesperidin potentially showed a beneficial cardiovascular effect by impacting the serum metabolomic profile based on the levels of urine and plasma metabolite, hesperidin 7-*O*-β-D-glucuronide, obtained from pre- and stage-1 hypertension patients who received 500 mL hesperidin-containing orange juice per day. The results showed blood pressure reduction and anti-inflammation.[10] Scoparone shows a PPARγ antagonic effect in 3T3-L1 preadipocyte differentiation suppression as researchers at University of Science and Technology in Korea shift their attention to obesity, using real time-PCR analysis which indicated down-regulated adipogenic genes.[11]

Problems with the chemical profile of Traditional Chinese medicines threaten to create a chasm between the quality assessment of herbal medicine and their clinical efficacy. Geographic factors have caused a fluctuation in the chemical constituents in plants. In the study associated with the geographic discrimination of *Lycium barbarum* Berry, Cossignani and his colleagues indicated that the Chinese region is a rich environment for Goji lipids, while Italian and Mongolian samples was ideal for phytosterols.[12] To understand the chemical components that are basis for quality control and assessment of herbal medicines, Salem and his colleagues collected *Zingiber officinale* from four different countries (including China, India, Pakistan, and Peru) and established an mass spectrometry (MS)-based metabolomic method to screen specific ingredients that have been important in the causing regional discrimination.[13] As they reported last year, the geographical origin influenced primary and secondary metabolite abundances, especially those of gingerols, amino acids, and organic acids.[13] *C. medica* L. var. sarcodatylis Swingle (or Foshou) has thrived in the south of China, including the Zhejiang, Fujian, Guangdong, Guangxi, Sichuan, and Yunnan provinces.[2] Southern China is especially favorable for the development of plant diversity because of complex terrain and changing climate. Thus, geographic origin has paramount importance and influence in the quality control and assessment of *C. medica* L. var. sarcodatylis. It is still not clear whether the geographic factors cause heterogeneity in phenolic compounds in *C. medica* L. var. sarcodatylis, despite several laboratories isolating coumarins or flavonoids from this species for evaluating their bioactivity.[14−16]

Methods that can reflect the molecular network and accurately quantify target ingredients in herbal medicinal plants are indispensable to their quality control and assessment. As researchers did in response to the purpose intended to discover therapeutic medicines, MS-based platforms are likely profiling phenolic components in samples to avoid inaccurate estimation challenges.[17] Ten compounds had been found for the first time as compositions of *Pistacia lentiscus* leaves among 46 main secondary metabolites obtained from an alcoholic extract based on liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), and Pacifico and his colleagues evaluated the antioxidant potential of the extract, demonstrating it to exert a remarkable cytoprotective response in oxidant-injury SK-N-BE(2)-C cell model.[18] According to a recent study, the significant antioxidant capacity of *Capparis spinosa* was found to be in fresh buds—showing higher phenolic amount determined through liquid chromatograohy-diode array detector coupled with tandem mass spectrometry (LC-DAD-ESI-MS/MS), characterized by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS parameters in principal component analysis—rather than berries.[19] Many studies ignored phenolic constitutes in *C. medica* L. var. sarcodatylis, preferring to focus on the crucial biological activities of essential oils and polysaccharides,[20−23] which is essential for the finger citrus processing industry. Given the significant geographic discrepancy between the planting regions, we are interested in the environment-driven variation of the chemical properties in *C. medica* L. var. sarcodatylis and the antioxidant activity of phenolic ingredients.

In the present work, the quality assessment of *C. medica* L. var. sarcodatylis samples collected from seven geographic regions of southern China was carried out using ultra-high-performance liquid chromatography (UHPLC) coupled with MS technique. The variations in total phenolic compounds, total flavonoids, and nine ingredients were also determined. Antioxidant activity analysis was employed to compare the antioxidant activity among the samples from different origins.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials. There were nine reference standards (including hesperidin, scopoletin, 5,6 dimethoxycoumarin, 5,7-dimethoxycoumarin, ferulic acid, diosmin, and vicenin), which were purchased from Shanghai Yuan Ye Biotechnology Co., Ltd. (Shanghai, China). Their purity was above 98% as tested using HPLC. Their identities were confirmed by NMR spectroscopic methods and HR ESI-MS in a separate publication. Methanol (MeOH) was bought from Chengdu Kelon chemical reagent factory (Chengdu, China). The MS grade solvents including acetonitrile (ACN) and formic acid (FA) were purchased from Sigma (St. Louis, MO, USA).

The plant materials were collected from seven different geographical regions in China and authenticated by Prof. Yingjiao Zhang. Fresh materials were dried by the following method. After air-drying, the sample was dried at −60 °C at a vacuum of 0.1 kPa using an FD-1D-50 freeze-dryer (Beijing Bilang Experimental Equipment Co., Ltd., Beijing, China). The dried slices were milled into fine powder and stored at −18 °C for the preparation of tests.

2.2. Sample Preparation. Sample powder (1 g) was taken to be homogenized with 80% methanol at a ratio of 1:25 (w/v) or water. Then, the mixture was extracted at 40 °C for 1 h with ultrasound assistance. The mixture was centrifuged at 6000 rpm for 15 min. All sample solutions were kept in −20 °C and filtered through 0.22 μm nylon membrane filters before injection into the UPLC-Q-TOF-MS system.

