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# Qualitative and quantitative analysis of chemical constituents of *Centipeda minima* by HPLC-QTOF-MS & HPLC-DAD

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## ABSTRACT:

A high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS) method in both positive and negative ion modes was established to investigate the major constituents in the ethanolic extract of *Centipeda minima* (EBSC). Twelve common components including flavones and their glycosides, phenolic and polyphenolic acids, and sesquiterpene lactone were identified in ten batches of samples based on comparison with the retention time and accurate mass of external standards (mass accuracy within 3 ppm) or the fragmentation patterns of tandem MS. Meanwhile, a simple, accurate and reliable HPLC-DAD method was also developed to determine the content of 10 chemical markers simultaneously. Results obtained from method validations including linearity, accuracy and precision showed that this new method is reliable and robust. Isochlorogenic acid A and brevilin A were found to be the most abundant in the ethanol extract of EBSC and could be served as markers for quality control of EBSC.

Keywords: *Centipeda minima*, quadrupole time-of-flight mass spectrometry (QTOF MS), quality control, herbal analysis, isochlorogenic acid A, brevilin A

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## 1. Introduction

Nasopharyngeal carcinoma (NPC), arising from epithelium of the nasopharynx, is one of the most common malignancies in southeast Asia and southern China and their incidence rates are nearly 100-fold higher than that in western countries [1, 2]. Currently, radiotherapy is still the first line treatment for NPC, especially at early stages. However, the treatment fails, very often, leading to recurrent and locoregionally advanced NPC patients [3, 4]. It is reported that the treatment of the NPC patients in both stages 1 and 2 can be improved by the combination of radiotherapy and chemotherapy [5]. Cisplatin is the most effective cytotoxic agent used in NPC treatment, however inherent drug resistance limits its applications in NPC chemotherapy [6]. Therefore, it is necessary to find and develop more suitable therapeutic agents for the NPC chemotherapy.

*Centipeda minima* (L.) A. Braun and Ascherson, is known as Ebushicao (EBSC) in Chinese. It is an annual herbaceous plant widely distributed in China and eastern tropical Asia. The whole plant is traditional Chinese medicine for the treatment of rhinitis, sinusitis, pain, swelling and cancer [7]. Recent pharmacological studies also showed that EBSC possessed anti-tumor [8], anti-proliferative [9], antioxidant, anti-inflammatory [10], and antibacterial activities [11]. Phytochemical studies isolated sesquiterpene lactones, oxygenated monoterpenes, flavonoids and organic acid from the herb [10, 12-14].

Our previous study has revealed that the ethanolic extract of EBSC showed significant anti-proliferative activities and apoptosis induction on human nasopharyngeal carcinoma CNE-1 cell line in vitro, its underlying mechanisms were associated with the up-regulation of Bax protein and down-regulation of Bcl-2 protein as well as inhibition on PI3K-Akt-mTOR signaling pathway [15, 16]. Characterization of the chemical constituents in the ethanolic extract of EBSC would help to identify the potential compounds responsible for the biological functions and provide hints for the further development of anti-cancer drug. Moreover, no quality control on characterizing the chemical profiles of EBSC has been proposed in the literature. The study of the active fraction of EBSC would provide a good quality control method relevant to biological activities. Thus, the objective of this study is to identify the chemical constituents and to develop a reliable analytical method for quantitative analysis of some of these compounds. We have examined the chemical composition of ethanolic extract of EBSC by time-of-flight mass spectrometer. We have also quantified ten markers, including 3 flavones and their glycosides, 6 phenolic and polyphenolic acids and 1 sesquiterpene lactone by HPLC-DAD. Validation parameters including sensitivity, linear range, precision and accuracy, have been also determined. Finally, the newly developed HPLC-DAD method was successfully applied to quantify ten chemical markers in 10 batches samples collected from various geographical locations. To our knowledge, this is the first report of an analytical method for simultaneous determination of flavones and their glycosides, phenolic and polyphenolic acids, as well as sesquiterpene lactone compounds in EBSC.

## 2. Materials and methods

### *2.1. Samples*

Ten authenticated samples were collected from different provinces of China and a list of EBSC samples collected for this study is shown in Table 1.The voucher specimens were deposited in the State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, P. R. China

## *2.2. Chemical analysis*

### *2.2.1. Chemical reagents*

Chlorogenic acid, cryptochlorogenic acid, caffeic acid, isochlorogenic acid A, isochlorogenic acid B and isochlorogenic acid C (purity > 98%) were purchased from Chengdu Must Bio-Technology Co., LTD (Chengdu, China) while rutin (purity > 98%) was purchased from National Institutes for Food and Drug Control of China (Beijing, China); kaempferol-3-O-rutinoside and 3-methoxyquercetin (purity > 98%) were purchased from Shanghai Tanto Biotech Co., LTD (Shanghai, China) and brevilin A(purity > 90%) was purchased from BioBiopha Co., LTD (Kunming, China). Brevilin A was further purified by preparative HPLC and the final purity of brevilinA was 97.7% as determined by HPLC-DAD at 224 nm. Arnicolide D and arnicolide C were obtained by isolation using preparative HPLC and the chemical structures were confirmed by $^{1}$H-NMR and $^{13}$C-NMR spectral analysis. HPLC grade acetonitrile and acetic acid were purchased from Thermo Fisher Scientific Inc., Tedia (USA) and Acros Organics, respectively. Ethanol (Guangdong Chemical Reagent Engineering-technological Research and Development Center, Guangzhou, China) was of analytical-reagent grade. Double deionized water was purified by Milli-Q water system (Millipore Corp., Bedford, MA, USA) and used to prepare all buffer and sample solutions.

