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Ultrasonic/microwave assisted extraction and diagnostic ion filtering strategy by liquid chromatography–quadrupole time-of-flight mass spectrometry for rapid characterization of flavonoids in *Spatholobus suberectus*

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ABSTRACT

Spatholobus suberectus is a widely used herb in traditional medicine for the treatment of blood stasis syndrome and related diseases. In this work, a potential ultrasonic/microwave assisted extraction (UMAE) method was developed for efficient sample pretreatment, and a diagnostic ion filtering strategy with liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–Q-TOF-MS) was established for rapid characterization of flavonoids in *S. suberectus*. The factors of UMAE influencing the extraction yield of flavonoids of *S. suberectus* were evaluated. The optimal conditions were determined as: microwave power of 300 W, extraction time of 450 s, 70% methanol as extraction solvent, solvent to solid ratio of 20 mL/g, ultrasound power of 50 W, extraction temperature of 80 °C, and one extraction cycle. Compared with commonly used extraction methods, UMAE showed higher efficiency and shorter extraction time for sample preparation. Subsequently, the major diagnostic ions and fragmentation pathways of flavonoids in Q-TOF-MS were summarized with available reference compounds. Using a new diagnostic ion filtering strategy, a rapid screening and identification of thirty-eight compounds was achieved in real *S. suberectus* samples. The results of this study clearly demonstrate the potential of UMAE for efficient extraction and LC–Q-TOF-MS for rapid and sensitive structural elucidation of flavonoids in *S. suberectus*, and open perspectives for similar studies on other medicinal herbs.

1. Introduction

Plants and herbs are the fundamental basis of sophisticated traditional medicines for thousands of years [1]. Plant natural products have also provided an unparalleled source of chemical diversity for discovery of interesting bioactive molecules [2]. Spatholobi Caulis, derived from the vine stem of *Spatholobus suberectus* Dunn (Leguminosae), is a widely used herb in traditional medicine and officially listed in the 2010 edition of the *Chinese Pharmacopoeia* with the name of Jixueteng. *S. suberectus* possesses a variety of functions, including blood circulation improvement [3], anti-inflammation [4], tyrosinase inhibition [5], and HIV-1-protease inhibition [6]. It has long been prescribed for the treatment of diseases such as rheumatism, anemia and menoxenia in Asian countries. Chemical and pharmacological investigations disclosed that flavonoids are the major ingredients of *S. suberectus* [7,8].

The first challenge in analyzing herbs is the complexity of the sample matrices. Efficient sample preparation can improve extraction and enrich the target analytes [9]. A diversity of sample preparation techniques with different solvents and methods has been developed for extraction of flavonoids from herbs [10,11]. More recently, newer methods that use less solvent, automate easily and take a short time have been described [12,13]. Ultrasound-assisted extraction (UAE) is a fast, low cost and efficient alternative to conventional liquid–solid extraction methods. Ultrasonic energy allows greater penetration of solvent into the herbal tissue, increases the contact surface area, and generates expansions–compressions, thus enhancing the extraction efficiency [14]. Microwave-assisted extraction (MAE) with an adsorption-wave technique can heat the sample in a very short time and accelerate the extraction. Compared with maceration, percolation and UAE, MAE enables a significant reduction in the extraction time and the consumption of organic solvent [15]. Each method has advantages and limitations, for example, MAE tends to cause inhomogeneous heating. Therefore, ultrasonic/microwave assisted extraction (UMAE), a complementary technique coupling UAE and MAE, has shown some more advantages [16–18].

In addition to the sample preparation, another challenge in analyzing herbal medicine is the secondary metabolite identification [19,20]. Thus, a wide range of analytical methods need to be used to fully characterize these 'magical components'. An emerging instrumental trend has been the application of MS

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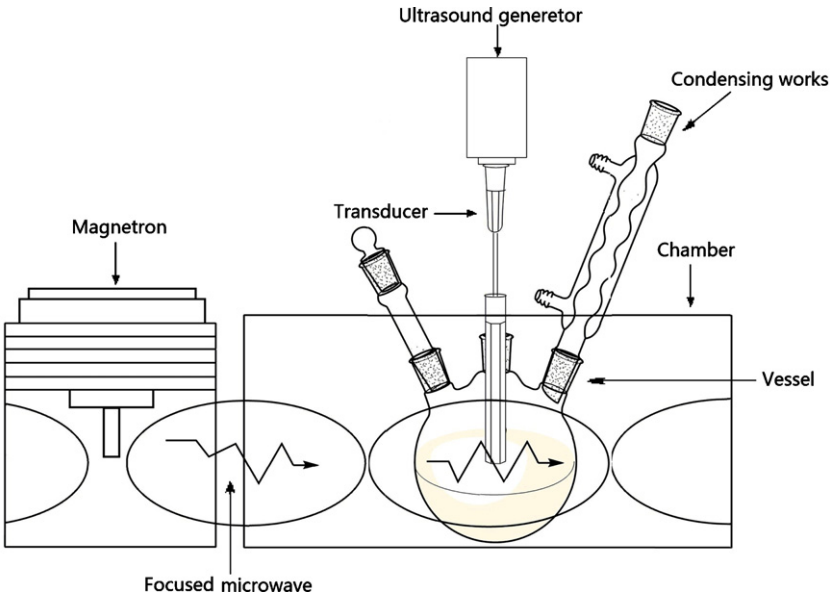


Fig. 1. Schematic diagram of simultaneous ultrasonic and microwave extracting apparatus.

and its hyphenation with chromatographic separation techniques. The most used hyphenated technique is high-performance liquid chromatography–mass spectrometry (HPLC–MS) [21,22]. Multistage MS (also called tandem MS or MS/MS or MS^n) combines the different designs of mass analyzers, giving advanced structural information and having the sensitivity, specificity, and versatility. Hybrid systems such as quadrupole time-of-fight mass spectrometry (Q-TOF-MS), which provide fragmentation information together with accurate mass measurements of product ions, are powerful tools for structural characterization. To the best of our knowledge, systematic investigation on flavonoids in *S. suberectus* has not been reported.

In this work, a potential combined method using ultrasonic/microwave assisted extraction (UMAE) is developed and extraction conditions were optimized through orthogonal array design experiments. Subsequently, a diagnostic ion filtering strategy with LC–Q-TOF-MS is proposed for rapid characterization of flavonoids in *S. suberectus*. The major diagnostic ions and fragmentation pathways of flavonoids in Q-TOF-MS are summarized with available reference compounds. Using a new diagnostic ion filtering strategy, rapid screening and identification of compounds is achieved in real *S. suberectus* samples. This study shows the potential of UMAE and LC–Q-TOF-MS method for extraction and structural characterization of flavonoids in *S. suberectus*, and open perspectives for similar studies on other medicinal herbs.

2. Experimental

2.1. Materials

The dried stems of *S. suberectus* were purchased from Simcere Pharmaceutical Co. (Nanjing, China), and authenticated by one of the authors, Dr. Ping Li. The voucher specimen was deposited in the department of Pharmacognosy, China Pharmaceutical University, Nanjing, China. Authentic standards of epigallocatechin, protocatechuic acid, catechin, epicatechin, procyanidin B2, hesperetin, ononin, daidzein, calycosin, liquiritigenin, genistein, naringenin, isoliquiritigenin, formononetin, medicagol and biochanin A were purchased from Shanghai Tauto Biotechnology Co., Ltd. The purities of the reference compounds were determined to be higher than 95% by HPLC-DAD.

Acetonitrile and formic acid, both MS grade, were obtained from Merck (Darmstadt, Germany). Ultrapure water was prepared by Milli-Q system (Milford, MA, USA). HPLC grade methanol and analytical grade ethyl acetate (Nanjing Chemical Reagent Factory, China) was used for sample preparation.

2.2. Extraction procedures

Dried stems of *S. suberectus* were powdered into a homogeneous size by a disintegrator (HX-200A, Yongkang Hardware and Medical Instrument Plant, China), and then sieved (60 mesh). The materials (1.0 g) were extracted with different techniques (ultrasonic/microwave assisted extraction, microwave-assisted extraction, ultrasound-assisted extraction, soxhlet extraction, and heat reflux extraction) individually for obtaining the optimized extraction procedure.

2.2.1. Ultrasonic/microwave assisted extraction (UMAE)

A simultaneous ultrasonic and microwave extracting apparatus (XO-SM50, Nanjing Xianou Instrument Manufacturing Co., Ltd., China) shown in Fig. 1 was applied for sample extraction. Several grams of drug powder varied according to the solvent to material ratio were put into the closed vessels designed for UMAE and extracted under different UMAE conditions. After extraction, the vessels were allowed to cool at room temperature. Microwave power (100–500 W), extraction time (100–900 s), extraction solvent (30–100% methanol, pure ethanol), solvent to solid ratio (10–50 mL/g), ultrasound power (30–250 W), extraction temperature (40–120 °C) and number of extraction cycles (1–3 times) were evaluated for the extraction of flavonoids from *S. suberectus*.

2.2.2. Microwave-assisted extraction (MAE)

MAE was carried out in the same apparatus as UMAE, while the ultrasound power was set at "off" mode. Accurately weighed drug powder (1 g) was introduced with 20 mL 70% methanol to the closed vessel. The extraction was carried out at 80 °C for 15 min

Table 1
The factors and levels for the orthogonal array design.