2.3. Qualitative Analysis. Characterization analyses were carried out using UHPLC system (Waters Corporation; Milford, MA, USA) with a PDA detector coupled to Waters Xevo G2-XS QTOF micro-mass spectrometer (Waters; Manchester, UK) fitted with an ESI source acting in negative and positive modes. The separation of the compounds was carried out on a Waters BEH C18 column (2.1 mm × 100 mm, 1.7 μm) operated at 30 °C. The mobile phase, which consists of 0.1% formic acid in water (A) and acetonitrile (B) as mobile phase, was delivered at a flow rate of 0.3 mL/min under a gradient program. The gradient system was 0−5 min, 5−10% B, 5−13 min, 10−20% B, 13−18 min, 20−40% B, 18−20 min, 40%−70%

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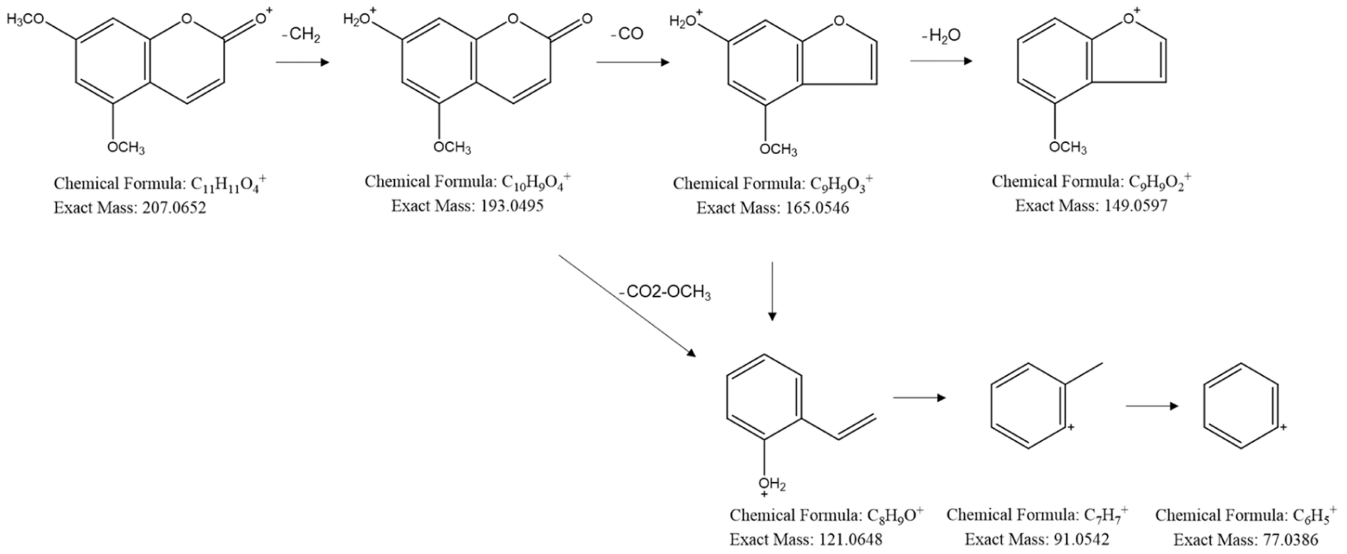

Figure 1. Fragmentation pattern of 5,7-dimethoxycoumarin.

B, 20−21 min 70−100% B, and 21−24 min, 100% B. The sample injection volume was 1 μL.

The mass spectrometer was operated in positive ESI mode, and mass spectra were recorded by scanning the mass range of m/z 100−1000 in both MS and MS/MS modes. The MS parameters were as follows: source temperature of 120 °C, desolvation temperature of 250 °C, cone gas flow of 50 L/h, desolvation gas flow of 600 L/h, source capillary voltage of 3.0 kV. For the MS/MS analysis, collision energy was set at 10 and 20 eV. The accurate mass data of the molecular ions were processed through the Mass Hunter version B 04.00 (Agilent Technology Santa Clara, CA, USA).

2.4. Quantitative Analysis. Quantitative analyses were performed using the Agilent 1290 UHPLC-DAD system equipped with a diode array detector (DAD). Using the following UHPLC condition, the separation of the compounds was carried out on a Poroshell 120 PFP column (4.6 × 100 mm, 2.7 μm particle size, Agilent, Santa Clara, CA, USA) operated at 30 °C. The mobile phase, which consists of 0.1% formic acid in water (A) and acetonitrile (B), was delivered at a flow rate of 0.3 mL/min under a gradient program. The gradient system was 0−10 min, 5−12% B, 10−20 min, 12−20% B, 20−36 min, 20−40% B, 36−37 min, 40%−45% B, 37−38 min, 45%−95% B, and 38−40 min, 95% B. The sample injection volume was 5 μL. The DAD was set to monitor at 280 nm (for hesperidin), 320 nm (for ferulic acid, scopoletin, 5,7-dimethoxycoumarin, and 6,7-dimethoxycoumarin), and 350 nm (for vicenin and diosmin). The purity of the standard that was larger than 98% calculated by NMR spectrum was dissolved in 80% of methanol (vol/vol) at a concentration of 1.5 mg/mL. Then, the stock solution was diluted to make a series of working standard solutions at appropriate concentrations in order to construct the calibration curves. Linear regression analysis was conducted by plotting the peak area against the concentrations.

The content of total flavonoids was determined using UV spectrophotometry. The curves implied good linearity, and the correlation coefficient was 0.9975 for rutin. A total of 20 μL of *C. medica* extract (80% MeOH or water) was treated with 15 μL of 5% $NaNO_2$ solution for 6 min at room temperature. 15 μL of 10% $Al(NO_3)_3$ solution was added and incubated for 5 min. After that, 4mL of 1M NaOH solution was added to it. The solution was allowed to stand for 15 min before analyzing against the blank solution. The UV detector was set to 510 nm.

The total phenol content in *C. medica* was measured by Folin−Ciocalteu method. A total of 20 μL of *C. medica* extract (80% MeOH or water) was treated with 15 μL of the Folin-Ciocalteu regent for 5 min. Then, 160 μL of 5% Na_2CO_3 solution was added, and the mixture solution was placed under the conditions of protection from light at room temperature for 1 h. The solution allowed was to stand for 15 min before analyzing against the blank solution. The UV detector was set to 765 nm.

