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Qualitative and quantitative analysis of sesquiterpene lactones in *Centipeda minima* by UPLC-qTOF-MS & UPLC-QQQ-MS

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Abstract

An ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-qTof-MS) method has been used to identify sesquiterpene lactones in the methanolic extract of *Centipeda minima*. Fifteen sesquiterpene lactones were tentatively identified based on retention time and accurate mass of external standards or exact accurate mass searching (within two ppm) by comparison of some previous isolated sesquiterpene lactones. Meanwhile, a rapid, sensitive, precise, and reliable ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UPLC-QQQ-MS) method has been developed to evaluate the quality of *Centipeda minima* through a simultaneous determination of five sesquiterpene lactones, namely Brevilin A ,Arnicolide C, Arnicolide D, Microhelenin C, Minimolide F. Chromatographic separation was achieved on a Waters Acquilty UPLC C18 column (2.1 × 50 mm, 1.7 m) with a mobile phase consisting of a) 0.1% formic acid and b) a mixture of acetonitrile and methanol 50:50v/v under an isocratic elution (42:58) manner. Positive electrospray ionization mode with multiple reaction monitoring was applied for the detection of the five sesquiterpene lactones. Method validation for linearity, accuracy and precision was also carried out. Finally, the method was successfully used for the analysis of 10 batches of *Centipeda minima* samples collected in China. Brevilin A and arnicolide D were the dominant sesquiterpene lactones in *Centipeda minima* and could be proposed as suitable markers for the quality control of *Centipeda minima*

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1. Introduction

Centipeda minima (L.) A. Braun and Ascherson, known as Ebushicao in Chinese, is an annual herbaceous plant widely distributed in China and eastern tropical Asia [1]. The use of the whole plant in Chinese folk medicine for treating cold, nasal allergy, diarrhea, malaria and asthma has a long history [2]. Previous pharmacological studies have demonstrated that extracts of *Centipeda minima* and some isolated compounds from the *Centipeda* minima possess anti-tumor [3, 4], anti-proliferative [5, 6], antioxidant and anti-inflammatory [7, 8] properties. Phytochemical investigation of this herbal material has led to the identification of various compounds such as sesquiterpene lactones, triterpenes, flavonoids, polyphenol, phenolic and organic acid [8-12]. Due to their abundant and commercial availability, phenolic compounds such as protocatechualdehyde, vanillic acid, isochlorogenic acid A and isochlorogenic acid C have been utilized as marker for the quality control of herbal material and extract [8, 9, 12]. However, these markers are also widely present in other plants, and are thus not so representative ingredients contributing to the biological activities of this herbal material.

In recent years, some sesquiterpene lactones such as brevilin A, arnicolide C and arnicolide D have been isolated and exerted excellent cytotoxic activity against colon adenocarcinoma CT26 cells [3] and human colon carcinoma HT-29 cell line [13]. It has been suggested that the double bond in the cyclopentane ring and the double bond at position 10 are the structural components that are responsible for the activity [13]. Thus, the use of sesquiterpene lactones as chemical marker for the quality control of *Centipeda minima* could have some comparative advantage.

Ultra-high performance liquid chromatography (UPLC), arising from the use of small particle diameter of column on the basis of HPLC, allows fast screening of a large number of phytochemicals, especially when it is coupled with mass spectrometry such as triple quadrupole and quadrupole time-of-flight. In our work, UPLC coupled with high-resolution orbitrap mass spectrometry was utilized for identification of fifteen sesquiterpene lactones in *Centipeda minima*. MS/MS scan mode was used to obtain their fragmentation pattern and further applied to identify other unknown sesquiterpene lactones. Meanwhile, a rapid, accurate, and reliable UPLC coupled with triple quadrupole mass spectrometry (QQQ-MS) method was developed to quantify five sesquiterpene lactones in ten batches of *Centipeda minima*. Multiple reaction monitoring (MRM) mode was used to effectively avoid the interference of peak overlapping and give extremely low detection and quantitation limits. Finally, method validation for linearity, accuracy and precision was also carried out. To our knowledge, this is the first report of an analytical method for simultaneous determination of sesquiterpene lactones in *Centipeda minima*.

2. Material and method

2.1 Plant material

Ten batches of *Centipeda minima* samples were collected from herbal markets in China and all samples were authenticated by Dr. Sibao Chen, based on the microscopic feature of the volume 7

of the Hong Kong Chinese Materia Medica Standards [14] and the latest version of the Chinese Pharmacopoeia [15].