Factors[a]	Microwave power (W) A	Extraction time (s) B	Extraction solvent C	Solvent to solid ratio (mL/g) D	Ultrasound power (W) E	Extraction temperature (°C) F
Levels						
1	100	100	30% methanol	10	30	40
2	200	300	50% methanol	20	50	60
3	300	450	70% methanol	30	70	80
4	400	600	100% methanol	40	100	100
5	500	900	100% ethanol	50	250	120

[a] Experiments were conducted in duplicate.

Table 2
Results of the six-factor-five-level orthogonal array design optimization of the UMAE method for flavonoids from *S. suberectus*.

Factor	Microwave power (W) (A)	Yield[a] (mg/g)	Extraction time (s) (B)	Yield (mg/g)	Extraction solvent (C)	Yield (mg/g)
Mean[b]	100	11.982	100	11.561	30% MeOH	11.231
	200	12.228	300	12.261	50% MeOH	12.114
	300	13.086	450	12.441	70% MeOH	12.601
	400	12.528	600	12.090	100% MeOH	12.016
	500	10.115	900	11.585	100% EtOH	11.977
Range[c]		2.971		0.880		1.370

Factor	Solvent to solid ratio (mL/g) (D)	Yield (mg/g)	Ultrasound power (W) (E)	Yield (mg/g)	Extraction temperature (°C) (F)	Yield (mg/g)
Mean[b]	10	11.249	30	11.763	40	11.428
	20	12.307	50	12.600	60	11.578
	30	12.298	70	11.972	80	12.404
	40	12.061	100	12.008	100	12.388
	50	12.024	250	11.596	120	11.946
Range[c]		1.058		1.004		0.976

[a] Extraction yield of total flavonoids. Experiments were conducted in duplicate.
[b] Mean extraction yield of total flavonoids of five treatments at each level for each factor.
[c] Range extraction yield of total flavonoids among the five levels for each factor.

Table 3
Analysis of variance.

Factors	Sum of squares	Degree of freedom	*F*-ratio	$F_{0.05}$	*p* value	Significance
A	25.310	4	7.962	6.390	$0.01 < p < 0.05$	Yes
B	3.179	4	1.000	6.390	–	No
C	4.830	4	1.519	6.390	–	No
D	3.753	4	1.181	6.390	–	No
E	2.900	4	0.912	6.390	–	No
F	3.945	4	1.214	6.390	–	No

for two cycles. The extracts were collected and forwarded to post treatment.

2.2.3. Ultrasound-assisted extraction (UAE)

The materials (1.0 g) were weighed and put into a conical flask. Then 60 mL of 70% methanol solution was added to the flask, and extracted in an ultrasonic bath (Kunshan Ultrasonic Instrument Co. Ltd., China) at 50 °C for 30 min with ultrasonic power of 150 W. This extraction process was repeated two cycles.

Table 4
Recoveries for UMAE of flavonoid standards and flavonoids from spiked powders of *S. suberectus*.

Flavonoids	Standards[a]		Spiked powders[b]	
	Recovery (%)	RSD%	Recovery (%)	RSD
Epigallocatechin	88.9	3.7	87.1	4.0
Catechin	89.1	2.5	88.0	3.8
Hesperetin	94.8	4.1	95.1	2.9
Naringenin	95.5	3.2	94.9	3.2
Genistein	98.3	2.4	100.3	2.5
Biochanin A	97.8	4.5	101.5	3.5

[a] Mean ± SD for recoveries relative to the reference ($n = 3$).
[b] Mean ± SD for recoveries relative to the amount spiked ($n = 3$).

2.2.4. Soxhlet extraction (SE)

Sixty mesh powder of 1.0 g was extracted with 80 mL 100% methanol using soxhlet apparatus for 6 h under a temperature of 80 °C. The extract was then concentrated using rotary vacuum evaporator.

2.2.5. Heat reflux extraction (HRE)

HRE was conducted in a water bath at 90 °C. An amount of 1.0 g drug powder were placed into a 100 mL glass flask with 60 mL 70% methanol and extracted for two 2 h cycles.

All of the obtained extract was cooled to room temperature and centrifuged ($5000 \times g$, 10 min). The supernatant was con-

Table 5
Comparison of UMAE and conventional extraction methods under the optimal conditions ($n = 3$).

Method	Extraction time	Extraction volume (mL/g)	Total flavonoids	
			Yield (mg/g)	RSD %
UMAE	450 s	20	18.79	1.2
MAE	0.5 h	40	13.66	2.4
UAE	1 h	120	9.85	2.2
SE	6 h	80	15.21	4.0
HRE	4 h	120	18.69	3.0

Table 6
Compounds identified in the extract of *Spatholobus suberectus*.

No.	Retention time (min)	$[M-H]^-$	(−)ESI-MS (*m*/*z*) Main fragment ions	Formula	Identification
1[a]	6.63	305.0675	261.0743; 219.0661; 179.0350; 165.0192; 137.0242; 125.0249	$C_{15}H_{14}O_7$	Epigallocatechin
2[a]	8.10	153.0190	109.0285	$C_7H_6O_4$	Protocatechuic acid
3	8.93	593.1298	467.1149; 425.0880; 407.0794; 305.0688; 289.0721; 245.0754; 179.0305; 165.0149; 137.0248; 125.0238	$C_{30}H_{26}O_{13}$	(Epi)gallocatechin–(epi)catechin
4	11.46	593.1316	467.0913; 425.0870; 407.0918; 305.0686; 289.0708; 245.0449; 179.0340; 165.0162; 137.0262; 125.0246	$C_{30}H_{26}O_{13}$	(Epi)gallocatechin–(epi)catechin
5	12.61	305.0671	261.0738; 219.0661; 179.0354; 165.0192; 137.0238; 125.0242	$C_{15}H_{14}O_7$	Gallocatechin
6	14.01	577.1342	559.1238; 451.1105; 425.0899; 407.0783; 289.0726; 245.0825; 203.0722; 179.0319; 165.0207; 137.0242; 125.0245	$C_{30}H_{26}O_{12}$	Epicatechin–epicatechin
7[a]	16.05	289.0714	245.0812; 203.0698; 179.0345; 165.0179; 137.0239; 125.0243	$C_{15}H_{14}O_6$	Catechin
8	17.62	561.1405	435.1044; 425.0868; 407.0795; 289.0721; 245.0844; 203.0706; 165.0182; 137.0248; 125.0230	$C_{30}H_{26}O_{11}$	(Epi)afzelechin–(epi)catechin
9	18.68	577.1354	451.1051; 425.0844; 407.0768; 289.0714; 245.0813; 203.0715; 179.0337; 165.0194; 137.0234; 125.0240	$C_{30}H_{26}O_{12}$	(Epi)catechin–(epi)catechin
10	19.71	577.1346	451.1029; 425.0862; 407.0768; 289.0714; 245.0813; 203.0708; 179.0341; 165.0202; 137.0233; 125.0238	$C_{30}H_{26}O_{12}$	Epicatechin–epicatechin
11	20.72	865.1989	739.1692; 713.1528; 695.1398; 577.1352; 451.1061; 425.0901; 407.0768; 289.0704; 287.0556; 261.0377; 245.0458; 125.0238;	$C_{45}H_{38}O_{18}$	(Epi)catechin–(epi)catechin–(epi)catechin
12[a]	21.40	289.0714	245.0809; 203.0702; 179.0342; 137.0234; 125.0236; 109.0288	$C_{15}H_{14}O_6$	Epicatechin
13	22.84	865.1995	739.1680; 713.1470; 695.1494; 577.1343; 451.1010; 425.0850; 407.0774; 289.0702; 287.0558; 261.0415; 245.0428; 125.0237	$C_{45}H_{38}O_{18}$	(Epi)catechin–(epi)catechin–(epi)catechin
14	23.47	561.1389	435.1093; 425.0870; 407.0772; 289.0712; 245.0817; 203.0722; 165.0193; 137.0249; 125.0238	$C_{30}H_{26}O_{11}$	(Epi)afzelechin–(epi)catechin
15	24.29	577.1371	451.1042; 425.0878; 407.0770; 289.0715; 245.0787; 203.0722; 179.0335; 165.0121; 137.0224; 125.0237	$C_{30}H_{26}O_{12}$	(Epi)catechin–(epi)catechin
16	24.97	865.1968	739.1733; 713.1470; 695.1494; 577.1343; 451.1078; 425.0872; 407.0788; 289.0711; 245.0466; 125.0223	$C_{45}H_{38}O_{18}$	(Epi)catechin–(epi)catechin–(epi)catechin
17	25.45	273.0758	255.0559; 229.0786; 187.0723; 179.0213; 165.0178; 137.0178; 125.0208	$C_{15}H_{14}O_5$	(Epi)afzelechin
18	25.88	849.2090	723.1772; 697.1607; 679.1439; 577.1295; 451.1083; 425.0832; 407.0809; 289.0704; 245.0411; 165.0184; 137.0297; 125.0246	$C_{45}H_{38}O_{17}$	(Epi)afzelechin–(epi)catechin–(epi)catechin
19	28.25	287.0557	251.2369; 213.0688; 187.0458; 165.7443; 145.0267; 135.0452; 123.0079; 107.0135	$C_{15}H_{12}O_6$	Plathymenin
20	29.64	577.1337	451.1052; 425.0887; 407.0767; 289.0727; 245.0815; 203.0703; 179.0367; 165.0197; 137.0241; 125.0244	$C_{30}H_{26}O_{12}$	(Epi)catechin–(epi)catechin
21	34.61	561.1447	435.1120; 425.0880; 407.0774; 289.0725; 245.0835; 203.0698; 165.0175; 137.0244; 125.0241	$C_{30}H_{26}O_{11}$	(Epi)afzelechin–(epi)catechin
22[a]	36.59	301.0719	286.0474; 268.0291; 259.0689; 257.0689; 224.0338; 151.0040; 123.0074;	$C_{16}H_{14}O_6$	Hesperetin
23[a]	40.82	475.1238	267.0661; 252.0396; 165.0197; 159.1387; 148.6859	$C_{22}H_{22}O_9$	Ononin
24[a]	43.46	253.0503	235.0324; 223.0365; 208.0541; 195.0498; 169.0637; 135.0078; 133.0282; 117.0376	$C_{15}H_{10}O_4$	Daidzein
25[a]	47.34	283.0622	268.0360; 239.0342; 212.0458; 223.0473; 196.0578; 156.0610	$C_{16}H_{12}O_5$	Calycosin
26[a]	51.06	255.0665	237.0538; 209.0600; 135.0066	$C_{15}H_{12}O_4$	Liquiritigenin
27	52.88	285.0773	270.0526; 252.0409; 242.0573; 224.0457; 151.0019; 123.0022	$C_{16}H_{14}O_5$	3,7-Dihydroxy-6-methoxyflavonol
28	53.89	271.0622	135.0440	$C_{15}H_{12}O_5$	Butein
29[a]	54.89	269.0458	224.0477; 159.0457; 133.0291	$C_{15}H_{10}O_5$	Genistein
30[a]	55.20	271.0629	253.0488; 225.0517; 151.0021; 125.0211; 107.0118	$C_{15}H_{12}O_5$	Naringenin
31	57.24	301.0711	284.0373; 271.0535; 255.0470; 239.0340; 228.0373; 183.0390; 167.0518; 149.0311	$C_{16}H_{14}O_6$	Suberectin
32	62.00	281.0462	253.0497; 239.0537; 227.0617; 225.0565; 209.0609; 195.0574; 161.0747; 135.0119	$C_{16}H_{10}O_5$	Pseudopaptigenin
33[a]	62.58	255.0659	135.0078; 119.0498	$C_{15}H_{12}O_4$	Isoliquiritigenin
34[a]	63.20	267.0668	252.0418; 223.0395; 195.0442; 132.0223; 135.0079	$C_{16}H_{12}O_4$	Formononetin
35	64.44	297.0401	282.0536; 267.0319; 245.0532; 223.0406; 211.8808; 179.0464; 165.0507; 151.0507	$C_{17}H_{14}O_5$	Afrormosin
36	68.81	283.0617	268.0377; 240.0406; 211.0564; 198.0290; 183.0442; 145.0277; 137.0766	$C_{16}H_{12}O_5$	Prunetin
37[a]	75.90	295.0247	267.0306; 249.0217; 223.0406; 211.0441; 167.042; 141.0315	$C_{16}H_8O_6$	Medicagol
38[a]	76.51	283.0630	268.0368; 240.0439; 211.0374; 159.5997; 135.0111	$C_{16}H_{12}O_5$	Biochanin A