2.5. Determination of Antioxidant Activity. *2.5.1. Free radical Scavenging by the DPPH Radical.* The DPPH assay is based on the scavenging of DPPH$^{\bullet}$ radicals, which change the color from deep violet to light yellow and consequently could be measured at 515 nm on a UV−vis spectrophotometer. Radical scavenging capability of plant extracts was determined by a slightly modified method by Miliauskas and his colleagues.[24] Briefly, the DPPH solution in methanol was prepared daily and incubated with appropriate volumes of plant extracts for 20 min at 37 °C in the dark. The absorbance was measured at 515 nm (A_a). A sample blank test using methanol in the DPPH$^{\bullet}$ solution was also performed (A_b). In addition, an appropriate solvent blank reading was recorded (A_t). All measurements were performed in triplicate. Radical scavenging capacity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = (1 - (A_a - A_t)/A_b) \times 100$$

2.5.2. Free Radical Scavenging by the ABST Radical Method. The 2,2-azinobis(3-ethyl-benzothiazoline-6-sulphonic acid) (ABST) radical scavenging activity of each extract was determined according to the method of Re and his colleagues[25] with some modifications, which is based on the ABST radical cation (ABST$^{+\bullet}$) reduction by antioxidants in Foshou extracts. ABST$^{+\bullet}$ was produced by reacting ABST solution with 6.73 mM potassium persulfate in the dark for 12 h at room temperature. For the study, the ABTS$^{+\bullet}$ working solution was diluted in phosphate-buffered saline to an absorbance of 0.704 at 734 nm. After the addition of 40 μL of methanol or aqueous plant extract solutions to 160 μL of ABTS$^{+\bullet}$ solution, the absorbance reading

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Table 1. Coumarins Identified in *Citrus medica*

peak no	R_t (min)	$(M + H)^+$ (m/z)	formula	fragment ions MS/MS (m/z)	identified compounds
C1	3.775	353.091	$C_{17}H_{20}O_8$	267,191,185,115	isomer of 7-ethoxycoumarin-glucoside
C2	4.534	393.085	$C_{20}H_{24}O_8$	231,185	osthenol 7-*O*-glucoside
C3	4.574	355.108	$C_{16}H_{18}O_9$	193,179,137,133,122	hydroxymethoxy coumarin-glucoside
C4	4.586	355.108	$C_{16}H_{18}O_9$	193,178,137,133,122	5,7-dihydroxy-4-methylcoumarin-glucoside
C5	4.988	193.0519	$C_{10}H_8O_4$	178,150,137,133,122,94,77	isomer of hydroxymethoxycoumarin
C6	5.002	355.1658	$C_{16}H_{18}O_9$	193,178,150,137,133,000	7-hydroxy-8-methoxycoumarin-glucoside
[a]C7	5.055	195.0894	$C_{10}H_{10}O_4$	138,123,110	ferulic acid
C8	5.324	193.0548	$C_{10}H_8O_4$	178,150,137,133,122,94,77	isomer of hydroxymethoxycoumarin
C9	5.476	355.1042	$C_{16}H_{18}O_9$	193,178,133	hydroxymethoxy coumarin-glucoside
C10	6.368	379.1024	$C_{18}H_{18}O_9$	217,185,177,149,117,89	5-methoxypsoralen
C11	7.098	193.0491	$C_{10}H_8O_4$	178,150,105,77	isomer of hydroxymethoxycoumarin
C12	7.548	367.1423	$C_{15}H_{26}O_{10}$	241,205,185	isomer of 1-*O*-(3-butenyl)-6-*O*-α-L-arabinose-β-D-glucopyranoside
C13	7.959	193.0519	$C_{11}H_{10}O_4$	178,150,137,133,94,77	isomer of hydroxymethoxycoumarin
C14	8.168	231.1078	$C_{14}H_{14}O_3$	203,189,147,133,115,91	osthenol
C15	8.569	369.1558	$C_{17}H_{20}O_9$	207,185	isomer of dimethoxycoumarin-glucoside
C16	9.134	369.1558	$C_{17}H_{20}O_9$	207,185	isomer of dimethoxycoumarin-glucoside
C17	9.322	369.1558	$C_{17}H_{20}O_9$	207,185	isomer of dimethoxycoumarin-glucoside
C18	10.692	219.0665	$C_{12}H_{10}O_4$	177,149,147,119,91	7-acetyl-4-methylcoumarin
C19	10.699	261.0812	$C_{15}H_{16}O_4$	201,171,145,115,102	5-prenyloxy-7-methoxycoumarin
C20	13.137	219.0695	$C_{12}H_{10}O_4$	177,149,119,91	8-acetyl-7-hydroxy-4-methylcoumarin
C21	15.653	355.0849	$C_{21}H_{22}O_5$	297,254,239	6′,7′-epoxybergamottin
C22	15.707	237.0791	$C_{12}H_{12}O_5$	222,207,193	6,7,8-trimethoxycoumarin
C23	16.231	237.0791	$C_{12}H_{12}O_5$	207,176,147,91	7-(2-hydroxyethoxyl)-6methoxycoumarin
C24	16.413	353.1602	$C_{17}H_{20}O_8$	191,185	isomer of 7-ethoxycoumarin-glucoside
[a]C25	16.953	207.0765	$C_{11}H_{10}O_4$	193,165,149,121,91,77	5,7-dimethoxycoumarin
[a]C26	17.260	217.0518	$C_{12}H_8O_4$	203,174,146,118,89	bergapten
C27	17.813	219.0695	$C_{12}H_{10}O_4$	177,147,119,91	8-acetyl-7-methoxycoumarin
C28	17.858	287.0959	$C_{16}H_{14}O_5$	203,159,147,131,119,91	oxypeucedanin
C29	18.522	339.0878	$C_{21}H_{22}O_4$	254,239	bergamottin

[a]Identified by the reference compound.

