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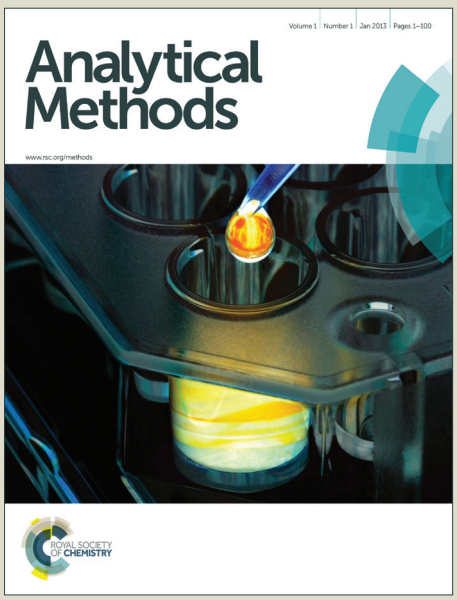

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Fingerprint analysis and quantitative determination of 16 constituents of Antike capsule by high-performance liquid chromatography-photodiode array detection

Linrui Duan, Hua Li, Jiani Yuan, Hang Zhao, Xiaokai Zhang, Xuanxuan Zhou, Qian Yang, Wei Cao, Yanhua Xie, Shan Miao, Siwang Wang*

Department of Natural Medicine, School of Pharmacy, Fourth Military Medical University, 169 West Changle Road, Xi'an 710032, Shaanxi, People's Republic of China

*Correspondence: Professor Siwang Wang; Tel.: +86 29 8477 2519; fax: +86 29 8322 4790; E-mail address: siwangwangyys@163.com

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Abstract

In this study, a novel, convenient, accurate, and valid method was developed by using high-performance liquid chromatography-photodiode array detection to obtain a chromatographic fingerprint of Antike capsule (AC). Using Computer Aided Similarity Evaluation software, 28 characteristic peaks in chromatograms of 10 batches analyzed samples were screened out and traced to the source of original materials toad skin and angelica, in which 16 of the peaks were identified as gamabufotalin, arenobufagin, telocinobufagin, desacetylcinobufotalin, bufotalin, cinobufotalin, bufalin, cinobufagin, resibufogenin, ferulic acid, n-butylidenephthalide, senkyunolide A, senkyunolide I, senkyunolide H, ligustilide, and coniferylferulate. At the same time, the fingerprint similarity was calculated and the contents of known ingredients were also determined simultaneously. This method demonstrated good precision, reproducibility, and stability (relative standard deviation [RSD] of relative retention time [RRT] < 2.0% and RSD of relative peak area [RPA] < 5.0%). Good linear behaviors over the investigated concentration ranges were observed for all the analytes (r^2 > 0.9994), the recoveries and RSD varied from 96.35% to 102.43% and 0.48% to 1.98%, respectively. The proposed method enabled fingerprint analysis and simultaneous identification and determination of 16 constituents in a single run. In addition, it provides a significant reference for the quality control of AC.

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1 Introduction

The application of Traditional Chinese medicine (TCM) in disease treatment has a long history throughout China. Thousands of years ago, Chinese ancestors collected herbs or animal resources to treat specific illnesses. With the continuous efforts of researchers from generation to generation, the peculiar theoretical system of TCM has formed. Attributed to the features of significant curative properties and low toxic effects, in recent years, utilization of new TCM drugs is more popular than ever before. However, the complexity and ambiguity of compositions have restricted the development of TCM, which has become the bottleneck that obstructs broad application of TCM all over the world [1, 2]. Therefore, it is indispensable and urgent to implement quality control of TCMs.