[a] Authentic standards.

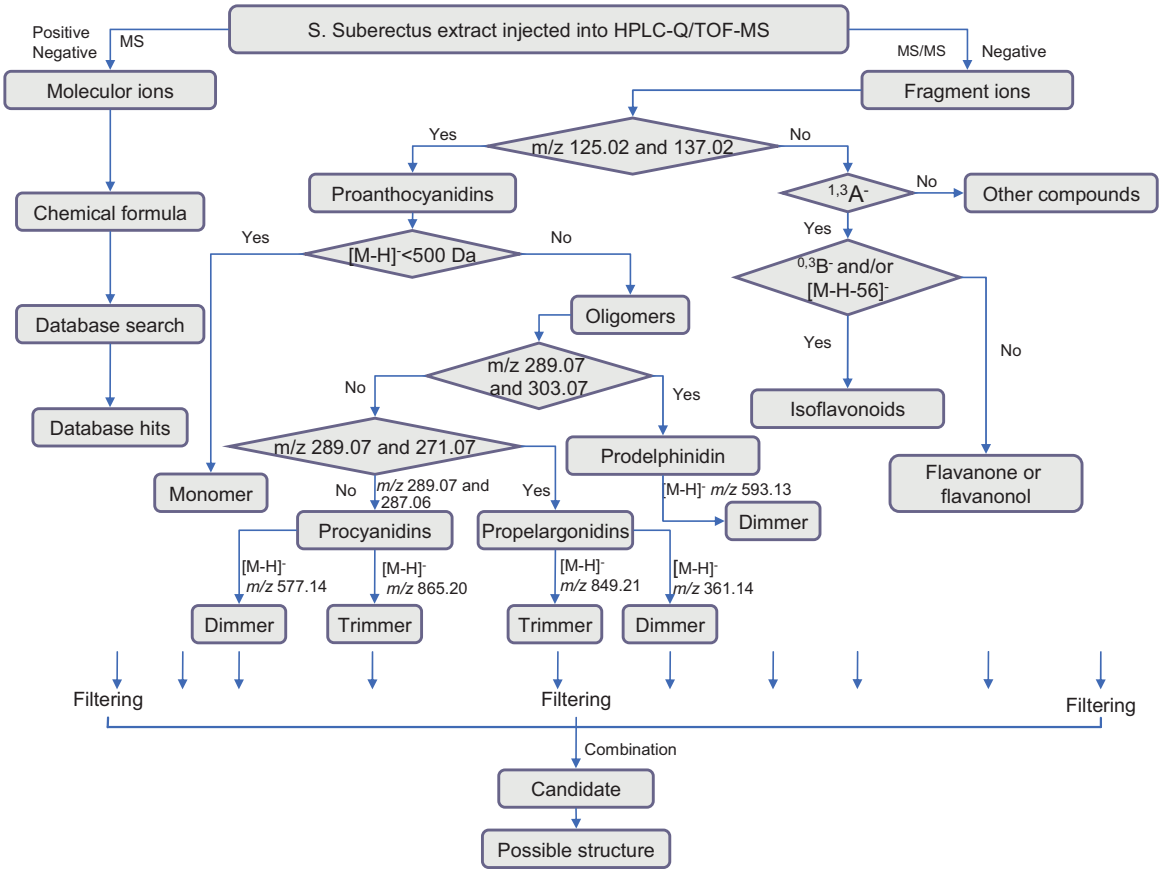


Fig. 2. General strategy for identification of flavonoids in *Spatholobus suberectus* samples by LC–Q-TOF-MS.

centrated, and the volume of extract was made up to 25 mL with methanol. The conditions for MAE, UAE, SE and HRE were all optimized by orthogonal array design experiments (data not shown).

2.3. Determination of total flavonoids

Total flavonoids were determined using a colorimetric method described by Heimler et al. [23] with a minor modification. Briefly, to 0.2 mL of each sample obtained as above, 0.8 mL of a 5% $NaNO_2$ solution, 0.15 mL of a freshly prepared 10% $AlCl_3$ solution, and 0.5 mL of 1 M NaOH solution were added. Deionized water was added to make the final volume to 2.5 mL. The absorbance was read at 510 nm after 15 min. The contents of total flavonoids were measured and then expressed as catechin equivalent (CE, mg catechin/g sample) through the calibration curve of catechin. The calibration curve range was 10–200 μg/mL ($R^2 = 0.9983$).

2.4. Extract post-treatment before chromatographic analysis

The extract collected in the UMAE extraction procedure at optimized conditions was evaporated to near dryness in a rotary evaporator. The residue was redissolved in 20 mL water and extracted with ethyl acetate (30 mL, 30 mL and 20 mL) for three times. The extracts were combined, evaporated close to dryness and redissolved in 5 mL methanol. The solution was centrifuged (13,000 × *g*, 10 min) prior to injection into HPLC system.

2.5. Identification of flavonoid compounds by LC–Q-TOF-MS

The qualitative study of the flavonoid compounds in extract was performed using LC–Q-TOF-MS. An Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, micro degasser, an auto plate-sampler and thermostatically controlled column oven was applied. Detections were performed using a 6520 quadrupole time-of-flight tandem mass spectrometer with mass resolution up to 40,000 (Agilent Technologies, Santa Clara, CA, USA).

Chromatographic separations were achieved on an Extent-C_{18} column of 250 mm × 4.6 mm, 5 μm particle size (Agilent Technologies, Santa Clara, CA, USA). The chromatographic conditions were as follows: flow rate of 1 mL/min, sample injection volume of 1 μL, column temperature of 25 °C and mobile phase A (0.1% formic acid) and mobile phase B (100% acetonitrile). The gradient profile was optimized as the following: 0–10 min, 8% B; 10–18 min, 8–15% B; 18–40 min, 15–25% B; 40–75 min, 25–45% B; 75–85 min, 45–70% B; 85–90 min, 70–100% B.