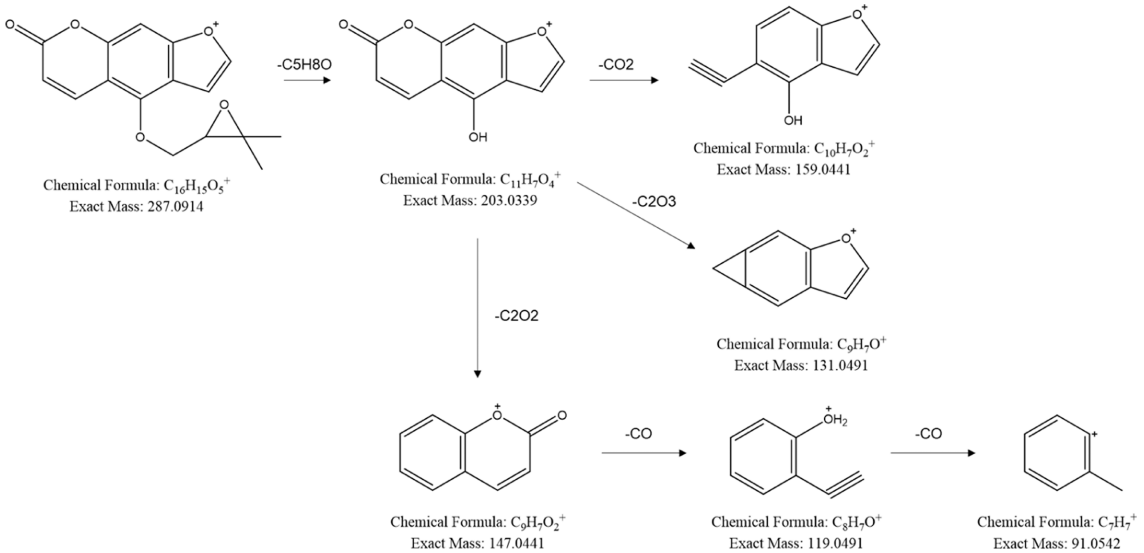

Figure 2. Fragmentation pattern of oxypeucedanin.

was taken at 30 °C, 6 min after initial mixing (A_r). An appropriate solvent blank reading was performed (A_t). 160 μL of $ABTS^{+\bullet}$ solution was allowed to react with 40 μL of methane or deionized water for 6 min (A_0). All solutions were prepared on the day, and all experiments were performed in triplicate. The $ABTS^{+\bullet}$ inhibition percentage of plant extracts was calculated as follows

$$\text{ABST radical scavenging activity (\%)} = (1 - (A_t - A_r)/A_0) \times 100$$

2.5.3. FRAP Assay. The ferric reducing capacity of plant extracts was measured by a modified version of ferric reducing antioxidant potential (FRAP) assay.[26,27] In this method, electron-donating antioxidants reduce the colorless ferric

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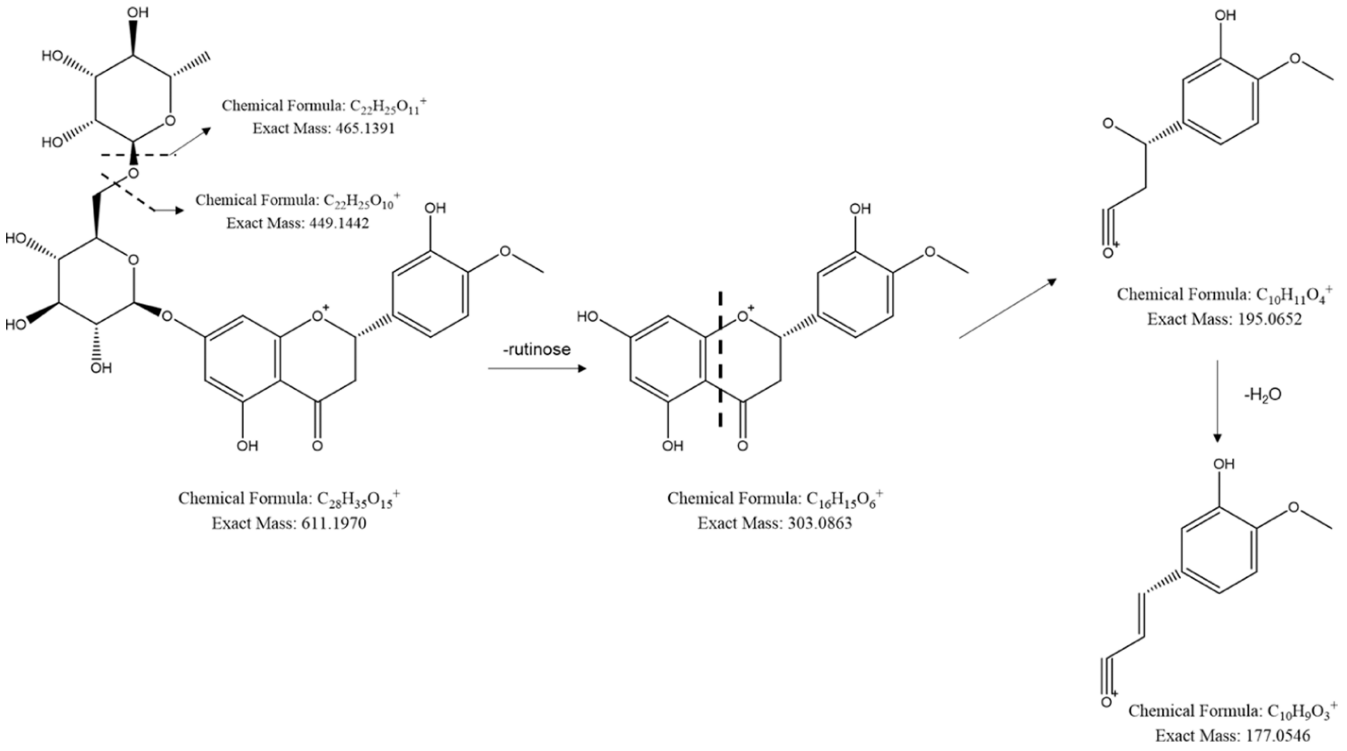

Figure 3. Fragmentation pattern of hesperidin.