The China Food and Drug Administration (CFDA) suggests that fingerprint technology be used in the process of establishing quality standards of TCM [3]. As one of the necessary components of TCM research, fingerprint analysis is considered as an effective method for controlling quality and plays an important role in guaranteeing the safety, efficacy, and stability of a product. In contrast to other methods, fingerprint analysis can reveal the total characteristics of TCMs in a relatively comprehensive way, rather than merely determining the contents of main components, which is appropriate for the features of complexity and ambiguity [4, 5]. Various separation and detection methods have been wildly utilized in fingerprint analysis. In recent years, chromatography has been used in separating the samples to obtain a characteristic fingerprint. The most common analytical separation technique

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for developing fingerprints is high-performance liquid chromatography (HPLC) [6, 7, 8]. In addition, gas chromatography (GC) [9], and capillary electrophoresis (CE) [10] have also adopted for developing fingerprints. In view of the sensitivity and selectivity of the detector, photodiode array detection (PAD) [11-13], evaporation light-scatter detection (ELSD) [14, 15], and mass spectrometry (MS) [15, 16] could be selected as required.

Antike capsule, a compound preparation consisting of the TCM materials toad skin (the dry skin of *Bufo bufo gargarizans cantor*) and angelica (*Angelica sinensis*), has been confirmed as an effective antineoplastic drug [17-19]. It obtained a new drug certificate [(95) Z-97] in China in 1996 and has been widely used in clinical treatment for many years [20-22]. Research indicates that the numerous monomeric compounds from toad skin play important anti-tumor roles *in vitro* and *in vivo*. For example, bufadienolides, such as bufalin, cinobufagin, resibufogenin, and telocinobufagin, are the major active compounds isolated from toad skin [23, 24]. Through inhibition of cell proliferation, induction of cell differentiation, induction of apoptosis, disruption of the cell cycle, inhibition of cancer angiogenesis, reversal of multi-drug resistance, and regulation of the immune response, these bufadienolides exhibit significant antitumor activity [25]. It was also demonstrated in a previous study that angelica had various important biological activities, such as hematopoiesis, immunomodulation, antitumor, antioxidant, radioprotection, and hypoglycemic activity [26]. By rational combination of these two components, Antike capsule possesses not only dramatic anti-tumor efficacy, but can strengthen the immune system with low side effects.

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Therefore, this new TCM heralds vast potential for development and considerable market prospects.

To date, a total of 39 bufadienolides have been authenticated and denominated [27]. In addition to this, 11 bufadienolides have been separated completely from toad skin: resibufogenin, cinobufagin, bufalin, telocinobufagin, bufotalin, desacetylcinobufotalin, hellebrigenin, arenobufagi, gamabufotalin, 11β-hydroxylresibufogenin, and cinobufotalin [28, 29]. Bioactive substances such as senkyunolide A, senkyunolide H, senkyunolide I, ligustilide, n-butylidenephthalide, ferulic acid, and coniferylferulate have also been isolated and identified from angelica [30, 31]. A large number of researchers continue to devote themselves to fingerprint research of toad skin and angelica. HPLC fingerprints of toad skin from 10 different regions of China were analyzed and 29 peaks were screened out as the characteristic peaks, of which 9 peaks corresponded to known bufadienolides [32]. In a separate study, the fingerprint of Chinese angelica, based on the consistent chromatograms of 40 samples and compared with 13 related herbs, included 4 Japanese Angelicae Root samples, 6 Szechwan Lovage Rhizome samples, and 3 Cnidium Rhizome samples [33]. These analyses can provide important references for the quality control of medicinal materials. Unfortunately, although many chemical constituents of toad skin and angelica have already been identified using fingerprinting, no chromatographic fingerprints for the quality control of the compound prescription have been reported until now. This is extremely adverse to constituent illustration and active component screening. Thus, with a view to the further development of Antike capsule, it is

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necessary to clarify its composition and establish quality standards.

2 Materials and methods

2.1 Chemicals and reagents

Reference compounds of 9 bufadienolides from toad skin (gamabufotalin, arenobufagin, telocinobufagin, desacetylcinobufotalin, bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin) and 7 ingredients from angelica (ferulic acid, n-butylidenephthalide, senkyunolide A, senkyunolide I, senkyunolide H, ligustilide, coniferylferulate) (99.0% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). The structures of the 16 reference compounds are presented in Fig.1.