The electrospray source of the MS was operated in both positive and negative modes and the operating parameters were: drying gas (N_2) flow rate, 10.0 L/min; drying gas temperature, 320 °C; nebulizer, 35 psig; capillary, 3000 V; Oct RFV, 750 V; and fragmentor voltage, 120 V. All the operations, acquisition, and analysis of data were monitored by Agilent LC–Q-TOF-MS MassHunter Acquisition Software Version A.01.00 (Agilent Technologies) and operated under MassHunter Acquisition Software Version B.02.00 (Agilent Technologies). Mass spectra were recorded across the range *m/z* 100–3000 with accurate mass measurement of all mass peaks. To optimize signals and obtain maximal structural information, the

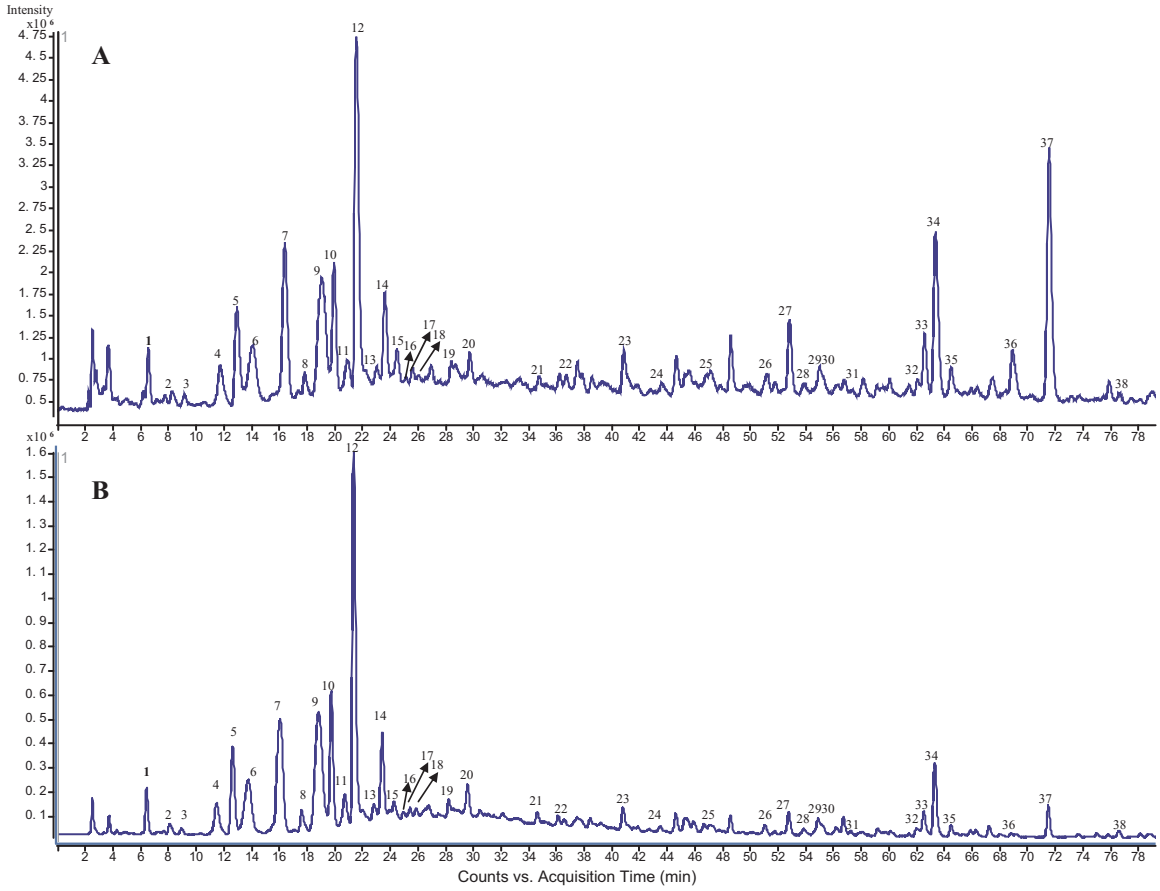


Fig. 3. Total ion chromatograms of *Spatholobus suberectus* extract in positive (A) and negative ion mode (B).

collision energy was adjusted from 5 V to 60 V for MS/MS experiments.

2.6. Statistical analysis

In orthogonal array design experimental arrangement and analysis, SPSS software (Version 13.0) was employed. Statistical significant was set at $p < 0.05$. Significance of the different was evaluated by orthogonal design ANOVA method.

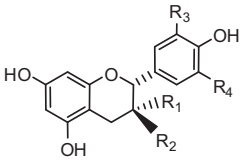
3. Results and discussion

3.1. UMAE method development

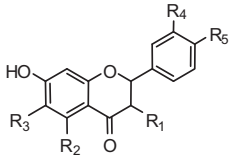
As a hybrid of microwave-assisted extraction and ultrasonic extraction, UMAE can provide energy by simultaneous irradiation with microwaves and ultrasound. Thus, it is necessary to optimize extraction conditions for a better usage of both types of energy. Six factors were optimized in this study using a six-factor-five-level orthogonal array design experiment (OAD) ($L_{25}(5^6)$), including microwave power (factor A), extraction time (factor B), extraction solvent (factor C), solvent to solid ratio (factor D), ultrasound power (factor E) and extraction temperature (factor F). The extraction yield of total flavonoids was selected as the evaluation criterion. All the levels and factors are shown in Table 1.

The mean extraction yield of total flavonoids at the five levels for each of the six extraction factors are shown in Table 2. As seen in Table 2, the range showed that the effects of the six factors on the extraction efficiencies in the order was A > C > D > E > F > B. The best combination of levels was A_3 B_3 C_3 D_2 E_2 F_3. With the greatest range among the five levels, microwave power was the most important factor influencing flavonoids extraction. The extraction yield increased as microwave power was increased from 100 W to 300 W, but decreased when the microwave power was increased to 400 W. Reduction in extraction yield at 500 W of microwave power was also observed. Microwave power corresponded to extraction temperature. A higher microwave powers would shorten the time to reach the maximum extraction temperature (e.g. the boiling point of the extraction solvents), and prolonged the exposure time of the analytes at the elevated temperatures. As a result, some labile flavonoids may have experienced thermal-degradation, thus extraction efficiency decreased. The other factors, namely extraction time, extraction solvent, solvent to solid ratio, ultrasound power and extraction temperature, showed less effect on extraction efficiencies for the flavonoids extraction (Table 2). The above results were further confirmed by variance analysis in Table 3. According Table 3, microwave power has significant effects ($p < 0.05$) on the extraction yield.

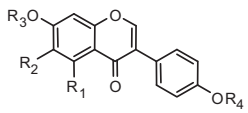
In summary, the optimum conditions of UMAE for extraction of flavonoids from *S. suberectus* were: microwave power of 300 W, extraction time of 450 s, 70% methanol as extraction solvent, solvent to solid ratio of 20 mL/g, ultrasound power of 50 W and extraction temperature of 80 °C. The effect of the number of extraction cycles (1–3 times) on extraction efficiency under the above optimized conditions was also investigated. The results suggested that the extraction yield obtained by extraction for one cycle (18.79 mg/g) was not significant different from those for two or three extraction cycles (18.86 and 18.89 mg/g respectively). Therefore, one extraction cycle was considered to be appropriate.



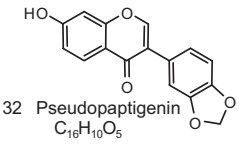
No.	Compound	Formula	R_1	R_2	R_3	R_4
1	Epigallocatechin	$C_{15}H_{14}O_7$	OH	H	OH	OH
5	Gallocatechin	$C_{15}H_{14}O_7$	H	OH	OH	OH
7	Catechin	$C_{15}H_{14}O_6$	H	OH	OH	H
12	Epicatechin	$C_{15}H_{14}O_6$	OH	H	OH	H
17	Afzelechin	$C_{15}H_{14}O_5$	H	OH	H	H
Compounds 6, 9, 10, 15, 20	(Epi)catechin-(epi)catechin					
Compounds 11, 13, 16	(Epi)catechin-(epi)catechin-(epi)catechin					
Compounds 8, 14, 21	(Epi)afzelechin-(epi)catechin					
Compound 18	(Epi)afzelechin-(epi)catechin-(epi)catechin					
Compounds 3, 4	(Epi)gallocatechin-(epi)catechin					



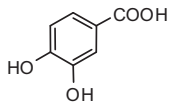
No.	Compound	Formula	R_1	R_2	R_3	R_4	R_5
19	Plathymenin	$C_{15}H_{12}O_6$	H	H	OH	OH	OH
22	Hesperetin	$C_{16}H_{14}O_6$	H	OH	H	OH	OCH_3
26	Liquiritigenin	$C_{15}H_{12}O_4$	H	H	H	H	OH
30	Naringenin	$C_{15}H_{12}O_5$	H	OH	H	H	OH
31	Suberectin	$C_{16}H_{14}O_6$	H	H	OCH_3	OH	OH
27	3,7-Pihydroxy-6-methoxyflavonol	$C_{16}H_{14}O_5$	OH	H	OCH_3	OH	OH



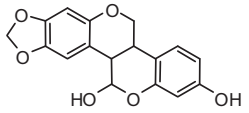
32 Pseudopaptigenin $C_{16}H_{10}O_5$



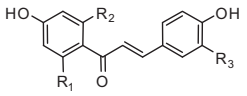
No.	Compound	Formula	R_1	R_2	R_3	R_4
23	Ononin	$C_{22}H_{22}O_9$	H	H	Glu	CH_3
24	Daidzein	$C_{15}H_{10}O_4$	H	H	H	H
25	Calycosin	$C_{16}H_{12}O_5$	H	H	H	CH_3
29	Genistein	$C_{15}H_{10}O_5$	OH	H	H	H
34	Formononetin	$C_{16}H_{12}O_4$	H	H	H	CH_3
35	Afrormosin	$C_{17}H_{14}O_5$	H	OCH_3	H	CH_3
36	Prunetin	$C_{16}H_{12}O_5$	OH	H	CH_3	H
38	BiochaninA	$C_{16}H_{12}O_5$	OH	H	H	CH_3



2 Protocatechuicacid $C_7H_6O_4$



37 Medicagol $C_7H_6O_4$



No.	Compound	Formula	R_1	R_2	R_3
28	Butein	$C_{15}H_{12}O_5$	OH	H	OH
33	Isoliquiritigenin	$C_{15}H_{12}O_4$	H	OH	H

Fig. 4. Chemical structures of compounds identified in the *Spatholobus suberectus* extract.