### *2.2.2. High-performance liquid chromatography – diode array detection*

To determine the contents of ten chemical markers, a HPLC – DAD method with gradient elution was developed and employed. Aglient1200 series HPLC apparatus, equipped with a quaternary solvent delivery system, an auto-sampler and UV detector, was used. Chromatography was carried out on a Grace Alltima column (250 mm × 4.6 mm, 5.0 μm) at room temperature. Detection wavelengths were set at 326 (0 – 55 min) and 224 nm (55 – 65 min). The mobile phase consisted of (A) 0.2% acetic acid and (B) acetonitrile (v/v) using a gradient elution. The gradient program is as follow: initial 90% (A), linear change from 90% to 89% at 0 – 13 min, linear change from 89% to 85% (A) at 13 – 18 min, linear change from 85% to 74% (A) at 18 – 40 min, linear change from 74% to 63% (A) at 40 – 45 min, linear change from 63% to 47% (A) at 45 – 52 min, keep 47% (A) at 65 min. Re-equilibration duration was 10 min between individual runs. The flow rate was 1.0 mL/min and aliquots of 5 μL were injected.

### *2.2.3. High performance liquid chromatography/time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS)*

QTOF/MS analysis was achieved by an Agilent series 1290 UHPLC instrument (Agilent, Waldbronn, Germany) coupled with a 6540 QTOF mass spectrometer equipped with an electrospray ionization interface (Agilent, Santa Clara, CA, USA). The mass-spectrometric conditions were optimized as follows: capillary voltage 3.5 kV, fragmentor voltage 175 V, pressure of nebulizer 40 psi, drying gas temperature 300 °C, sheath gas temperature 350 °C. Nitrogen was used as the sheath gas and the drying gas with flow rate of 11.0 and 8.0 L/min, respectively.

All the acquisition and data analysis were carried out using the Mass Hunter software (Agilent Technologies). The data was acquired in the scan mode from *m*/*z* 100 to 1700 with 1.0 spectra/s. For real time mass correction in the positive and negative switching modes, 121.05087, 922.00979 and 119.03632, 955.97192 external mass standards were used, respectively. Data from both modes have been used for identification of flavones and their glycosides, phenolic and polyphenolic acids, and sesquiterpene lactones in this study.

### *2.3. Sample preparation*

0.3 g powdered sample, accurately weighted and placed into a 50 mL centrifuge tube, was sonicated (540W) at room temperature with 10 mL 50% ethanol for 30 min. Then, the mixture was centrifuged at *ca*. 3000 × *g* for 5 min and then filtrated into a 25 mL volumetric flask. The extraction procedure was repeated for one more time. Afterward, the residue was washed by 3 mL 50% ethanol and the solution was transferred to the volumetric flask. Finally, the sample solution was marked up to 25 mL and was filtrated through a 0.45 μm syringe filter before HPLC analysis.

## 3. Results and Discussion

### 3.1. Identification of major constituents

Twelve components including phenolic, polyphenolic acid, flavones and their glycosides, and sesquiterpene lactones were identified among ten batches samples by comparing their retention time and accurate mass with those of external standards or data reported in literature. A summary of the mass spectra obtained was shown in Table 2.

### 3.1.1. Flavones and their glycosides

One flavonol aglycone and two flavonol glycosides were detected in both positive and negative modes. In positive ion mode, flavonol aglycone only showed up as $[M+H]^+$ while flavonol glycosides showed up as $[M+H]^+$ and $[M+Na]^+$. In the negative mode, both appeared as the deprotonated $[M-H]^-$ and $[2M-H]^-$ ions. Both positive and negative modes provide good intensity, but positive mode gives much clearer fragmentation pattern for structural identification. For flavonol glycosides, the product ions due to the cleavage at the glycosidic linkages were detected. The fragments of *m*/*z* 449.1054 and *m*/*z* 287.0517 were produced directly from the parent ion *m*/*z* 595.1648 of kaempferol-3-*O*-rutinosidewhile *m*/*z* 465.1026 and *m*/*z* 303.0498 were produced directly from the parent ion *m*/*z* 611.1607 of rutin (Fig 2a & b).

### 3.1.2. Phenolic and polyphenolic acid

Six phenolics and polyphenolic acids were detected in both positive and negative modes and the response in negative mode was much higher than that in positive mode. Among them, three isomeric forms of dicaffeoylquinic acid, including isochlorogenic acid A, B and C, were all present with high content. The fragmentation pattern of this type of compounds, using isochlorogenic acid A as an example consists of the parent ion $[M-H]^-$ at *m/z* 515.1200 ($C_{25}H_{24}O_{12}$), fragments at *m/z* 353.0874 ($C_{16}H_{17}O_9$), and *m/z* 191.0562 ($C_7H_{11}O_6$), and a fragment at *m/z* 173.0451 ($C_9H_7O_4$) after sequential loss of water molecule. Isochlorogenic acid B and C showed similar fragmentation pattern with high ratio of *m/z* 173.0451 ($C_9H_7O_4$) / *m/z* 191.0562 ($C_7H_{11}O_6$), which is consistent with the previous literature [17].