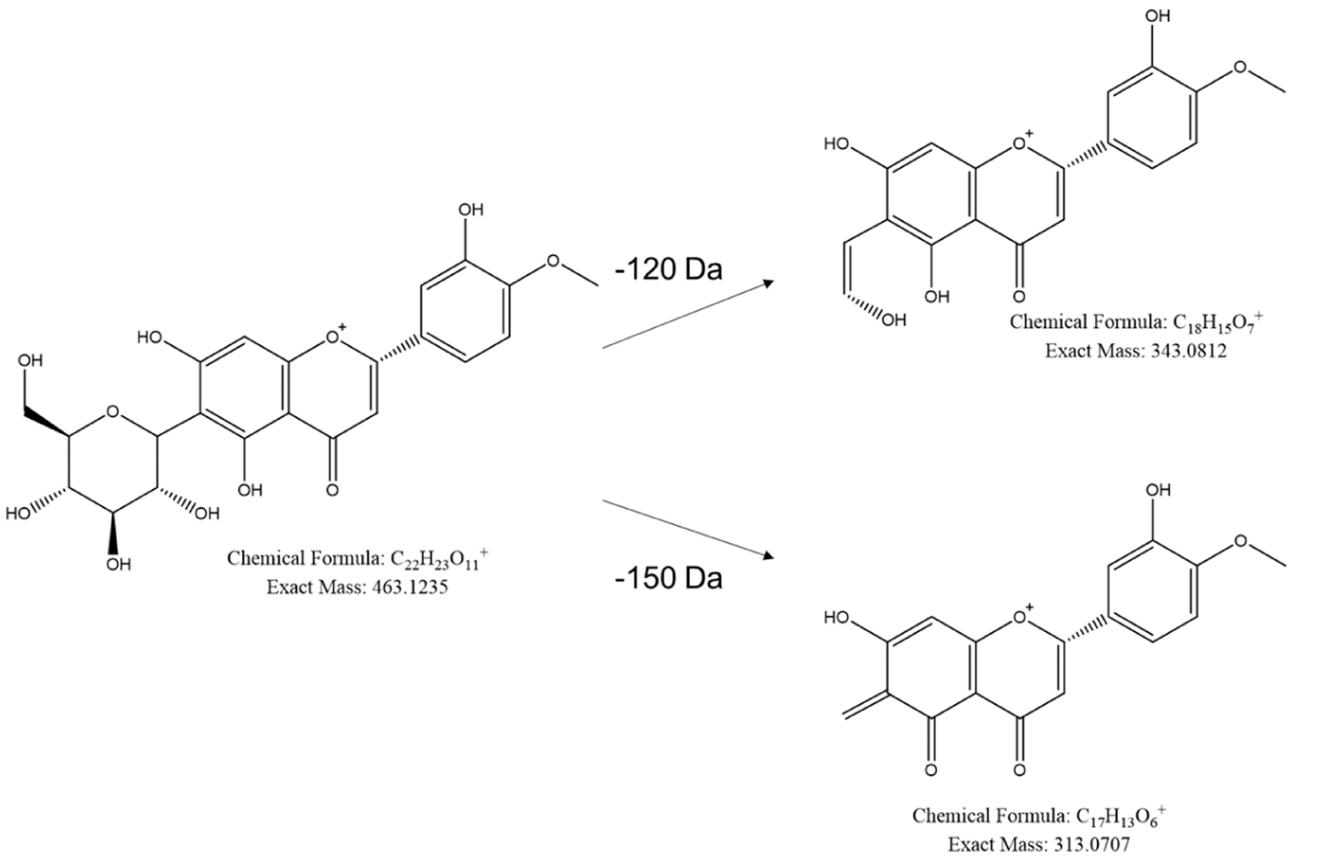

Figure 4. Fragmentation pattern of diosmetin-8-C-glucoside.

complex (Fe^{3+}-tripyridyltriazine) to a blue-colored ferrous complex (Fe^{2+}-tripyridyltriazine) at low pH condition, which is monitored spectrophotometrically at 593 nm. The working FRAP reagent was prepared by mixing acetate buffer, 2,4,6-tri(2-pyridyl)-*s*-triazine (in hydrochloric acid), and ferric chloride at a ratio of 10:1:1. The standard curve was established using

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Table 2. Flavonoid Derivatives Identified in *Citrus medica*