Acetonitrile (MeCN) (HPLC-grade) and methanol (MeOH) (HPLC-grade) were purchased from Honeywell (Muskegon, MI, USA). Acetic acid (HOAc) (HPLC-grade) was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Ultra pure water was prepared with a Millipore water purification system (Milford, MA, USA) and filtered with a 0.22 μm microporous membrane that was purchased from Kexun experimental equipment Co., Ltd (Guangzhou, China). Phosphoric acid (H_3PO_4) and potassium dihydrogen phosphate (KH_2PO_4) used in the mobile phase were of AR-grade and were purchased from Hongyan Chemical Reagent Co., Ltd (Tianjin, China).

Ten batches of Antike capsule were purchased from the Changchun Yuanda Guoao Pharmaceutical Co., Ltd. The product lot numbers were S-1 (150105), S-2 (150115), S-3 (150205), S-4 (150301), S-5 (150315), S-6 (150329), S-7 (150415), S-8 (150428),

Analytical Methods Accepted Manuscript

S-9 (150505), and S-10 (150520). In addition to this, the raw materials of Antike capsule, which mainly consists of toad skin and the roots of angelica, were also obtained from this company and identified by Professor Jianbo Wang (Institute of Materia Medica, School of Pharmacy, Fourth Military Medical University, Xi'an, China).

2.2 Preparation of standard solutions

A standard stock solution containing the 16 components (gamabufotalin 160 μg mL^{-1}, arenobufagin 220 μg mL^{-1}, telocinobufagin 160 μg mL^{-1}, desacetylcinobufotalin 100 μg mL^{-1}, bufotalin 280 μg mL^{-1}, cinobufotalin 480 μg mL^{-1}, bufalin 500 μg mL^{-1}, cinobufagin 400 μg mL^{-1}, resibufogenin 180 μg mL^{-1}, ferulic acid 240 μg mL^{-1}, n-butylidenephthalide 1800 μg mL^{-1}, senkyunolide A 400 μg mL^{-1}, senkyunolide I 240 μg mL^{-1}, senkyunolide H 80 μg mL^{-1}, ligustilide 400 μg mL^{-1} and coniferylferulate 240 μg mL^{-1}) was prepared in chromatographic pure methanol and stored away from light at 4°C. Working standard solutions containing the 16 compounds were prepared by appropriate dilution of the stock solution.

2.3 Preparation of sample solutions

The capsule shells were stripped and 2.0 g of each of the 10 powdered samples of Antike capsule was extracted with methanol (20 mL, HPLC-grade) by reflux for 1.0 h at 70°C. After cooling, methanol was added to the decoctions to make up the initial weight. The resulting extract was centrifuged for 15 min at 12,000×g and was filtered with a 0.45 μm microporous membrane (Kexun experimental equipment Co., Ltd, Guangzhou, China) prior to analysis. In the same way, 1.0 g of toad skin and angelica

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powdered samples were also prepared as comparison. Aliquots (20 μL) of sample solution were injected into the HPLC system for analysis.

2.4 Apparatus and chromatographic conditions

The analyses were performed using a Shimadzu Prominence LC-20A HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-20AD quaternary pumps, SPD-M20A PDA detector, SIL-20AC HT autosampler, CTO-20A thermostat compartment and LC-solution software. The samples were separated on an Agilent Zorbax SB-C_{18} column (4.6×250 mm 5 μm, Agilent Technologies, USA) guarded by an Agilent Zorbax SB-C_{18} 4.6×12.5 mm analytical guard column. The mobile phase consisted of MeCN and 0.1% HOAc-0.5% KH_2PO_4 aqueous solution (adjusted to pH=2.4 with H_3PO_4). A multistep gradient program was used as follows: 8% MeCN (0min), 30% MeCN (20 min), 40% MeCN (45 min), 50% MeCN (70 min), 40% MeCN (75 min), and 8% MeCN (80 min). The flow rate was 0.8 mL min^{-1}, column temperature was maintained at 30°C, the wavelength of PAD ranged from 200 to 800 nm, and the chromatograms were recorded at 296 nm.