2.2 Chemicals and reagents

Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstad, Germany) while formic acid (HPLC grade) was purchased from Sigma Aldrich (Steinheim, Germany). Reference compounds of brevilin A ,arnicolide C, arnicolide D, microhelenin C, minimolide F, and deoxydlephantopin (Figure 1) were purchased from YONGJIAN Bio-technology CO. Ltd (Jiang Su, China) and the purity of all reference compounds was determined to be >98% by high performance liquid chromatography diode array detection. Double deionized water was purified by the Milli-Q water system (Millipore Corp., Bedford, MA, USA). All other reagents and chemicals used were of analytical grade.

2.3 Sample preparation

0.1g powder of herbal material was accurately weighed, sonicated for 30 min with 10 mL methanol (with spike of an internal standard) at room temperature. Then the solution was centrifuged at 13000 g for 10 min and the supernatant was transferred into a 25 mL volumetric flask. The extraction was repeated once and marked-up with methanol. The solution was filtered through a 0.22 μm PTFE syringe filter to an auto-sampler vial and an aliquot of 2 μL was injected into the MS system for simultaneously analysis.

2.4 Ultra-high performance liquid chromatography coupled to high resolution mass spectrometry

Chromatographic analysis was performed on a Waters Acquity UPLC system (Waters Co. Milford, MA, USA) equipped with a binary pump solvent management system, an online degasser, an auto-sampler and a column temperature maintainer set at 40℃. Chromatographic separation was conducted on an Acquity UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 μm) with BEH C18 guard column (2.1mm x 5 mm, 1.7 μm). The mobile phase was a mixture of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in a mixture of 50% acetonitrile and 50% methanol, v/v) at a flow rate of 0.3 mL/min with linear elution gradient as follows: 0-1 min, 5% B; 10-13 min, 95% B. A 3-min post-run time was set to fully equilibrate the column. The auto-sampler was conditioned at 6 ^{o}C.

Mass spectrometry analysis was achieved by a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer equipped with a heated electrospray ionization (H-ESI) interface (Thermo Fisher, Waltham, MA, USA). The mass-spectrometric conditions were optimized as follows: spray voltage, 3500V in positive H-ESI mode; ion transfer tube and vaporizer temperature, 300 ^{o}C and 320 ^{o}C respectively. Nitrogen gas was used as the sheath gas and the aux gas with a flow rate of 35 units and 10 units, respectively. The instrument was operated in data-dependent acquisition mode, with full MS scan mode over mass to charge ratio from 100 to 1200 and with Orbitrap at 120,000 resolution and RF Lens at 30% as detection. Mass fragmentation was carried

out for peak identification at normalized collision energy of 30±10% higher energy collisional dissociation with detection in the Orbitrap (30,000 resolution) MS2 detection. All the data were evaluated using Thermo Xcalibur Qual Browser software (Thermo fisher Scientific).

2.5 Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry

Chromatographic analysis was performed on a Waters Acquity UPLC system (Waters Co. Milford, MA, USA) equipped with a binary pump solvent management system, an online degasser, an auto-sampler and a column temperature maintainer set at 40℃. Chromatographic separation was conducted on an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm).The mobile phase consists of 0.1% formic acid water (A) and mixture of 0.1% formic acid ACN: MeOH (50:50, *v*/*v*) (B) at a flow rate of 0.30 mL/min using a gradient elution of 42% B at 0-8 min, 42-95% B at 8-10 min. A 3-min post-run time was set to fully equilibrate the column. The auto-sampler was conditioned at 4ºC.

Mass spectrometry detection was performed using XEVO triple quadrupole MS (Waters Co. Milford, MA, USA) with electrospray ion source (ESI). Quantification was performed using positive ion multiple reaction monitoring mode (MRM) with deoxydlephantopin as the internal standard (IS). Nitrogen was employed as curtain gas (CUR), nebulizer gas (GS1), heater gas (GS2) and collision gas. MS conditions were optimized as follows: capillary voltage 3.0 kv, source temperature 150 ºC, desolvation temperature 350 ºC, cone gas flow 45L/h, desolvation gas flow 800L/h. Cone voltage (CV) and collision energy (CE) of each analyte were optimized and summarized in Table 2. The Masslynx V4.1software (Waters) was used for instrument control, data acquisition and handling.