### *3.1.3. Sesquiterpene lactones*

For sesquiterpene lactones, only positive mode was able to give good signals and the parention present in the form of $[M+H]^+$ and $[M+Na]^+$. In this work, arnicolide D, arnicolide C and brevilin A were identified as the protonated species $[M+H]^+$ at 333.1694, 335.1857 and 347.1856. Fig 3 indicated the fragmentation pattern of brevilinA observed. The daughter ion *m/z* 247.1324 ($C_{15}H_{19}O_3$) was detected due to the cleavage of the C-O bond at $C_6$ directly from the parent ion *m/z* 347.1856 $[M+H]^+$and *m/z* 369.1669 $[M+Na]^+$. Then, subsequent 5-membered ring breaking yields fragments at *m/z* 229.1224, 201.1273 and 173.0961. Similarly, the MS obtained for arnicolide D at 57.5 min and arnicolide C at 58.3 min indicated that these two compounds followed the same fragmentation pathway. Arnicolide D and arnicolide C are not commercially available, isolation was carried out in this study to obtain the compounds and their identities were confirmed by H-NMR and C-NMR.

Arnicolide C was isolated as colorless crystal and the $^{1}$H and $^{13}$C NMR spectroscopic data are shown as below: $^{1}$H-NMR (400 MHz, CDCl$_3$) δ : 7.71 (1H, dd, *J* = 1.5, 6.0 Hz, H-2), 6.10 (1H, dd, *J* = 3.0, 6.0 Hz, H-3), 5.43 (1H, s, H-6), 4.70 (1H, m, H-8), 3.08-3.10 (2H, m, H-11, 1), 2.85 (1H, m, H-7), 2.50 (1H, m, H-9a)，2.45 (1H, m, H-17), 2.25 (1H, m, H-10), 1.63 (1H, m, H-9b), 1.55 (3H, d, *J* = 7.5 Hz, H-13), 1.25 (3H, d, *J* = 6.5 Hz, H-14), 1.10 (3H, d, *J* = 7.0 Hz, H-18 or 19), 1.08 (3H, d, *J* = 7.0 Hz, H-18 or 19), 1.05 (3H, s, H-15).$^{13}$C-NMR (400 MHz，CDCl$_3$) δ : 209.5 (C-4), 179.1 (C-12), 175.7 (C-16), 162.1 (C-2),129.5 (C-3), 79.5 (C-6), 71.8 (C-8), 54.9 (C-1), 54.0 (C-5), 48.9 (C-7), 41.0 (C-9), 40.5 (C-11), 33.9 (C-17), 25.8 (C-10), 19.7 (C-18), 18.9 (C-19), 18.6 (C-13), 17.6 (C-14), 10.9 (C-15).

Arnicolide D was isolated as colorless crystal and the $^{1}$H and $^{13}$C NMR spectroscopic data are shown as below: $^{1}$H-NMR (400 MHz, CDCl$_3$)δ: 7.71 (1H, dd, *J* = 1.5, 6.0 Hz, H-2), 6.11 (1H, dd, *J* = 3.0, 6.0 Hz, H-3), 5.90 (1H, s, H-18a), 5.55 (1H, s, H-6), 5.50 (1H, s, H-18b), 4.80 (1H, m, H-8), 3.10-3.20 (2H, m, H-11, 1), 2.95 (1H, m, H-7), 2.55 (1H, m, H-9a), 2.25 (1H, m, H-10), 1.90 (3H, s, H-19), 1.68 (1H, m, H-9b), 1.58 (3H, d, *J* = 7.5 Hz, H-13),1.25 (3H, d, *J* = 6.5 Hz, H-14), 1.10 (3H, s, H-15). $^{13}$C-NMR (400 MHz, CDCl3) δ: 209.4 (C-4), 179.0 (C-12), 166.0 (C-16), 162.0 (C-2), 136.0 (C-17), 129.5 (C-3), 126.0 (C-18), 79.5 (C-6), 72.5 (C-8), 55. (C-1), 54.1 (C-5), 48.7 (C-7), 41.0 (C-9), 40.5 (C-11), 25.9 (C-17), 19.7 (C-10), 18.2 (C-13), 17.6 (C-14), 11.0 (C-15).

### *3.2. Method development for marker quantitation*

In the HPLC-DAD analysis, various mobile phase compositions and chromatographic conditions were examined to optimize the chromatographic conditions. To achieve best detection sensitivity for these three classes of compounds, two detection wavelengths were employed for detection, which 326 nm and 224 nm were used in 0 – 55 min and 55 – 65 min, respectively. In addition, different mobile systems consisting of acetonitrile-water and methanol-water with the presence of different acids, including formic acid, acetic acid and trifluoroacetic acid of various concentrations (0.05-0.2%) were evaluated to optimize the separation and to achieve good peak shape. Congested overlapping peak clusters were observed in the first 10 min if no acid present in the mobile phase. Finally, 0.2% acetic acid – acetonitrile system provides the best separation for these compounds. We also examined two columns, Alltech Alltima $C_{18}$ and Grace Vision HT $C_{18}$ and the results showed that Alltima $C_{18}$ provides less tailing effect in the peaks detected.