2.5 Data analysis

Data analysis was performed using the Computer aided Similarity Evaluation (CASE) software, which was developed by the Research Center for the Modernization of Traditional Chinese Medicines (Central South University, Changsha, China) [34], and mainly applied in the similarity study of chromatographic and spectral patterns. The CASE software can normalize the chromatogram and match the identical peaks in each chromatogram automatically. With this software, based on median or average

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data, the correlation coefficient and congruence coefficient were calculated to evaluate the similarity of each sample [35].

3 Results and discussion

3.1 Optimization of extraction conditions

With the purpose of optimizing the extraction conditions, which can fully show the overall extraction of Antike capsule, in this study, multiple related extraction conditions were evaluated. The following experimental factors were involved. First, extraction method (ultrasonication, reflux); second, extraction solvent (methanol, acetonitrile or ethyl alcohol); third, solvent composition (20, 40, 60, 80 or 100%, v/v); and finally, extraction time (0.5, 1, 2 or 4 h). Sum numbers and areas of characteristic peaks in each chromatogram were used as evaluation criteria for each experimental factor. After multiple factor-independent and integrated comparisons, the efficiency and feasibility were taken into consideration, and the optimal extraction conditions of Antike capsule were finally achieved.

3.2 Optimization of HPLC conditions

In order to achieve the best separation, various factors were examined, including chromatographic column, mobile phase, elution mode, detection wavelength, flow rate, and column temperature. The Ultimate XB C_{18} column (4.6×250 mm 5 μm, Welch Technologies, Shanghai, China), Phenomenex Luna 5u C_{18} column (4.6×250 mm 5 μm, Phenomenex Technologies, USA), Yilite Hypersil BDS C_{18} column (4.6×250 mm 5 μm, Yilite Technologies, Dalian, China), Yilite SinoChrom ODS-BP C_{18} column (4.6×250 mm 5 μm, Yilite Technologies, Dalian, China) and Agilent

Analytical Methods Accepted Manuscript

Zorbax SB-C_{18} column (4.6×250 mm 5 μm, Agilent Technologies, USA) were employed. Compared with methanol, acetonitrile combined with phosphoric acid and potassium dihydrogen phosphate buffer remarkably improved the separation of the major constituents in toad skin. In addition, the addition of acetic acid had substantial effects on the selectivity and efficiency of some compounds. Under different gradient elution modes, variations of pH, flow rate, and column temperatures were also compared. The recorded wavelength was selected according to the characteristic UV profiles. The maximum absorption wavelength of gamabufotalin, arenobufagin, telocinobufagin, desacetylcinobufotalin, bufotalin, cinobufotalin, bufalin, cinobufagin, resibufogenin, ferulic acid, n-butylidenephthalide, senkyunolide A, senkyunolide I, senkyunolide H, ligustilide and coniferylferulate were at 300 nm, 298 nm, 300 nm, 295 nm, 294 nm, 294 nm, 300 nm, 294 nm, 299 nm, 322 nm, 236 nm, 279 nm, 276 nm, 276 nm, 327 nm, and 317 nm, respectively. Considering the maximum absorption wavelength of each ingredient, and to obtain the optimal chromatogram, 296 nm was finally chosen as the recorded wavelength. After many tests, the Agilent Zorbax SB-C_{18} column with the acetonitrile and 0.1% acetic acid-0.5% potassium dihydrogen phosphate aqueous solution (adjusted to pH 2.4 with phosphoric acid) using gradient elution was selected for the simultaneous separation and determination.

3.3 Method validation of the fingerprints

With the established extraction and HPLC conditions, sample 6 (130310) was analyzed to validate the method, which involved precision, reproducibility, and stability experiment. The injection precision was evaluated by successive analysis of

Analytical Methods Accepted Manuscript

the same sample solution five times, the reproducibility was evaluated with five independently prepared sample solutions, and the analysis of the same sample solution at different times (0, 2, 4, 6, 8, 12 and 24 h) was used to evaluate the stability of sample solutions within 24h. The characteristic peak of NO.22 was selected as a reference to calculate the relative retention time (RRT) and relative peak area (RPA) of each characteristic peak (Fig.2A). The relative standard deviations (RSDs) of RRT and RPA of characteristic peaks were used to reflect the precision, reproducibility, and stability respectively. The results demonstrated that the RSDs of injection precision were below 1.25% (n=5) for RRT and 4.75% (n=5) for RPA. The RSDs of reproducibility were below 1.88% (n=5) for RRT and 4.44% (n=5) for RPA. The RSDs of stability were below 1.42% (n=7) for RRT and 4.63% (n=7) for RPA. These results confirmed that the method of HPLC for the fingerprint analysis was valid and satisfactory (Table.1).