3. Results and discussion

3.1 Identification of sesquiterpene lactones by ultra-high resolution mass spectrometry

Positive ion ultra-high resolution mass spectrometry full analysis and sequential fragmentation by MSn of sesquiterpene lactones were first investigated. Figure 2 showed the based peak chromatogram of the methanolic extract of *Centipeda minima*. Brevilin A, arnicolide D and arinicolide C were detected via high performance liquid chromatography coupled with a diode array detector and mass spectrometry in previous work and their fragmentation pattern via mass spectrometry had been proposed [9]. In this work, the fragmentation patterns of others minor sesquiterpene lactones that were present in the extract of *Centipeda minima* including minimolide F and microhelenin C were discussed in more detail.

Minimolide F was isolated by the use of supercritical fluid extraction technology [16], and it consisted of an epoxide carbon ring in the skeleton of sesquiterpene lactones itself. A fragment m/z 317.1745, originated from the removal of H_2O at the O ring position, was intensely shown in the spectrum while 247.1329 m/z was observed due to the cleavage of 5-membered rings directly

from the parent ion m/z 335.1851 $[M+H]^+$ and m/z 357.1671 $[M+Na]^+$. Due to the uniqueness of this fragment m/z 317.1745 $[M-H_2O+H]^+$, it was chosen as precursor ion for further MSn analysis and the results were shown in Figure 3. Due to the cleavage of the C-O bond, this fragment m/z 229.1223 was the most prominent. Subsequently, breaking of the 5-membered rings yielded m/z 183.1168 and m/z 157.1012.

Similarly, minimolide E was tentatively identified in the extract of *Centipeda minima* at retention time of 7.29min. Not only two adduct parent ions 347.1852 m/z $[M+H]^+$ and 369.1671 m/z $[M+Na]^+$ were observed, but also m/z 329.1746, which originated from the removal of H_2O at the O ring position of an epoxide carbon ring in the skeleton of sesquiterpene lactones, was intensely shown in the spectrum. Furthermore, the presence of m/z 229.1223, m/z 183.1169 and m/z 157.1012 could help confirm the core skeleton of sesquiterpene lactones.

Microhelenin C, a structural isomer of brevilin A, was also present in the extract of *Centipeda minima* and two adduct parent ions m/z 347.1852 $[M+H]^+$ and m/z 369.1671 $[M+Na]^+$, were observed. MSn analysis of protonated adduct was carried out and the obtained fragments were m/z 247.1328, m/z 229.1222, m/z 201.1273, m/z 173.0960 and m/z 145.1011, which were in good agreement with the MS data of brevilin A. Moreover, it is worth mentioning that the above fragments belonged to the core skeleton of sesquiterpene lactones and served as a diagnostic fragment for allowing the detection of other unknown sesquiterpene lactones compounds.

Table 1 summarized that 15 sesquiterpene lactones were tentatively identified in the extract of *Centipeda minima*. Five sesquiterpene lactones including, arnicolide D, arinicolide C, minimolide F, microhelenin C and brevilin A were confirmed by external standards while other 10 sesquiterpene lactones were tentatively identified by exact accurate mass searching via the comparison of some previous isolated sesquiterpene lactones[16-22]. Information regarding their characteristic mass fragments from Msn analysis was further strongly supported their identities and details of their mass fragment was also included in Table 1.

3.2 Method development of quantitative analysis of five sesquiterpene lactones by UPLC-QQQ-MS

3.2.1 Optimization of UPLC and MS/MS condition

For optimization of MS conditions, full scan MS method was used to examine the target analytes in positive ionization mode. All the compounds were then determined respectively in direct infusion mode to optimize a proper transition for the MS/MS detection. $[M+Na]^+$ and $[M+H]^+$ were the basic protonated ions for all sesquiterpene lactones under positive ion mode. $[M+H]^+$ was selected as precursor ion for all sesquiterpene lactones because $[M+Na]^+$ was very stable. The response of product ion was very low even though high-energy collision-induced was applied. Then, the conditions of multiple reaction monitoring (MRM) determination including cone voltage and collision were optimized according to the highest sensitivity and specific ion

pairs. The MRM transitions and parameters of sesquiterpene lactones compounds are shown in Table 2.