The 95% ethanol extract of EBSC has been shown to be active against the growth of human nasopharyngeal carcinoma cell CNE-1 [15]. The effect of the extraction on the chemical composition was further evaluated in this study to develop a procedure which extracts more compounds and retains all the major chemical constituents of the ethanol extract used in the previous study. To optimize the extraction procedure, 0.3 g of sample was extracted using different concentration of ethanol (50%, 70% and 100%) and comparing the peak areas of the HPLC chromatograms obtained. 100% ethanol and methanol gave poor extraction efficiency for all three classes of constituents while 50% ethanol showed the best extraction efficiency for all of them. Besides, ultrasonication and heat-reflux with 30 min were compared and the results indicated that ultrasonication and heat-reflux methods had similar performance. The number of successive extraction on the extraction efficiency was also examined and 2 extraction cycles are sufficient to extract all ten chemical markers from EBSC. Thus, the optimal extraction condition for the active ingredients of ESBC samples is two cycles of 30 min ultrasonication extraction with 50% ethanol.

The HPLC method developed was validated according to the International Conference on Harmonization (ICH) guidelines and the results of linearity, precision, accuracy and stability were tabulated in Table 3 and 4. The calibration curves of the 10 standards exhibited good linearity ($r^2 > 0.999$) within the test range. The limit of detection (LOD) and limit of quantification (LOQ) values were determined based on the basis of the signal-to-noise ratio (S/N) of 3 and 10, respectively. LODs and LOQs are in the range of 0.05 – 0.213 µL/mL and 0.167–0.745 µL/mL, respectively. In order to examine the recovery of the extraction method, accurate amounts of the standards were added to a sample and analyzed as described in Section 2.2 and 2.3. The recoveries were calculated by the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%. The developed analytical method had good accuracy with overall recovery 100 ± 7.5% ($n = 3$) for all 10 chemicals in EBSC samples. Finally, stability of testing solutions was examined for the standard solutions and sample extract that stored at room temperature for 2 days. The intra-day and inter-day variabilities ($n = 3$) of all ten standards in the herbal extracts were less than 5% and all analytes were found to be stable within the duration of the extraction and analysis. All the results suggested that this assay showed good reproducibility.

### *3.3. Content of the markers in EBSC samples*

The proposed HPLC-DAD method was applied to analyze 10 chemicals markers in 10 batches of ESBC samples from different geographical locations. Figure 4 shows typical chromatograms of the standard solution and of an extract of EBSC. The retention times of chlorogenic acid, cryptochlorogenic acid, caffeic acid, rutin, isochlorogenic acid B, kaempferol-3-*O*-rutinoside, isochlorogenic acid A, isochlorogenic acid C, 3-methoxyquercetin and brevilin A are 13.93 min, 14.51min, 21.24 min, 32.31min, 35.71min, 36.64 min, 37.85 min, 40.13 min, 50.29 min, and 61.26 min, respectively.

Correlation coefficient is used as a measure for similarity between chromatograms. The chromatogram of a standardized extract is recommended to serve as the reference profile, but unfortunately it is rarely available. Therefore, the mean or median chromatographic profile of the data set is usually used. Table 5 showed the similarity index for the chromatogram of each sample, only sample 6 is below 0.8. It was because some large peaks being detected at 24-29 min and 42-47 min only presented in the chromatogram of the sample 6.

As shown in Table 5, the actual content of the components showed a large variations among the 10 samples studied. The data suggested that all the samples contain these markers and they would be appropriate marker(s) for quality control of the herbs. In the Chinese Pharmacopeia [18], there is no assay requirement for EBSC. Among 10 chemical markers identified, isochlorogenic acid A and brevilin A were the most abundant and their average contents were above 1.0 mg/g. Moreover, isochlorogenic acid A has been reported to show potent anti-hepatitis B activity [19] and brevilinA has been reported to be anti-cancer by inhibiting the activity of Janus Kinase and blocking STAT3 signaling in cancer cell [20]. There is no report of the instability of isochlorogenic acid A and brevilin A.  Thus, isochlorogenic acid A and brevilin A are proposed as suitable markers for quality control of EBSC.

## 4. Conclusion

Chemical profiling of EBSC were examined by an effective HPLC-DAD-QTOF-MS and 12 common constituents including flavones and their glycosides, polyphenolic acid and sesquiterpene lactones were identified based on their mass and fragmentation patterns. Isochlorogenic acid A and brevilin A are found to be abundant among ten batches samples and could be served as suitable markers for quality control of ESBC. Also, the obtained chemical fingerprint could also provide another quality control measure for this herb.

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**Table 1.** A list of sample collections

| Batch No. | Geographical location | Collection time |
|---|---|---|
| S1 | Xinyang, Henan | 13/06/2011 |
| S2 | Huaibin, Henan | 15/10/2010 |
| S3 | Linquan, Anhui | 16/10/2010 |
| S4 | Suizhou, Hubei | 10/06/2011 |
| S5 | Nanning, Guangxi | 10/10/2010 |
| S6 | Jingxi, Guangxi | 12/11/2010 |
| S7 | Panan, Zhejiang | 21/09/2010 |
| S8 | Yulin, Guangxi | 06/04/2011 |
| S9 | Xiangfan, Hubei | 06/06/2011 |
| S10 | Yingshang, Anhui | 12/06/2011 |