peak no	R_t (min)	$(M + H)^+$ (m/z)	formula	fragment ions MS/MS (m/z)	identified compounds
F1	6.138	727.2155	$C_{32}H_{38}O_{19}$	595,565,433,367,343	vitexinxyloside- or isovitexinxyloside-arabinoside
F2	6.434	565.1668	$C_{26}H_{28}O_{14}$	433,415,337,313,283	vitexinxyloside
F3	6.468	595.1798	$C_{27}H_{30}O_{15}$	577,457,379,337,325	apigenin-6,8-di-C-glucoside
F4	6.966	625.1916	$C_{28}H_{32}O_{16}$	607,589,487,469,409	diosmetin 6,8-di-C-glucoside
F5	7.305	595.1748	$C_{27}H_{30}O_{15}$	433,415,379,337,313	vitexin-glucoside
F6	7.365	625.1865	$C_{28}H_{32}O_{16}$	607,589,487,469,409	diosmetin 6,8-di-C-glucoside
F7	8.458	625.1916	$C_{28}H_{32}O_{16}$	607,463,409,367,343	diosmetin-C-glucoside-*O*-glucoside
F8	8.530	595.1798	$C_{27}H_{30}O_{15}$	433,415,337,313,283	isovitexin-glucoside
F9	8.710	595.1748	$C_{27}H_{30}O_{15}$	433,415,397,337,313	isovitexin-glucoside
F10	8.895	565.1668	$C_{26}H_{28}O_{14}$	433,415,337,313,283	isovitexinxyloside
[a]F11	8.90	433.1187	$C_{21}H_{20}O_{10}$	337,313,283,165	vitexin
[a]F12	9.144	611.1701	$C_{27}H_{30}O_{16}$	465,303,257,229,153	rutin
F13	9.189	565.1619	$C_{26}H_{28}O_{14}$	433,415,337,313,283	isomer of vitexinxyloside or isovitexinxyloside
F14	9.193	579.1849	$C_{27}H_{30}O_{15}$	433,415,337,313,283	vitexin or isovitexin-rhamnoside
F15	9.262	433.1187	$C_{21}H_{20}O_{10}$	415,397,337,313,283	isovitexin
F16	9.651	595.1798	$C_{27}H_{30}O_{15}$	449,287,153	kaempferol-rutinoside
F17	10.05	595.1748	$C_{27}H_{30}O_{15}$	463,445,367,343,313	diosmetin-C-glucoside-*O*-xyloside
F18	10.27	595.1699	$C_{27}H_{30}O_{15}$	463,445,367,343,313	diosmetin-C-glucoside-*O*-xyloside
F19	10.361	463.1313	$C_{22}H_{22}O_{11}$	445,427,343,313,298	diosmetin-8-C-glucoside
F20	10.608	463.1313	$C_{22}H_{22}O_{12}$	445,427,343,367,313	diosmetin-6-C-glucoside
F21	10.745	595.2297	$C_{27}H_{30}O_{15}$	449,287,153	kaempferol-3-*O*-rutinoside
F22	11.200	317.0717	$C_{16}H_{12}O_7$	303,285,153	isorhamnetin
F23	11.204	625.1967	$C_{28}H_{32}O_{16}$	479,317,302,285,229	isorhamnetin-3-rutinoside
F24	11.286	595.1649	$C_{27}H_{30}O_{15}$	449,287	kaempferol-3-*O*-neohesperidoside
F25	11.398	579.1849	$C_{27}H_{30}O_{15}$	433,271,153	apigenin-7-*O*-neohesperidoside
[a]F26	11.968	609.1945	$C_{28}H_{32}O_{15}$	463,301,286,258	diosmin
F27	12.338	301.0737	$C_{16}H_{12}O_6$	287,258,229,203,153	diosmetin
F28	12.346	463.1313	$C_{22}H_{22}O_{13}$	301,286,258,229,153	chrysoeriol 7-*O*-glucoside
F29	12.367	609.1945	$C_{28}H_{32}O_{15}$	463,301,286,258	diosmetin-rutinoside
F30	12.566	303.0895	$C_{15}H_{10}O_7$	177,153,145,117,89	quercetin
[a]F31	12.584	611.2055	$C_{28}H_{34}O_{15}$	465,449,303,195,177	hesperidin
F32	12.603	465.1409	$C_{21}H_{20}O_{12}$	303,258,177,153	isoquercitrin
F33	13.051	303.0931	$C_{16}H_{14}O_6$	177,153,145,117,89	hesperidin hesperetin
F34	15.341	609.1743	$C_{28}H_{32}O_{15}$	433,313,285,283,177	vitexin or or isovitexin-glucuronide
F35	16.843	361.1001	$C_{19}H_{20}O_7$	346,331,316,303,275	trihydroxy-trimethoxylflavone
F36	17.641	361.0962	$C_{19}H_{20}O_7$	346,315,287,183,161	trihydroxy-trimethoxylflavone
F37	17.926	361.0962	$C_{19}H_{20}O_7$	346,345,315,287, 161, 135	trihydroxy-trimethoxylflavone
F38	18.106	361.0962	$C_{19}H_{20}O_7$	346,331,303,285	trihydroxy-trimethoxylflavone

[a]Identified by the reference compound.

different concentrations of $FeSO_4{\cdot}7H_2O$. All solutions were used on the day of preparation. The sample solution was incubated with FRAP reagent for 30 min at 37 °C in a water bath, and then the absorbance was measured at 593 nm. A sample blank test using acetate buffer was also performed. In this assay, the reduction activity of the plant extract was calculated with reference to the signal given by the Fe^{2+} solution. FRAP values were expressed as mg Fe^{2+}/g of sample. All experiments were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Characterization of the Major Metabolites in *C. medica*. Currently, over 100 compounds have been identified and characterized in *C. medica*; most of these compounds are coumarins and flavonoids.[28,29] A private database of coumarins and flavonoids was established by using Agilent MassHunter Workstation according to the reported structure. A total of 67 compounds including 29 coumarin derivatives and 38 flavonoids were tentatively identified by UPLC-Q-TOF-MS.

5,7-Dimethoxycoumarin (reference compound) was taken as an example to show the fragmentation pattern of the coumarin derivatives. In Figure 1, the loss of 28 and 44 are attributed to the carbonyl group and lactone in benzopyran. The common fragmentation ions of them are m/z 91 and m/z 77, which are generated in response to methylbenzene and benzene, respectively. This allows us to rapidly distinguish coumarins and non-coumarins during chemical profiling. The high-resolution mass data and MS/MS fragment ions of coumarins are listed in Table 1. According to the fragmentation ions, m/z 149, m/z 147, m/z 91, and m/z 77 could be regarded as the diagnostic ions of coumarins in *C. medica.*

The target peaks based on their product ions were divided into three groups (basic coumarins, coumarin-glucosides, and furocoumarins). For compounds C1-4, 6,9, and 24, except for diagnostic ions, the loss of 162 was also observed in MS/MS fragment ions, which indicated that these compounds are coumarin-glucosides. Besides, some compounds show the feature of furocoumarins. For example, compound 28 (the accurate molecular weight of 287.0959) showed the fragmenta-

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Table 3. Content (μg/g) of Nine Compounds in *C. medica*

geographic origin	site	vicenin	diosmin	7-hydroxycoumarin	scopoletin	ferulic acid	6,7-dimethoxycoumarin	hesperidin	5,7-dimethoxycoumarin	bergaptene
Guangdong	Zhaoqing	[a]	316.03± 9.26	[a]	2.45 ± 0.32		[a]	221.4 ± 9.96	974.9 ± 45.3	[a]
Guangxi	Guilin	38.43 ± 0.82	578.26 ± 49.23	8.57 ± 0.34			4.26 ± 0.71	396.5 ± 5.54	487.1 ± 10.6	8.97± 0.39
Sichuan	Renshou	56.51 ± 0.81	455.17 ± 8.29	[a]	7.23 ± 0.51	7.40 ± 0.16	8.18 ± 0.09	413.3 ± 9.43	1039.7 ± 8.42	77.73 ± 2.01
	Shimian	48.97 ± 1.16	689.79± 61.64	5.69 ± 0.45	[a]	11.17± 0 0.47	3.39 ± 0.12	785.4 ± 29.3	824.6 ± 21.2	38.29 ± 1.25
	Hejiang	38.43 ± 1.75	604.19 ± 36.13	12.11 ± 0.59	3.8 0 ± 0.51	[a]	8.28 ± 0.51	942.1 ± 29.3	1609.2 ± 65.1	27.70 ± 1.76
Yunnan	Kunming	38.93 ± 0.11	267.87 ± 4.47	[a]	[a]	[a]	[a]	158.7 ± 2.11	394.5 ± 4.55	1.21 ± 0.68
Zhejiang	Jinhua	[a]	428.17 ± 27.95	[a]	[a]	[a]	4.87 ± 0.65	290.9 ± 20.1	1168.33 ± 67.7	[a]

[a]Not detected.