3.4 Fingerprint analysis of Antike capsule

In order to establish the representative HPLC fingerprint of Antike capsule, 10 different batches of samples were analyzed, and each chromatogram was used to construct the reference chromatograms. On the premise of achieving optimal chromatographic resolution and peak pattern, 28 peaks were screened as characteristic peaks (Fig.2A). The reference chromatograms of fingerprints derived with CASE software are shown in Fig.2B. The chromatograms of 10 samples were compared with the corresponding reference chromatograms, and their similarity was evaluated with correlation coefficients and congruence coefficients, which were calculated from

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median and average data (Table.2). Evaluation of their similarity using fingerprinting showed that the quality of sample 7 was lower than that of the other samples. Despite of this, the lowest similarity value was only 0.9873, which indicated that these samples had a high similarity and there was no obvious differences in the quality of the 10 batches of Antike capsule.

The characteristic peaks clearly revealed the fingerprint of Antike capsule, however, as a compound preparation composed of toad skin and angelica, the fact that only the characteristic peaks were investigated is inadequate. Further investigation was required found out which raw material they came from. Based on the fingerprint, the chromatograms of toad skin and angelica powdered samples were also compared with the Antike capsule (Fig.2C). After comparison, 28 characteristic peaks were traced to the source of original raw materials, in which peaks NO.3, 5, 6, 7, 9, 10, 12, 20, 22, 24, 26, 27, and 28 were derived from angelica, peaks NO.4, 8, 11, 13, 14, 15, 16, 17, 18, 19, 21, 23, and 25 were derived from toad skin, and peaks NO.1 and 2 were derived from common compositions of toad skin and angelica.

3.5 Identification of the characteristic peaks

To further illustrate the characteristic peaks and chemical constitution of Antike capsule, the characteristic chromatograms were compared with the chromatograms of reference compounds (Fig.3). According to the consistence in retention times and UV absorption, there were 16 peaks among the 28 characteristic peaks that were unambiguously identified: gamabufotalin, arenobufagin, telocinobufagin, desacetylcinobufotalin, bufotalin, cinobufotalin, bufalin, cinobufagin, resibufogenin,

Analytical Methods Accepted Manuscript

ferulic acid, n-butylidenephthalide, senkyunolide A, senkyunolide I, senkyunolide H, ligustilide, and coniferylferulate (Fig.3 and Table.3).

3.6 Method validation of quantitative determination

3.6.1 Calibration curves and the limit of detection

All calibration curves were plotted based on linear regression analysis of the integrated peak areas (x) versus concentrations (y, μg mL^{-1}) of the 16 identified constituents in the standard solution at 9 different concentrations. Each concentration was analyzed using 6 paralleled samples. The regression equations, correlation coefficients, and linear ranges for the analysis of the 16 identified constituents are shown in Table.4.

The limit of detection (LOD) value was calculated as the number of the injected samples that gave a signal-to-noise ratio of 3 (S/N=3). The LOD values of the method for the 16 constituents are also listed in Table.4.

3.6.2 Precision and accuracy

The precision and the accuracy of the assay were evaluated with standard solutions at low, medium, and high concentrations and measured by RSD. The intra- and inter-day precision was determined six times during a single day and on six consecutive days, respectively. The results are summarized in Table.5. The intra- and inter-day precision calculated as the RSD were less than 2.0%. The accuracies of 16 constituents were within the range of 95.61-104.09%. These results meet the requirements of TCM content determination [36].

3.6.3 Recovery

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Three different concentrations (low, medium, and high) of authentic standards were added into samples (S-2). The resultant samples were processed and analyzed as described in Section 2.3. The quantity of each analyte was subsequently obtained from the corresponding calibration curve. Recovery of all 16 constituents were within the range of 94.86-103.10%, with an RSD between 0.30% and 1.80% (n=6). The corresponding values met with the standard requirements [36] (Table.6).