**Table 2**. Characterization of compounds from the ethanolic extract of EBSC

| Peak | Retention time (min) | Identification | MS (+) in TOF (error, ppm) | MS (-) in TOF (error, ppm) |
|---|---|---|---|---|
| 1 | 13.931 | chlorogenic acid | 355.1023 (0.28) | 353.0874 (1.13) |
| 2 | 14.514 | cryptochlorogenic acid | 355.1019 (1.4) | 353.0873 (1.42) |
| 3 | 21.241 | caffeic acid | 181.0451 (1.1) | 179.0348 (1.12) |
| 4 | 32.311 | rutin | 611.1607 (0.0) | 609.1462 (0.16) |
| 5 | 35.706 | isochlorogenic acid B | 517.1339 (0.38) | 515.1193 (0.39) |
| 6 | 36.641 | kaempferol-3-O-rutinoside | 595.1648 (1.51) | 593.1508 (0.67) |
| 7 | 37.851 | isochlorogenic acid A | 517.1338 (0.58) | 515.1200 (0.97) |
| 8 | 40.134 | isochlorogenic acid C | 517.1340 (0.19) | 515.1203 (1.55) |
| 9 | 50.285 | 3-methoxyquercetin | 317.0654 (0.63) | 315.0510 (0.64) |
| 10 | 61.256 | brevilin A | 347.1856 (0.84) | Na |
| A | 57.550 | arnicolide D | 333.1694 (0.90) | Na |
| B | 58.330 | arnicolide C | 335.1857 (1.20) | Na |

**Table 3.** Linear regression data, LOD and LOQ of the investigated compounds ($n$ = 3)

| Compounds | Linear range (µg/mL) | $r^2$ | LOQ (µg/mL) | LOD (µg/mL) | Recovery (%) |
|---|---|---|---|---|---|
| chlorogenic acid | 0.5 – 60.0 | 0.9997 | 0.614 | 0.184 | 98.71 |
| cryptochlorogenic acid | 0.3 – 36.0 | 0.9998 | 0.640 | 0.192 | 99.72 |
| caffeic acid | 0.1 – 12.0 | 0.9999 | 0.199 | 0.060 | 99.64 |
| rutin | 0.5 – 60.0 | 0.9997 | 0.745 | 0.224 | 104.00 |
| isochlorogenic acid B | 0.3 – 36.0 | 0.9998 | 0.303 | 0.091 | 102.72 |
| kaempferol-3-O-rutinoside | 1.0 – 120.0 | 0.9999 | 0.711 | 0.213 | 97.32 |
| isochlorogenic acid A | 2.0 – 240.0 | 0.9999 | 0.314 | 0.094 | 101.89 |
| isochlorogenic acid C | 1.5 – 180.0 | 0.9999 | 0.405 | 0.122 | 102.79 |
| 3-methoxyquercetin | 0.3 – 36.0 | 0.9998 | 0.167 | 0.050 | 98.10 |
| brevilin A | 0.8 – 96.0 | 0.9998 | 0.388 | 0.116 | 97.30 |

**Table 4**. Results of precision

| Compounds | Precision | | | |
|---|---|---|---|---|
| | Intra-day | | Inter-day | |
| | Content (mg/g) | RSD (%) | Content (mg/g) | RSD (%) |
| chlorogenic acid | 2.101 | 2.46 | 2.101 | 1.64 |
| cryptochlorogenic acid | 0.515 | 3.34 | 0.515 | 2.86 |
| caffeic acid | 0.281 | 0.31 | 0.281 | 3.56 |
| rutin | 1.089 | 0.59 | 1.089 | 1.14 |
| isochlorogenic acid B | 0.916 | 0.65 | 0.916 | 1.98 |
| kaempferol-3-O-rutinoside | 1.155 | 0.55 | 1.155 | 4.75 |
| isochlorogenic acid A | 9.789 | 0.51 | 9.789 | 0.92 |
| isochlorogenic acid C | 5.667 | 0.53 | 5.667 | 1.76 |
| 3-methoxyquercetin | 0.250 | 2.74 | 0.250 | 3.20 |
| brevilin A | 1.613 | 2.82 | 1.613 | 2.10 |

**Table 5.** Results of markers content and similarity index among 10 batches samples

| Batch No. | Content (mg/g) | | | | | | | | | | Similarity Index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| S1 | 0.249 | 0.063 | 0.036 | 0.206 | 0.100 | 0.427 | 0.959 | 0.348 | 0.261 | 4.708 | 0.937 |
| S2 | 0.320 | 0.103 | 0.100 | 0.499 | 0.148 | 0.665 | 1.452 | 0.662 | 0.179 | 2.514 | 0.970 |
| S3 | 0.222 | 0.082 | 0.061 | 0.349 | 0.097 | 0.388 | 0.931 | 0.462 | 0.173 | 2.652 | 0.985 |
| S4 | 0.200 | 0.058 | 0.031 | 0.142 | 0.084 | 0.325 | 0.763 | 0.273 | 0.233 | 4.617 | 0.918 |
| S5 | 0.328 | 0.161 | 0.059 | 0.684 | 0.362 | 0.986 | 4.220 | 2.143 | 0.443 | 2.327 | 0.801 |
| S6 | 2.102 | 0.515 | 0.281 | 1.089 | 0.916 | 1.155 | 9.789 | 5.668 | 0.250 | 1.613 | 0.542 |
| S7 | 0.992 | 0.105 | 0.103 | 0.594 | 0.141 | 0.536 | 2.693 | 0.613 | 0.226 | 3.686 | 0.958 |
| S8 | 0.422 | 0.177 | 0.067 | 0.666 | 0.098 | 0.363 | 1.293 | 0.471 | 0.193 | 2.521 | 0.961 |
| S9 | 0.206 | 0.059 | 0.032 | 0.183 | 0.075 | 0.299 | 0.717 | 0.323 | 0.192 | 5.253 | 0.892 |
| S10 | 0.390 | 0.114 | 0.164 | 0.479 | 0.126 | 0.598 | 1.411 | 0.625 | 0.212 | 2.748 | 0.987 |