Table 4. Content of Total Flavonoids and Phenolic Compounds in *C. medica*

geographic origin	site	extract	total flavonoids (mg/g)	total phenols (mg/g)
Guangdong	Zhaoqing	methanol	1.26 ± 0.09	2.19 ± 0.18
Guangxi	Guilin	methanol	1.93 ± 0.21	3.19 ± 0.16
Sichuan	Renshou	methanol	1.94 ± 0.19	3.42 ± 0.23
	Shimian	methanol	2.17 ± 0.12	3.74 ± 0.17
	Hejiang	methanol	2.39 ± 0.14	3.92 ± 0.13
Yunnan	Kunming	methanol	2.39 ± 0.17	2.41 ± 0.11
Zhejiang	Jinhua	methanol	1.72 ± 0.11	2.88 ± 0.32
Guangdong	Zhaoqing	aqueous	[a]	0.53 ± 0.02
Guangxi	Guilin	aqueous	[a]	0.90 ± 0.07
Sichuan	Renshou	aqueous	[a]	0.69 ± 0.02
	Shimian	aqueous	[a]	0.55 ± 0.03
	Hejiang	aqueous	[a]	0.90 ± 0.01
Yunnan	Kunming	aqueous	[a]	0.38 ± 0.04
Zhejiang	Jinhua	aqueous	[a]	1.60 ± 0.05

[a]Not detected.

tion ions *m*/*z* 203, 147, and 131, which could be tentatively identified as the furocoumarins. The loss of 84 Da MS2 spectrum of oxypeucedanin could be severed as (3,3-dimethyl-2-oxiranyl)methyl group, according to the investigation of phytochemicals of *C. medica*. The fragmentation of oxypeucedanin, an example of furocoumarins, is presented in Figure 2.

Except for coumarins, flavonoids are also rich in *C. medica*. In this case, compound F31 (*m*/*z* 611.2055) and F7 (*m*/*z* 625.1916) were selected as examples for the characterization of flavonoid derivatives. A series loss of 146 and 162 was observed at the disaccharide structure (rhamnose and glucose), which corresponds to *m*/*z* 465 ($(M-C_6H_{10}O_4+H)^+$) and *m*/*z* 449 ($(M-C_6H_{10}O_5+H)^+$),respectively, (Figure 3).

The fragmentation ion *m*/*z* 303 corresponds to hesperetin, the flavonoid aglycone. Additionally, both the neutral losses of 120 and 150 Da were detected in the MS/MS Fragment ions of compounds F1−F11 and F17−F20, indicating classic C-glycosidic structures.[30,31] A classic fragmentation pattern is shown in Figure 4. A series of 18 Da and 15 Da are corresponding to the hydroxyl group and methyl group in flavonoid derivatives. Other flavonoid derivative compounds are displayed in Table 2.

3.2. Quantification. The intrinsic quality of *C. medica* showed low similarity in the content of nine major metabolites, total flavonoids and total phenolic constituents. As shown in Table 3, diosmin, hesperidin, and 5,7-dimethoxycoumarin are detected in *C. medica* from all geographic origins, indicating that these three compounds could be regarded as the common quality standards for the evaluation of *C. medica*. The content of these is in the range of 158.7 ± 2.11−1609.2 ± 65.1 μg/g. Interestingly, the highest average contents of the three compounds are detected in the samples from Sichuan province. The highest average contents of hesperidin and 5,7-dimethoxycoumarin are detected in samples from Hejiang (Sichuan province), whereas the samples from Shimian (Sichuan province) showed the highest average content of diosmin. The samples with the lowest average content of the three compounds are collected from Kunming (Yunnan province). Furthermore, vicenin and bergaptene are absent in the samples from Guangdong and Zhejiang provinces. This result indicated that the accumulation of phytochemicals in *C. medica* may have a

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Table 5. Antioxidant Activity of *C. medica L. var sarcodatylis* Extracts[a]

geographic origin	geographic origin	extract	DPPH inhibition %	ABTS inhibition %	Fe^{2+} (mg/g)
Guangdong	Zhaoqing	methanol	18.05 ± 1.41	19.22 ± 0.35	0.57 ± 0.01
Guangxi	Guilin	methanol	37.61 ± 5.28	37.59 ± 0.98	0.98 ± 0.02
Sichuan	Renshou	methanol	34.22 ± 4.57	38.54 ± 2.44	1.00 ± 0.01
	Shimian	methanol	41.44 ± 2.41	47.54 ± 0.55	0.75 ± 0.01
	Hejiang	methanol	29.14 ± 3.63	46.54 ± 0.52	0.98 ± 0.02
Yunnan	Kunming	methanol	32.62 ± 1.14	28.64 ± 0.66	2.22 ± 0.06
Zhejiang	Jinhua	methanol	22.73 ± 1.97	32.43 ± 1.04	1.40 ± 0.01
Guangdong	Zhaoqing	aqueous	7.50 ± 0.77	2.17 ± 0.17	0.12 ± 0.002
Guangxi	Guilin	aqueous	7.60 ± 0.92	12.20 ± 0.82	0.21 ± 0.008
Sichuan	Renshou	aqueous	8.22 ± 1.33	4.78 ± 0.59	0.20 ± 0.0008
	Shimian	aqueous	8.12 ± 0.30	3.74 ± 0.24	0.19 ± 0.003
	Hejiang	aqueous	7.70 ± 0.21	5.09 ± 0.55	0.18 ± 0.005
Yunnan	Kunming	aqueous	5.44 ± 0.16	2.38 ± 0.07	0.14 ± 0.007
Zhejiang	Jinhua	aqueous	6.00 ± 0.24	3.57 ± 1.00	0.19 ± 0.005

[a]The relationship between antioxidant activity and constituents.