Table 1 and Figure 2 showed that there were many sesquiterpene lactones isomers present in the methanolic extract of *Centipeda minima*. It is a challenging task to quantify these isomers, especially those with the same precursor/product ion pairs, via liquid chromatography coupled with mass spectrometry system. In our work, different mobile systems that consisted of acetonitrile – 0.1% formic acid and methanol – 0.1% formic acid were tried to separate the five sesquiterpene lactones. However, incomplete separation of arinicolide C and minimolide F was observed. Mixture of methanol and acetonitrile in different ratios were further utilized and methanol and acetonitrile (50:50 v/v) – 0.1% formic acid with isocratic elution (42:58, v/v) achieved good peak shape with well resolution among the five sesquiterpene lactones.

3.2.2 Optimization of extraction

To optimize the extraction procedure, samples were extracted using different concentrations of ethanol and methanol (50%, 70% and 100%) and the peak areas of the UPLC-QQQ-MS chromatograms obtained were compared. 100% methanol gave the best extraction efficiency for all sesquiterpene lactones. In addition, the number of successive extraction on the extraction efficiency was also examined and two extraction cycles were sufficient to extract all sesquiterpene lactones. Thus, it was decided that two cycles of 30-min ultrasonication extraction with 100% methanol were the optimal extraction condition for sesquiterpene lactones of *Centipeda minima* samples.

3.2.3. Method validation

The UPLC-QQQ-MS method developed was validated for its accuracy, linearity and precision. Accuracy studies were performed by spiking the known amount of standard with low, medium and high level to the extract of *Centipeda minima* before the extraction began. The recoveries were calculated by the formula: recovery (%) = (amount found – original amount) / amount spiked x 100%. Table 3 listed the results of recovery and our developed analytical method showed good accuracy with a wide range of concentrations (overall > 90%) for five sesquiterpene lactones. As for the linearity, all five sesquiterpene lactones exhibited good linearity ($r^2 > 0.995$) within the tested range. Results of the limit of detection (LOD) and limit of quantification (LOQ) were also summarized in Table 4. Finally, stability of extract was examined at 4ºC for two days. The inter-day (n=3) and intra-day (n=6) stability of sample extracts were less than 5% (shown in Table 5) and all analytes were found to be stable within the duration of the whole analysis. All the obtained results indicated that UPLC-QQQ-MS was precise, accurate and sensitive for quantitative determination of the five sesquiterpene lactones.

3.3 Sample analysis

The developed UPLC-QQQ-MS method was applied to study the content of the five

sesquiterpene lactones in the ten batches of *Centipeda minima* collected in China. Figure 4 showed the typical chromatograms of the standard solution and of an extract of *Centipeda minima*, in which the retention time of arnicolide D, arinicolide C, minimolide F, microhelenin C and brevilin A are 3.32min, 3.92min, 4.47min, 4.96min and 5.62min respectively.

In the latest version of Chinese Pharmacopoeia, assay requirement is not included in the medical usage of *Centipeda minima*. To ensure the quality and efficacy of herbal materials, stringent quality control standards that require the help of quantitative analysis of suitable markers are required. The class of sesquiterpene lactones has been reported as active ingredients that possess anti-cancer properties [3-6]. As shown in table 6, the contents of the five sesquiterpene lactones in the ten batches of samples collected varied greatly. Brevilin A and arnicolide D were the most abundant and their average contents were 0.58mg/g and 1.88mg/g, respectively. Due to their biological activities, abundants and commercial availability of chemical markers, these two reference chemicals will be suitable for the quality control of this herbal material.

4. Conclusion

A total of 15 of sesquiterpene lactones were tentatively identified in methanol extract of *Centipeda minima* by ultra-high resolution mass spectrometry. Furthermore, five sesquiterpene lactones, namely arnicolide C, arnicolide D, minimolide F, microhelenin C and brevilin A were determined simultaneously by the developed ultra-high liquid chromatography coupled with triple-quadrupole mass spectrometry (UPLC-QQQ-MS). Results revealed that brevilin A and arnicolide D were the dominant sesquiterpene lactones in the ten batches collected in China and could serve as suitable markers for quality control of *Centipeda minima*.