3.2. The stability of flavonoids under optimized UMAE conditions

The stability of flavonoid compounds was evaluated under the optimized UMAE conditions. The results are presented in Table 4. The mean recoveries (relative to the reference) of the analyzed flavonoid standards were between 88.9 and 98.3%, indicating all the analytes were observed to be stable under the conditions used. The recovery of these compounds from real samples was also determined under the same conditions as those applied to the standards. Drug powders were spiked with different amounts of the standards, and then extracted by UMAE. Recovery was obtained by dividing the difference between the flavonoids amount in the spiked sample and the original amount in sample by the amount of added standards. The mean recoveries of the flavonoid compounds were in the range of 87.1–101.5%. The above recovery data obtained by UMAE was acceptable, and UMAE technique is considered to be viable for the extraction of flavonoids.

3.3. Comparison of UMAE with conventional extraction methods

In this work, UMAE and conventional extraction methods including MAE, UAE, SE and HRE were compared for their extraction efficiency. The extraction yields of total flavonoids obtained by five extraction methods under the optimal conditions are summarized in Table 5. The extraction time of UMAE, MAE, UAE, SE and HRE were 450 s, 0.5 h, 1 h, 6 h and 4 h, respectively. The extraction solvent of UMAE, MAE, UAE and HRE was 70% methanol, and the extraction solvent of SE was 100% methanol.

The extraction yields of flavonoids obtained using UMAE and HRE methods were higher than other methods (Table 5). The highest extraction yields using HRE was obtained after 4 h extraction under 90 °C. While the extraction completeness by HRE depended to a large extent on the extraction time and the extraction solvent volume, the completed extraction was achieved only in 450 s by UMAE with 20 mL 70% methanol. The excellent extraction efficiency of the UAME method compared to the conventional extraction methods may benefit from its synergistic effect by coupling two energetic radiations [24]. Microwave radiation provides energy through the dielectric mechanism, and forms bulk heating. However, the ultrasound effect resulted from cavitation, a phenomenon of the formation, growth, and implosive collapse of bubbles in the solvent. By coupling microwave and ultrasound radiations in the UAME technique, the extraction process was accelerated by improved dielectric heating, effective agitation, and enhanced mass transfer. The extraction time was thus dramatically reduced and the extraction efficiency was considerably improved. These results confirmed that UMAE had great potential and high efficiency for the extraction of flavonoids from *S. suberectus*.

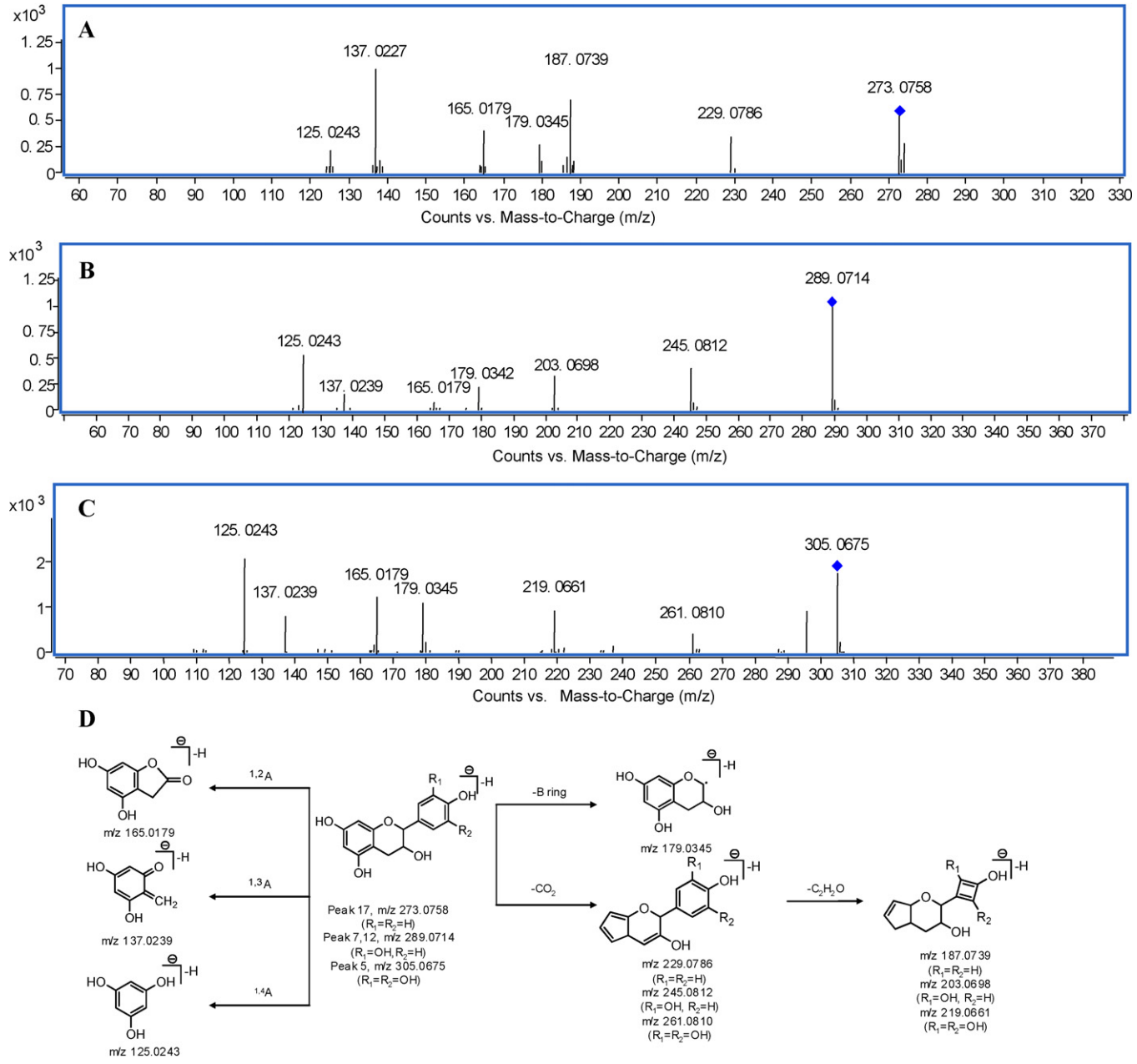


Fig. 5. (−)ESI-MS/MS mass spectra of afzelechin (peak **17**, A), catechin (peak **12**, B) and gallocatechin (peak **5**, C) and their proposed fragmentation pathways (D).

3.4. Diagnostic ion and characteristic pathways of flavonoids

To study the fragmentation pathways of different flavonoid aglycones, (−)ESI-MS/MS spectra of available reference compounds were obtained using the deprotonated ion as precursor ion. Most of fragment ions arise from retro-Diels-Alder (RDA) reactions, and are characteristic for flavonoid identification. In this study, the RDA fragment ions containing intact A and B ring are designated as $^{i,j}A^-$ and $^{i,j}B^-$ respectively. The superscripts i and j indicate the cleavage position of C-ring bonds. Among these fragment ions, the $^{1,3}A^-$ ion, observed for all flavonoid groups, was readily formed and often constituted the most abundant fragment ion. Losses or successive losses of small neutral molecules were also commonly observed, including CO (28 Da), CO_2 (44 Da), C_2H_2O (42 Da). The flavonoids with two OH groups in *ortho* positions could undergo a loss of H_2O. While the OMe-substituted flavonoids gave the $[M-H-CH_3]^-$ ion as their base peaks in the product ion spectra, then fragmented in the similar pathways to the OH-substituted ones.