A list of Figure

**Fig.1.** Chemical structures of 1) chlorogenic acid, 2) cryptochlorogenic acid, 3) caffeic acid, 4) rutin, 5) isochlorogenic acid B, 6) kaempferol-3-O-rutinoside, 7) isochlorogenic acid A, 8) isochlorogenic acid C, 9) 3-methoxyquercetin,10) brevilin A, A) arnicolide D, B) arnicolide C

**Fig.2.** Mass spectra and proposed fragment pathways of a) kaempferol-3-*O*-rutinoside, b) rutin and c) isochlorogenic acid A

**Fig.3.** Proposed fragmentation pathway for brevilin A and its mass spectrum

**Fig.4.** Typical chromatograms of the chemical markers in a) standards solution and b) extract of EBSC: (1) chlorogenic acid, (2) cryptochlorogenic acid, (3) caffeic acid, (4) rutin, (5) isochlorogenic acid B, (6) kaempferol-3-O-rutinoside, (7) isochlorogenic acid A, (8) isochlorogenic acid C, (9) 3-methoxyquercetin, (10) brevilin A, (A) arnicolide D and (B) arnicolide C.

Fig.1

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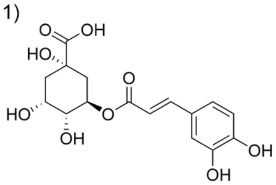

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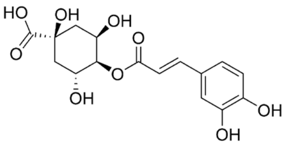

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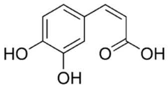

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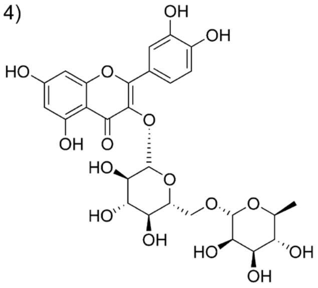

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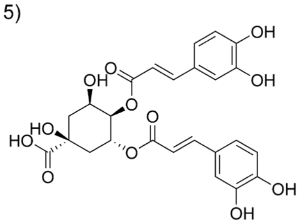

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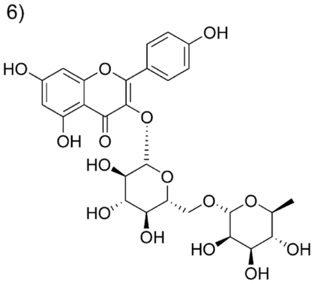

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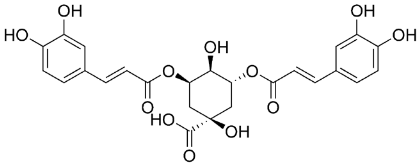

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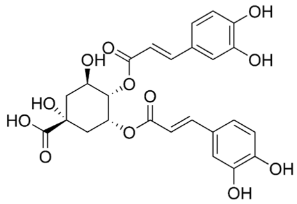

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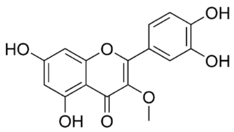

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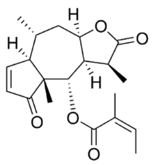

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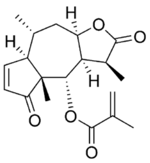