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A list of tables

Table 1: UPLC-ESI-qTof-MS identified sesquiterpene lactones compounds in *Centipeda minima*

No	Name	Retention Time (min)	Molecular formula	(m/z) mass accuracy (ppm)		Fragment ions observed
				$[M+Na]^+$	$[M+H]^+$	
1	Arnicolide A	5.43	$C_{17}H_{22}O_5$	329.1359 (0)	307.1539 (-0.33)	247.1328, 229.1222, 201.1273, 173.0960, 145.1011, 131.0855
2	Minimolide C	6.11	$C_{20}H_{28}O_6$	387.1777 (-0.26)	365.1947 (-3.29)	265.1434, 247.1328, 201.1274, 173.0961
3	Minimolide A	6.24	$C_{20}H_{28}O_6$	387.1778 (0)	365.1948 (-3.01)	265.1434, 247.1328, 201.1275, 173.0962
4	Arnicolide D	6.43	$C_{19}H_{24}O_5$	355.1515 (-0.28)	333.1696 (0.30)	247.1328, 229.1222, 201.1274, 173.0961,159.1186, 145.1012, 131.0856
5	Minimolide H	6.51	$C_{20}H_{26}O_6$	385.1621 (-0.26)	363.1801 (-0.27)	247.1328, 229.1222, 201.1275, 173.0962, 145.1012, 131.0856
6	Arnicolide C	6.61	$C_{19}H_{26}O_5$	357.1672 (0)	335.1852 (-0.30)	247.1328, 229.1222, 201.1273, 183.1168, 157.1012, 131.0856
7	Minimolide F	6.76	$C_{19}H_{26}O_5$	357.1671 (-0.28)	335.1851 (-0.60)	317.1745, 229.1223, 183.1168, 157.1011, 131.0855
8	Microhelenin C	6.84	$C_{20}H_{26}O_5$	369.1671 (-0.27)	347.1852 (-0.29)	247.1328, 229.1222, 201.1273, 173.0960, 145.1011, 131.0855
9	Brevilin A	6.95	$C_{20}H_{26}O_5$	369.1671 (-0.27)	347.1851 (-0.58)	247.1327, 229.1222, 201.1273, 173.0960, 145.1011, 131.0855
10	6-O-tigloyhelenalin	7.09	$C_{20}H_{24}O_5$	367.1515 (-0.27)	345.1697 (0)	247.1329, 229.1223, 201.1275, 173.0691, 145.1012
11	Arnicolide B / Microhelenin B	7.14	$C_{20}H_{28}O_5$	371.1827 (-0.54)	349.2007 (-0.86)	247.1328, 229.1225, 201.1274, 173.0960, 145.1011, 131.0854
12	Minimolide E	7.29	$C_{20}H_{26}O_5$	369.1671 (-0.27)	347.1852 (-0.29)	329.1746, 229.1223, 183.1169, 157.1012, 143.0856
13	Minimolide D	7.42	$C_{20}H_{28}O_5$	369.1671 (-0.27)	347.1852 (0.29)	247.1328, 229.1224, 201.1274, 173.0962
14	Arnicolide G	8.36	$C_{18}H_{24}O_5$	343.1514 (-0.58)	321.1695 (-0.62)	233.1693, 163.0753, 145.0648
15	2β-(Isobutyryloxy) florilenalin	8.74	$C_{19}H_{26}O_5$	357.1670 (-0.56)	335.1853 (0)	163.0751, 145.0648

Table 2: The information for MRM parameters.

No.	Compounds	Precursor	Production	Cone voltage	Collision energy	Retention time
1	Arnicolide D	333.1$[M+H]^+$	247.1	20	8	3.32
2	Arnicolide C	335.1$[M+H]^+$	247.1	20	8	3.92
3	Minimolide F	335.1$[M+H]^+$	229.1	20	12	4.47
4	Microhelenin C	347.1$[M+H]^+$	247.1	20	10	4.96
5	Brevilin A	347.1$[M+H]^+$	247.1	20	10	5.62
6	Deoxyelephantopin (IS)	347.1$[M+H]^+$	259.1	25	10	2.3

Table 3: Results of recovery

Compounds	Spiked concentration (ng/ml)	Recovery		Mean
		Average(%)	RSD(%)	
Arnicolide D	20	83.07%	4.36%	91.71 ± 3.82
	500	92.10%	5.38%	
	3000	99.95%	1.72%	
Arnicolide C	20	82.14%	5.39%	93.26 ± 4.04
	500	94.24%	5.21%	
	3000	103.40%	1.53%	
Minimolide F	20	82.61%	5.82%	93.73 ± 3.05
	500	96.60%	1.72%	
	3000	101.99%	1.61%	
Microhelenin C	20	77.20%	7.60%	93.68 ± 3.62
	500	89.38%	2.11%	
	3000	97.98%	1.14%	
Brevilin A	60	83.31%	5.09%	92.11 ± 4.10
	1500	90.74%	7.61%	
	9000	100.17%	1.99%	