For flavan-3-ols (catechins), intense C-ring cleavages in bonds 1/3 and 1/4 generated $^{1,3}A^-$ and $^{1,4}A^-$ ions. The ion $^{1,3}A^-$ at *m/z* 137.02 was the base peak. Along with the ion $^{1,4}A^-$ at *m/z* 125.02, $^{1,3}A^-$ ion was a marker of flavan-3-ols. In addition to the same fragments of neutral losses as those described above, characteristic $^{0,3}B^-$ RDA fragment ion and $[M-H-56]^-$ ion (a loss of two CO from the $[M-H]^-$) were observed for the isoflavonoids. This observation was in agreement with previous reports [25,26]. Flavanones yielded $^{1,3}A^-$ ions as base peaks with a few other RDA fragments in MS/MS analysis. For flavanonol aglycones with an OH group on C-3, RDA fragmentation leading to the $^{1,3}A^-$ or $[^{1,3}A^- - CO_2]$ ions was dominant. Unlike the rest of flavonoid families, chalcones do not present a heterocycle C, but an open and unsaturated 3-C chain. The fragmentation patterns of chalcones were characteristic for their cleavage at the bond between the carbon from the keto group and

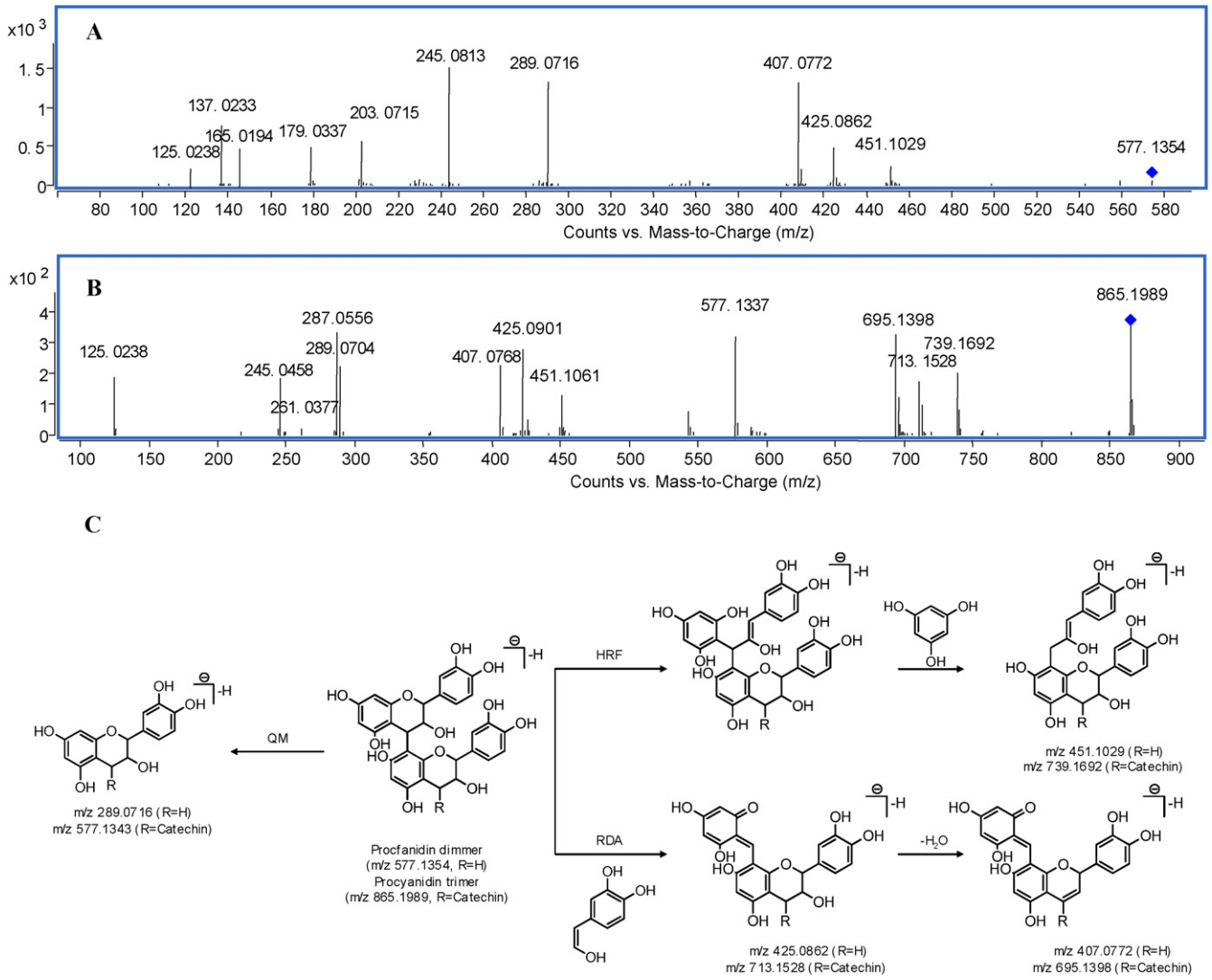


Fig. 6. (−)ESI-MS/MS mass spectra of procyanidin dimmers (A) and trimers (B) and their proposed fragmentation pathways (C).

α carbon to carbonyl, which provided important information on the substitution of OH or OMe groups on the A- and B-rings.

3.5. Diagnostic ion filtering strategy for rapid characterization of flavonoids in S. suberectus

After obtaining characteristic fragmentation pathways and diagnostic ions with available reference compounds, the complex *S. suberectus* extract was tested. Target compounds can be unequivocally identified by comparison of accurate retention times, molecular ions, and characteristic fragment ions with those of the reference compounds. Identification of untargeted compounds from herbal medicines is challenging. Since components contained in herbal medicines can usually be classified into families, and a certain family of components share common mother moieties, thus producing identical fragment ions in tandem mass spectrometry. Bearing this in mind, a diagnostic ion filtering strategy shown in Fig. 2 was proposed for characterization of unknown flavonoids in *S. suberectus*.

Firstly, the molecular ions of chromatographic peaks in extract were determined using both negative and positive ion mode for complementary information. Then, the accurate molecular formula of each peak was calculated and applied to subsequent screening for a hit against various chemical databases. Secondly, the extract was subjected to MS/MS experiment for fragment ions. The characterized diagnostic ions obtained with the reference compounds can then be used to filter and classify the untargeted compounds. In the first step, all the collected spectra were separated into two groups based on the fragment ions *m*/*z* 125.02 and 137.02. The group with the two ions was termed as proanthocyanidins (flavan-3-ols), which was further separated into monomer and oligomers based on $[M-H]^-$ ions. For oligomers, if the MS^2 spectra could generate ions *m*/*z* 289.07 and 303.07, the chemicals could be identified as prodelphinidins. If not, the compounds were termed as propelargonidins with *m*/*z* 289.07 and 271.07 ions, or procyanidins with *m*/*z* 289.07 and 287.06 ions. The dimmers and trimers of prodelphinidin, propelargonidins or procyanidins were separately identified on the basis of characterized $[M-H]^-$ ions. For the compounds without *m*/*z* 125.02 and 137.02 in the MS^2 spectra, $^{1,3}A^-$ was further applied for filtering flavonoids and other compounds. Then, the flavonoids were separated into isoflavonoid groups and flavanone or flavanonol groups based on the $^{0,3}B^-$ RDA fragment ion and $[M-H-56]^-$ ion. Finally, the database hits and filtering results were combined to locate the candidates. By fragmentation screening and matching, the most possible structure of unknown compounds can then be obtained.

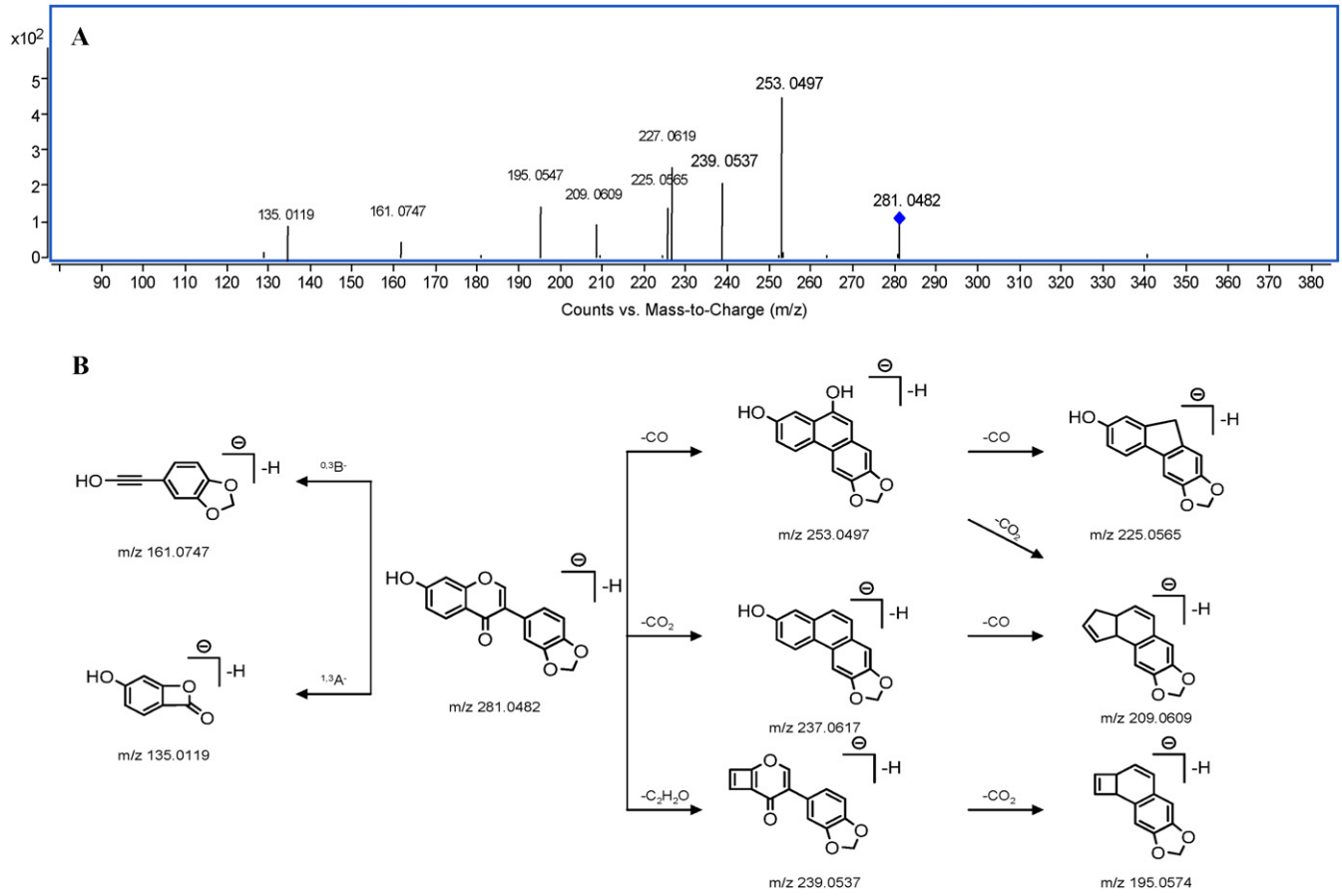


Fig. 7. (−)ESI-MS/MS mass spectrum (A) and proposed fragmentation pathways (B) of pseudopaptigenin.