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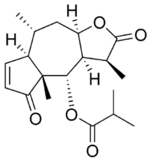
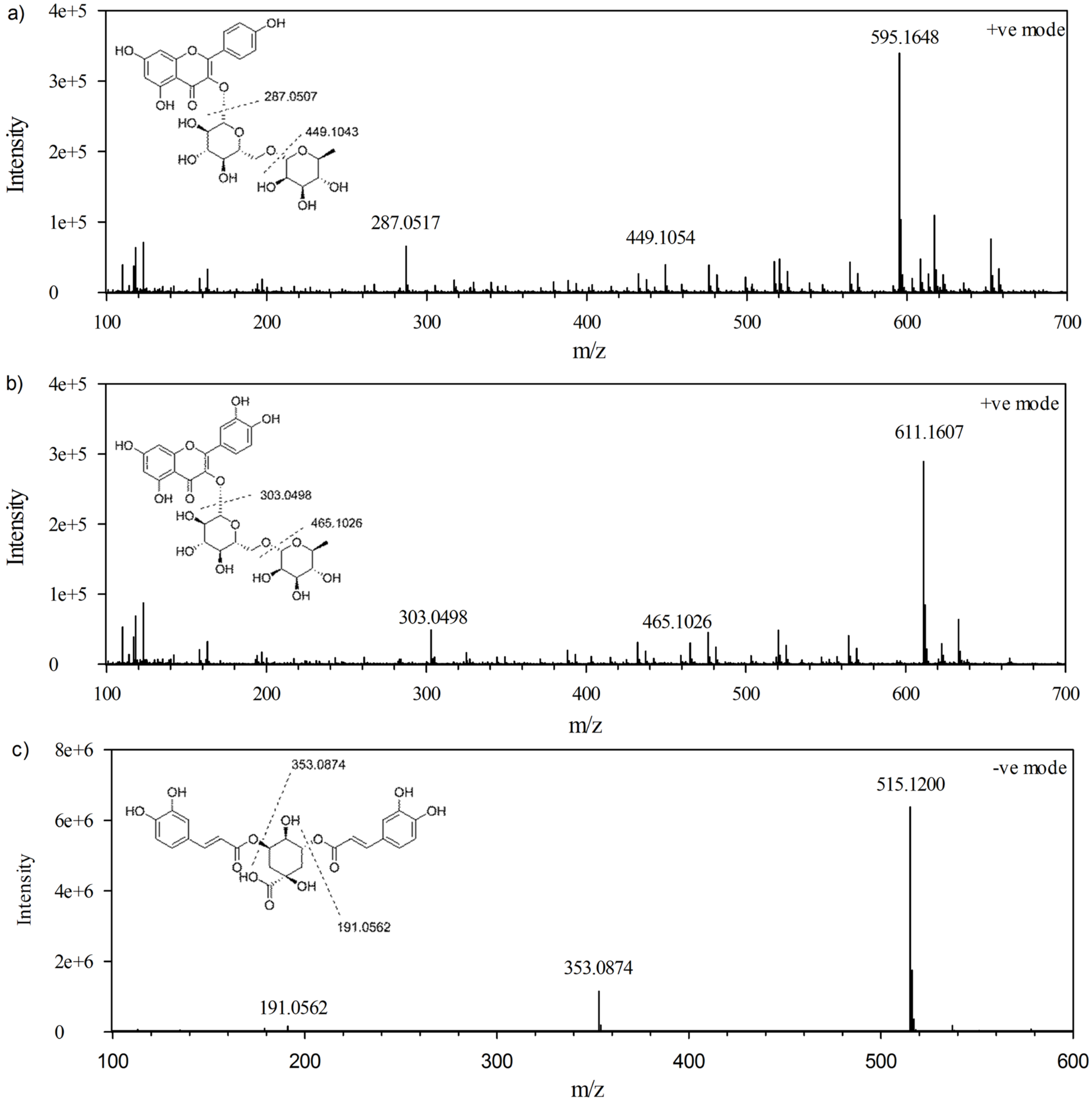