Recovery = (Detected amount/Spiked amount) × 100; data are expressed in triplicate

Table 4: Linearity, limit of detection (LOD) and limit of quantitation (LOQ) of analytes

Compounds	Linear ranges (ng/mL)	R^2	LOQ (ng/mL)	LOD (ng/mL)
Arnicolide D	2 - 6000	0.9976	2.0	0.7
Arnicolide C	2 - 6000	0.9975	2.0	0.7
Minimolide F	7 - 4000	0.9972	7.0	2.0
Microhelenin C	2 - 6000	0.9973	2.0	0.7
Brevilin A	3 - 20000	0.9972	3.0	1

Table 5: Results of intra-, inter-day precision and recovery

Compounds	Intra-day (n = 6)		Inter-day (n = 3)	
	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)
Arnicolide D	0.8079	0.73	0.8165	1.62
Arnicolide C	0.4649	1.17	0.4611	1.25
Minimolide F	0.0666	3.15	0.0657	2.39
Microhelenin C	0.1731	0.66	0.1734	0.88
Brevilin A	2.9371	1.09	2.9244	3.74

RSD = (SD of amount detected)/(Mean of amount detected) × 100; data are the mean of three experiments.

Table 6: Quantitative determination of sesquiterpene lactones in *Centipeda minima*

Batch no.	Content (mg/g) [a]				
	Arnicolide D	Arnicolide C	Minimolide F	Microhelenin C	Brevilin A
S1	0.716	0.416	0.061	0.155	2.557
S2	1.135	0.431	0.089	0.089	1.345
S3	0.610	0.255	0.061	0.135	2.312
S4	0.297	0.157	0.030	0.067	1.170
S5	0.466	0.160	0.053	0.096	1.472
S6	0.440	0.197	0.039	0.094	1.721
S7	0.524	0.239	0.047	0.112	2.056
S8	0.797	0.406	0.077	0.186	2.950
S9	0.499	0.226	0.047	0.108	1.921
S10	0.358	0.172	0.037	0.073	1.245
Average	0.584	0.266	0.054	0.112	1.875

Data are expressed in mean ± SD (n = 3); the RSD is less than 5%.

A list of figures

Figure 1. Chemical structures of a) arnicolide D; b) arnicolide C; c) minimolide F; d) microhelenin C; e) brevilin A; f) deoxydlephantopin (IS).

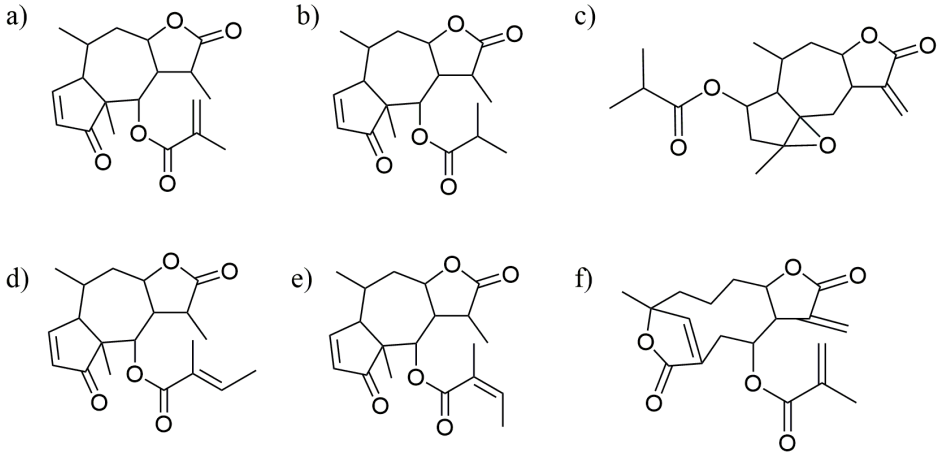

Figure 2: Base peak chromatogram of UPLC-ESI-qTof-MS of extract of *Centipeda minima*