3.6. Identification of flavonoids in *S. suberectus*

With the diagnostic ion filtering strategy described above and the obtained fragment pathways, thirty-six flavonoids and two other compounds were identified in real *S. suberectus* sample by LC–Q-TOF-MS. The TICs of the extract in positive and negative ion mode are presented in Fig. 3(A) and (B), respectively. The retention times and MS data of the characterized compounds are summarized in Table 6, and their chemical structures are shown in Fig. 4. The identification of each type of compounds is outlined below.

3.6.1. Flavanols

As polyhydroxyflavan-3-ols, proanthocyanidins includes monomers, oligomers and polymers. Previous studies on the chemical isolation of *S. suberectus* indicated the occurrence of catechin, epicatechin and gallocatechin [27]. However, up to date, few literatures regarding to other proanthocyanidins, particularly the oligomers, have been reported for this plant. In this study, a total of 19 proanthocyanidins were detected and identified. As far as we know, these compounds except for catechin, epicatechin and gallocatechin, were reported in *S. suberectus* for the first time.

3.6.1.1. Monomers. As presented in Table 6, peaks **1** (t_R = 6.63 min) and **5** (t_R = 12.61 min) had identical MS data. Peak **1** was definitely identified as epigallocatechin by comparing with corresponding standard. Peak **5** was tentatively deduced as gallocatechin according to a previous report [28]. Peaks **7** and **12** exhibited identical molecular $[M-H]^-$ and identical product ions. They were respectively identified as catechin and epicatechin by comparison with standards. In negative ion mode, peaks **7** exhibited a high-intensity ions at *m*/*z* 289.0714, *m*/*z* 245.0812 and 203.0698, representing $[M-H]^-$, $[M-H-CO_2]^-$ and $[M-H-CO_2-C_2H_2O]^-$, respectively. In addition, characteristic RDA ions $^{1,3}A^-$ (*m*/*z* 137.0239) and $^{1,4}A^-$ (*m*/*z* 125.0243), as well as fragment ions [M−H−B ring]$^-$ (*m*/*z* 179.0345) and $^{1,2}A^-$ (*m*/*z* 165.0179), were also observed. Peak **17** generated deprotonated ion at *m*/*z* 273.0758, 16 Da (O) less than that of catechin. For MS/MS spectrum, product ions representing $[M-H-CO_2]^-$, $[M-H-CO_2-C_2H_2O]^-$, $^{1,2}A^-$, $^{1,3}A^-$, $^{1,4}A^-$ and [M−H−B ring]$^-$ were detected, indicating that peak **17** fragmented in similar pathways as catechin. As a proanthocyanidin monomer, peak **17** was tentatively assigned as (epi)afzelechin [29]. Peaks **5**, **12** and **17** showed similar MS behaviors. Their mass spectra and elucidated fragmentation pathways are presented in Fig. 5.

3.6.1.2. Oligomers. Procyanidins are composed of flavan-3-ol units, epicatechins and/or catechins, linked mainly through C4–C8 bond. The *m*/*z* 577 and 865 in (−)ESI were indicative of procyanidin dimmers and trimers. For dimmers/trimers, the heterocyclic ring fission (HRF), retro-Diels-Alder fission (RDA) and quinine methide fission cleavge (QM) were the favored fragmentation, producing *m*/*z* 451/739, *m*/*z* 425/713 and *m*/*z* 289/577 ions (Fig. 6). The loss of H_2O after RDA fission of the heterocyclic rings gave product ion at *m*/*z* 407 for dimmer and *m*/*z* 695 for trimer. It is worth noting that the fragmentation pattern of the ion *m*/*z* 577 is the same as that of procyanidin B2. In this work, five procyanidin dimmers (peaks **6**, **9**, **10**, **15**, **20**) and three trimers (peaks **11**, **13**, **16**) were detected in *S. suberectus* (Table 6). These dimmers or trimers were stereoisomers, giving rise to almost the same mass fragment pattern. Peak **10** was unambiguously identified as procyanidin B2 (epicatechin–epicatechin) by comparing with reference compound. For other compounds, due to the absence of authentic

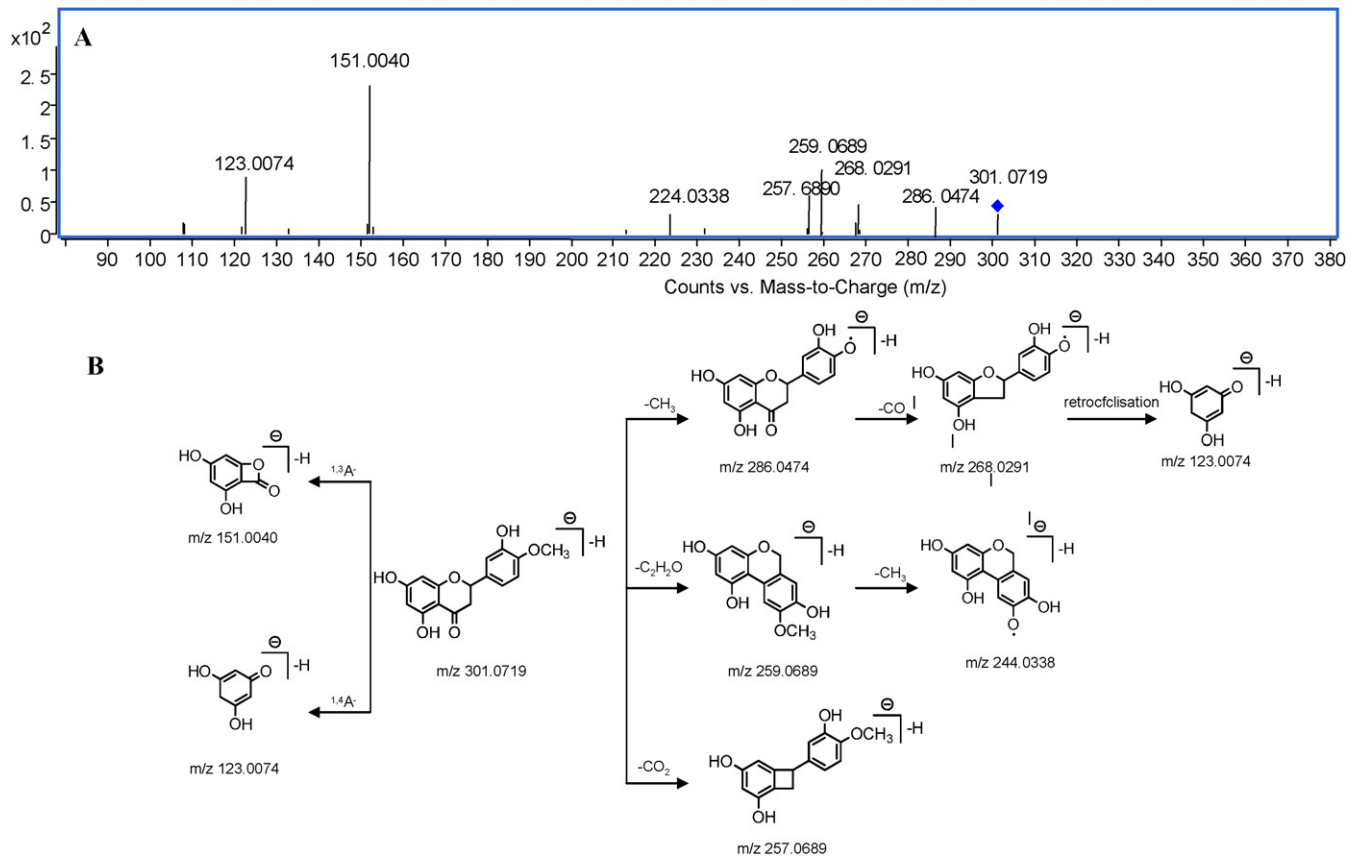


Fig. 8. (−)ESI-MS/MS mass spectrum (A) and proposed fragmentation pathways (B) of hesperetin.