Table 6. Pearson's Correlation Coefficients Among Flavonoids, Phenolic Compounds and Antioxidant Activity in *C. medica* Extracts

		total flavonoids	total phenol	DPPH inhibition	ABTS inhibition	Fe^{2+}
methanol	total flavonoids	1	0.110	−0.215	0.175	0.197
	total phenol		1	0.601	0.975[a]	−0.343
	DPPH inhibition			1	0.718	0.062
	ABTS inhibition				1	−0.187
	Fe^{2+}					1
aqueous	total phenol		1	0.645	0.318	−0.199
	DPPH inhibition			1	0.386	−0.159
	ABTS inhibition				1	−0.863[b]
	Fe^{2+}					1

[a]$p < 0.01$ (2-tailed). [b]$p < 0.05$ (2-tailed).

close relationship with the geographic origins. The chemical diversity of the samples from southwest and southeast of China could be regarded as their chemotaxonomy markers.

The content of total flavonoids and phenolic compounds in Foshou samples (80% MeOH extracts and water extracts) are listed in Table 4. The phenolic and flavonoids are mainly distributed in 80% MeOH extracts. Notably, total flavonoids are not detected in all samples of water extracts. Similarly, the samples with the top three contents of total phenolic compounds are collected from Sichuan province. Among them, the samples from Hejiang showed 3.92 ± 0.13 mg/g, followed by the samples from Shimian (3.74 ± 0.17 mg/g) and those from Renshou (3.42 ± 0.23 mg/g). The lowest content of total phenolic compounds is found in the samples from Zhaoqing (Guangdong province, 2.19 ± 0.18 mg/g). Moreover, the order of content of total flavonoids was different. The highest content (2.38 mg/g) is detected in the samples from Kunming (Yunnan province) and Hejiang (Sichuan province). The lowest content of total phenolic compounds (1.26 ± 0.09 mg/g) is also noted in the samples from Zhaoqing (Guangdong province).

3.3. Antioxidant Activity. In the present study, the antioxidant ability of extracts (methanol and water) of *C. medica* L. var sarcodatylis from seven geographic origins were assessed by a common reaction mechanism, as the single electron transfer (SET). The SET strategy included DPPH, ABST, and FRAP tests. As shown in Table 5, the free radical scavenging activity evaluated through three SET methods displayed a parallel trend among the samples from seven geographic origins, in spite of distinct absolute values. Generally, the samples from Sichuan province (Renshou, Shimian and Hejiang) showed highest free radical scavenging capacity. The DPPH, ABTS, and FRAP values of samples from Sichuan Province were 29.14 (±2.97)−41.44 (±1.97)%, 38.54 (±2.44)−47.54 (±0.55)%, and 0.75 (±0.01)−1.00 (±0.01) mg/g, respectively, and the samples from Guangxi (Guilin), Yunnan (Kunming), and Zhejiang (Jinhua) provinces showed comparable antioxidant capacity to those from Sichuan Province. Samples from Guangdong Province (Zhaoqing) exhibited inferior free radical scavenging ability. Moreover, we observed lower ABTS scavenging and ferric reducing abilities among the aqueous extracts compared with methanol extracts. However, this antioxidant activity initiated a strong DPPH response—one better suited for aqueous extracts. From the chemical quantification point of view, there were high contents (>1.90 ± 0.18 mg/g) of total flavonoids in the samples from Sichuan Province (Renshou, Shimian, and Hejiang). The low contents (<1.35 ± 0.12 mg/g) were found in the samples from the Guangdong Province (Zhaoqing). 5,7-Dimethoxycoumarin (824.57 ± 21.2−1609.15 ± 65.1 μg/g) was the predominant ingredient in Foushou samples regardless of the geographic origin or extract solvents. The low contents of the rest of compounds existed in all the samples, particularly the scopoletin (<7.3 ± 0.50 μg/g) and ferulic acid (<11.2 ± 0.46 μg/g) which could not be detected even in samples from Yunnan, Zhejiang, and Guangxi provinces. Signed into the study by Luo and his colleagues,[16] they stated that finger citron flavonoids had a more potent effect on scavenging ABTS radical than DPPH radical at the same

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concentration and made them potentially useful for patients to challenge the oxidative stress-mediated diseases. Combining the content data mentioned above, we speculated that flavonoids and phenolics were the main antioxidant compounds in Foushou samples. Other components that include enormously rich and powerfully antioxidative compounds such as alkyl glycosides, limonoids, and volatile ingredients should be considered. Thus, further study should be established to evaluate this.

The associations between the contents of total flavonoids, total phenolic compounds, and antioxidant activities were analyzed through Pearson's correlation coefficient analysis (Table 6). The highest positive correlations were between the content of total phenolic compounds and the antioxidant capacity measured by ABST radical scavenging assay ($r = 0.975$, $p < 0.01$, methanol extract), indicating that the higher the total phenolic compound content, the stronger the antioxidant activity. Positive correlations were also observed between total flavonoids or phenolic compounds and DPPH free radical scavenging activity. Discussing in the study associated with antioxidant activity of grains of hulless barley, Deng[32] suggested that phenolic compounds were the major ingredients of ABST free radical scavenging activity. However, no significant correlation between total flavonoids or phenolic compounds and ferric reducing capacity was observed in the present study. Because only limited published information about the secondary metabolites in Foshou is available, the analysis of connections between total flavonoids or phenolic compounds and antioxidant activity was limited in this study.

4. CONCLUSIONS

Sixty-seven constituents composed of 29 coumarin derivatives and 38 flavonoids were simultaneously identified in *C. medica* via UPLC-Q-TOF-MS. Among them, nine constituents were quantified using HPLC-DAD to reveal the differences between samples from seven geographic origins. Samples with higher content of flavonoids and phenolics showed better antioxidant activity, indicating that flavonoids and phenolics were the main antioxidant compounds in Foushou samples. These results provide insight into the relationship of antioxidant activity, geographic origins, and phytochemicals, which could be ideal for the quality control of *C. medica*.

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Notes

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