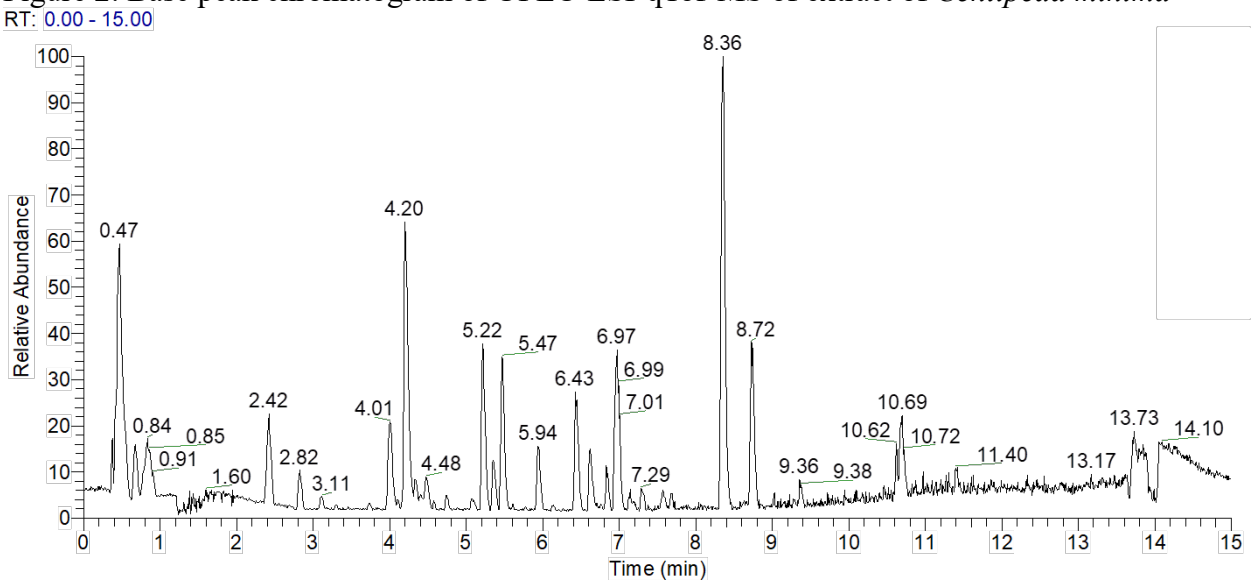

Figure 3: a) Mass spectrum of minimolide F, b) Mass spectrum of the precursor ion of m/z 335.1851, and c) proposed fragmentation pathway of minimolide F

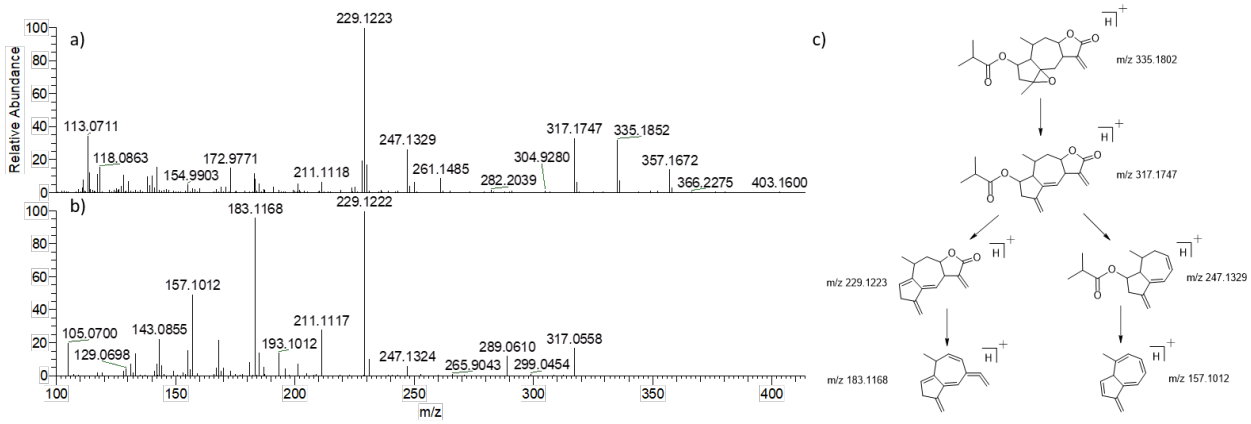

Figure 4: Extracted ion chromatograms from sesquiterpene lactones (a) a mixture of reference compounds, (b) a sample of *Centipeda minima* (1, arnicolide D; 2, arnicolide C; 3, minimolide F; 4. microhelenin C; 5, brevilin A; 6, deoxydlephantopin (IS)).