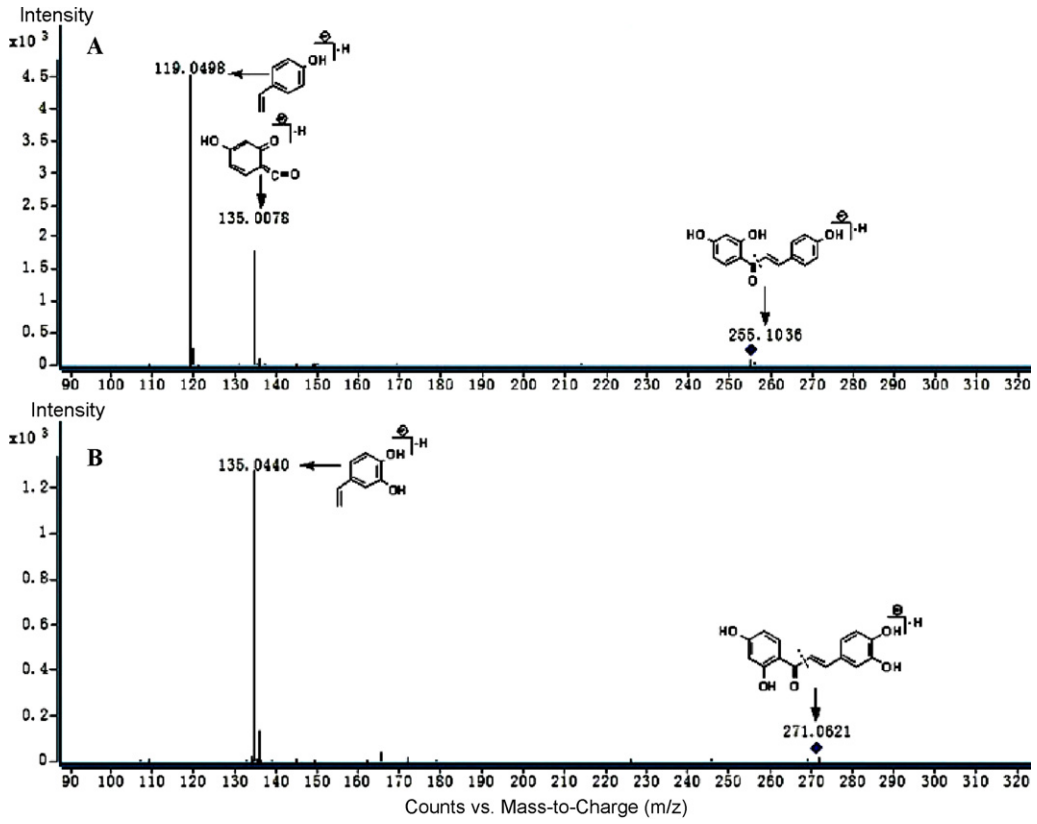


Fig. 9. (−)ESI-MS/MS mass spectra of isoliquiritigenin (A) and butein (B).

compounds, the position and the stereochemistry of the interflavan linkage (C4 → C6 or C4 → C8) cannot be explained by mass spectrometry.

Several propelargonidins were also detected in the extract of *S. suberectus*. In negative ion mode, the *m/z* 561 is 16 Da less than *m/z* 577, suggesting the existence of a subunit with one less hydroxyl group than the procyanidin. The HRF and RDA fragmentation ions at *m/z* 435 and 425 were dominant. The QM fragment ions *m/z* 271 and 289 signed the extension unit of this dimmer was (epi)afzelechin and terminal unit was (epi)catechin. According to Verardo et al. [30], peaks **8**, **14** and **21** were assigned to be (epi)afzelchin–(epi)catechin dimmer isomers. The trimer of (epi)afzelchin–(epi)catechin–(epi)catechin at *m/z* 850.2168 (molecular formula $C_{45}H_{38}O_{17}$) was detected at t_R of 25.88 min (peak **18**) and the same spectrum was reported by Gu et al. [31].

As depicted in Table 6, two dimmers of prodelphinidins ($[M-H]^-$ *m/z* 593) were detected at t_R of 8.93 min (peak **3**) and 11.46 min (peak **4**). The MS/MS analysis indicated that the fragmentation of prodelphinidins followed the same scheme as procyanidins. According to the literature [32], the dimmer was identified as (epi)gallocatechin–(epi)catechin. The (epi)gallocatechin position was determined on the basis of the RDA (*m/z* 425), HRF(*m/z* 467) and QM (*m/z* 305) fragment ions.

3.6.2. Isoflavonoids

Peaks **23**, **24**, **25**, **29**, **34** and **38** were definitely identified as ononin, daidzein, calycosin, genistein, formononetin and biochanin A by comparison with the reference standards. Peak **32** produced a $[M-H]^-$ ion at *m/z* 281.0642 and abundance fragment ions, such as ions at *m/z* 253.0497, 239.0537, 237.0617, 225.0565, 209.0609 and 195.0574 (Fig. 7A). The product ions at *m/z* 225.0565 and 209.0609 were generated from the elimination of CO and CO_2 from *m/z* 253.0497 ion. The characteristic fragment ions at *m/z* 161.0747 ($^{0,3}B^-$) and 135.0119 ($^{1,3}A^-$) were also observed by the C-ring RDA fragment. Peak **32** was tentatively identified as pseudobaptigenin and previously described in *S. suberectus* by Yoon et al. [33]. The fragment pathways of peak **32** are proposed in Fig. 7B.

Peak **35** gave $[M-H]^-$ ion at *m/z* 297.0401. Its MS^2 spectrum gave $[M-CH_3-H]^-$ and $[M-2CH_3-H]^-$ ions at *m/z* 282.0536 and 267.0319 as the base peaks, suggesting the presence of two methoxyl groups. Based on RDA fragment ions *m/z* 179.0464 ($^{0,3}B^-$) and 165.0507 ($^{1,3}A^-$), one methoxy group was assumed to attach to the A-ring and the other was attached to B-ring. The characteristic losses of CO (28 Da), CO_2 (44 Da) and successive loss of two CO (56 Da) from *m/z* 267.0319 were also observed, resulting in product ions at *m/z* 245.0532, 223.0406, and 211.8808. Peak **35** was tentatively ascribed to afrormosin which was previously reported in this herb [28]. Peak **38** had the same molecular formula and showed similar fragmentation pathways as peak **35**. It was assigned as prunetin [34].

3.6.3. Flavanones and flavanonols

Five flavanones and one flavanonol were identified in *S. suberectus*. Peaks **26** and **30** were identified as liquiritigenin and naringenin with available reference compounds. Peak **19** exhibited $[M-H]^-$ at *m/z* 287.0557, $[M-H-2H_2O]^-$ at *m/z* 251.2369, $^{1,3}B^-$ at *m/z* 135.0452 and $[^{1,3}A\text{-}CO]^-$ at *m/z* 123.0079. This compound was identified as plathymenin and previously reported in *S. suberectus*. [28]. Peak **22** was assigned to hesperetin and confirmed with standard compound. Hesperetin yielded a $[M-H]^-$ signal at *m/z* 301.0719 and a main fragment ion at *m/z* 286.0474 by the loss of methyl radical. Several neutral losses of CO, C_2H_2O and CO_2 were also observed. The RDA fragmentations involving the 1 and 3 bond and the 1 and 4 bond produced abundant ions at *m/z* 151.0040 ($^{1,3}A^-$) and *m/z* 123.0074 ($^{1,4}A^-$). These results were in agreement with a previous report [35]. The mass spectrum and proposed fragment pathways of peak **22** are shown in Fig. 8.

Compound **27**, exhibiting $[M-H]^-$ at *m/z* 285.0773, showed the elemental composition of $C_{16}H_{17}O_5$. The fragment ion at *m/z* 270.0526, producing by the elimination of CH_3 from $[M-H]^-$, was readily observed as the base peak. Further losses of CO_2, CO or H_2O formed ions at *m/z* 252.0409, 242.0573 and 224.0457. Compound **27** was tentatively identified as 3, 7-dihydroxy-6-methoxyflavonol [7]. As seen in Table 6, peak **31** had the same molecular ions but different fragment ions compared with peak **23**. Peak **31** was tentatively assigned as 7, 3′, 4′-trihydroxy-6-methoxyflavanone [7].

3.6.4. Chalcones and other compounds

Two chalcones, peaks **33** and **28**, were also detected. The $[M-H]^-$ ion at *m/z* 255.0659 of peak **33** gave two major product ions at *m/z* 119.0498 and 135.0078 (Fig. 9A). This compound was identified as isoliquiritigenin with a reference compound. As shown in Fig. 9B, peak **28** fragmented in similar pathway as isoliquiritigenin, and its molecular formula was determined as $C_{15}H_{12}O_5$. Peak **28** was tentatively assigned as butein [7]. The major product ion at *m/z* 135.0440 further confirmed this identification. Two non-flavonoids, peaks **2** ($[M-H]^-$ at *m/z* 153.0290) and 37 ($[M-H]^-$ at *m/z* 153.0190), were also detected. They were characterized as protocatechuic acid and medicagol respectively by comparison with reference compounds.

4. Conclusions

Sample preparation and analysis are two key steps towards the quality, safety and efficacy of herbal medicines. Whether an analytical technique is suitable depends on its capability for extraction and characterization of the target and non-target chemical constituents in an herbal sample. In this work, a potential UMAE method was developed for rapid and efficient extraction of total flavonoids in *S. suberectus*. Because of its higher efficiency and shorter extraction time for sample preparation compared with conventional extraction methods, UMAE may turn out to be a highly useful technique to extract target constituents in herbs, as well as a valuable addition to present analytical tools. This work also proposed a new diagnostic ion filtering strategy with LC–Q-TOF-MS for rapid characterization of chemical compounds in herbs. By matching diagnostic ions and fragmentation pathways, a total of thirty-eight compounds including thirty-six flavonoids and two non-flavonoid compounds were detected and characterized from the real *S. suberectus* extracts. The inherent characteristics of Q-TOF in accurate mass measurements, full-scan spectra, and abundant fragment ions make this analyzer very attractive in the qualitative analysis. The results of this study clearly demonstrate the potential of UMAE for efficient extraction and LC–Q-TOF-MS for rapid and sensitive structural elucidation of flavonoids in *S. suberectus*, and open perspectives for similar studies on other medicinal herbs.

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