Fig.2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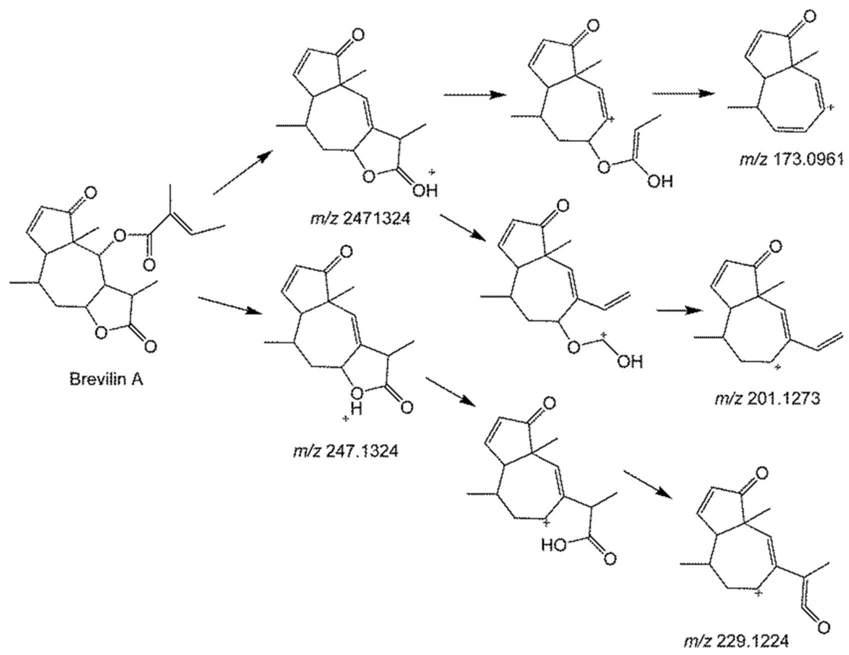
3e+5
2e+5
1e+5
0
Intensity
+ve mode
595.1648
287.0517
449.1054
287.0507
449.1043
HO
OH
O
100
200
300
400
500
600
700
m/z
b)
+ve mode
611.1607
303.0498
465.1026
303.0498
465.1026
c)
8e+6
6e+6
4e+6
2e+6
-ve mode
515.1200
353.0874
191.0562
353.0874
191.0562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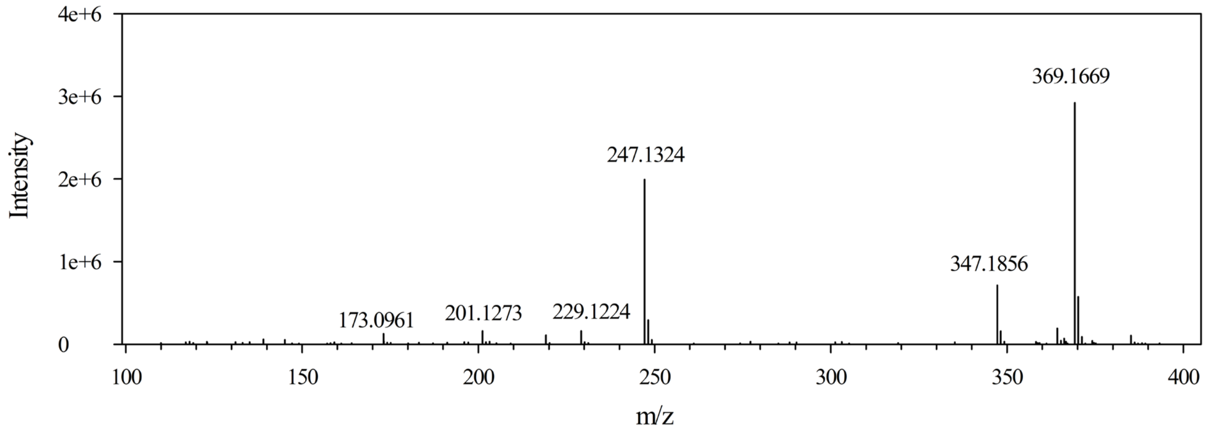
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3e+6
2e+6
1e+6
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100
150
200
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300
350
400
m/z
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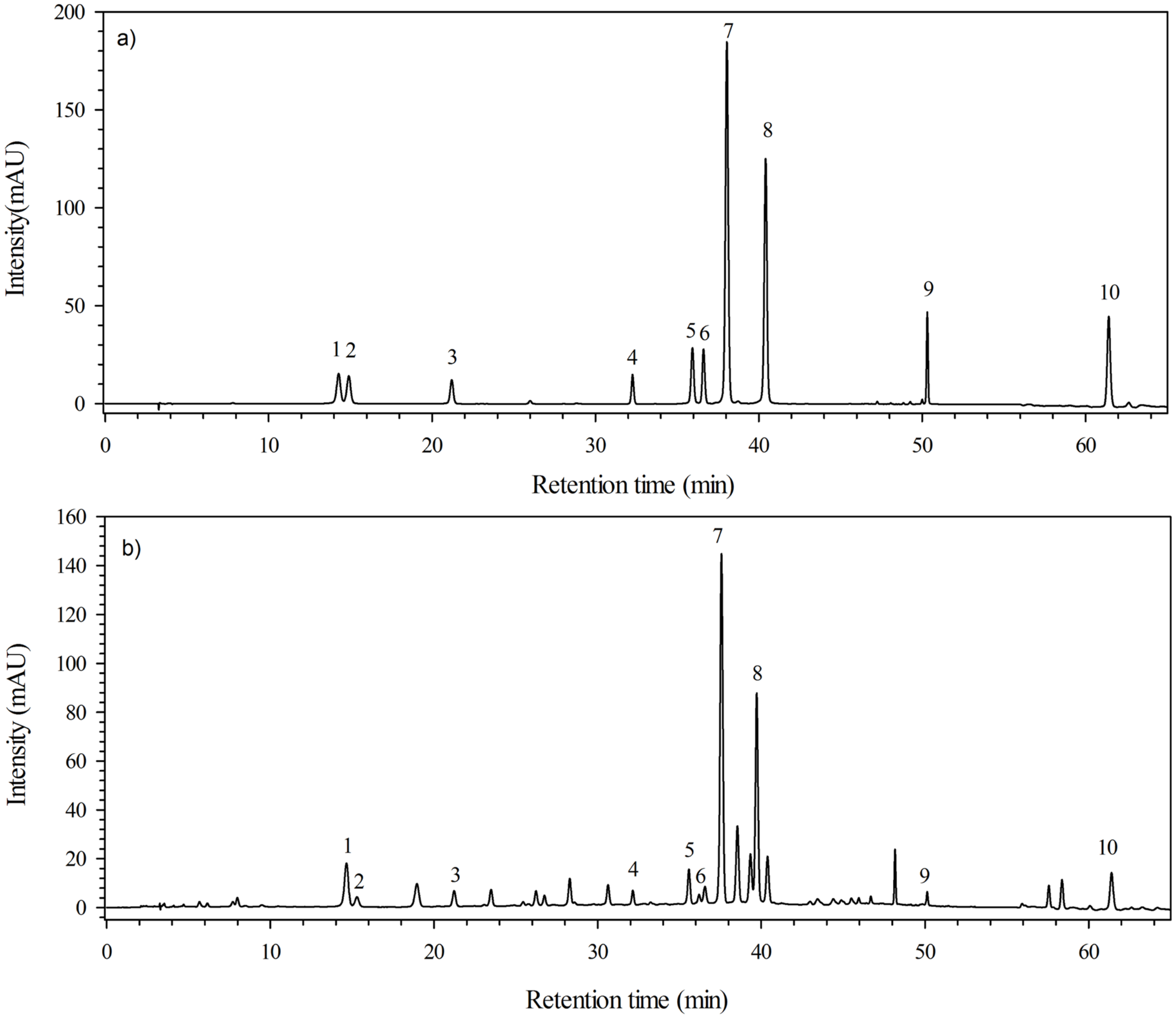
201.1273
229.1224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Intensity(mAU)
200
150
100
50
0
1 2
3
4
5 6
7
8
9
10
0
10
20
30
40
50
60
Retention time (min)
b)
Intensity (mAU)
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
Retention time (min)