3.6.4 Reproducibility and stability

For the reproducibility test, from the same batch of Antike capsule (S-2), 6 samples prepared in the same way were analyzed, and the RSD values of the peak area were at a range of 0.48-1.90% (meeting the requirement of < 2.0% per the Pharmacopoeia of the People's Republic of China [36]). For stability, after preparation of 0, 2, 4, 6, 8, 12, and 24 hours, the same sample solution was analyzed at room temperature. The RSD value of the peak area was no more than 1.96%. The results of the reproducibility and stability tests showed that the method manifested good reproducibility and the solution was considered to be stable within 24 h.

3.7 Quantitative determination of 16 identified ingredients in 10 batches of Antike capsule

The newly established method identified 16 constituents in 10 different batches of Antike capsule. As shown in Fig.3 and Table.7, under the analytical conditions, the 16 constituents included 9 compounds derived from toad skin (gamabufotalin, arenobufagin, telocinobufagin, desacetylcinobufotalin, bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin) and 7 compounds derived from angelica

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(ferulic acid, n-butylidenephthalide, senkyunolide A, senkyunolide I, senkyunolide H, ligustilide and coniferylferulate). In light of its high simplicity, precision, accuracy, and reliability, this method was concluded to be suitable for the routine analysis and quality control of commercial Antike capsule.

4 Concluding remarks

In the present research, we focused on developing a simple, convenient, accurate, and reproducible chromatographic fingerprint method to provide references for the quality control of Antike capsule by HPLC-PAD. By making use of the CASE software, multi-sample batches of samples were examined to generate the representative standard fingerprints, and characteristic peaks were screened out and traced to the source of original raw materials. Compared with the retention time and UV adsorption of reference compounds, characteristic peaks of known constitutes were identified, and the similarity of each sample was calculated and the known ingredients were quantitatively determined.

The method established in this study had already been validated and manifested high simplicity, precision, accuracy, recovery, reproducibility, and stability. In this study, this method was determined to be suitable for the routine analysis of Antike capsule and its components (toad skin, angelica). The HPLC fingerprint analysis and quantitative determination in this research could provide an important reference to establish the quality control standards for commercial Antike capsule. Furthermore, the revelation of major constituents in this study has made great contribution toward the further screening of the active ingredients in Antike capsule.

Analytical Methods Accepted Manuscript

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Conflict of Interest The authors report no conflict of interest.

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Table 1. Analytical method validation results for the fingerprint analysis

Peak NO.	RSD of relative retention time (%)			RSD of relative peak area (%)		
	Precision (n=5)	Reproducibility (n=5)	Stability (n=7)	Precision (n=5)	Reproducibility (n=5)	Stability (n=7)
1	0.82	0.96	1.29	3.65	4.24	4.23
2	0.86	1.19	1.16	3.28	4.14	2.41
3	0.95	1.00	0.76	2.87	2.77	2.46
4	1.25	1.88	1.42	3.77	1.81	3.83
5	0.74	1.29	1.27	2.77	3.78	2.75
6	0.53	0.89	0.69	3.05	3.55	3.00
7	0.28	0.84	0.36	1.95	2.60	1.82
8	0.14	0.71	0.30	3.32	2.10	2.29
9	0.15	0.64	0.23	1.47	2.06	1.68
10	0.12	0.56	0.18	4.34	1.61	2.90
11	0.12	0.45	0.20	1.92	3.09	2.11
12	0.13	0.39	0.21	3.10	3.54	4.63
13	0.23	0.16	0.16	4.01	2.19	2.28
14	0.24	0.16	0.15	1.58	2.10	2.44
15	0.27	0.30	0.20	2.02	4.44	2.55
16	0.26	0.36	0.23	4.75	2.94	2.87
17	0.24	0.16	0.17	4.30	3.32	3.32
18	0.22	0.30	0.21	3.11	2.48	2.58
19	0.19	0.11	0.18	3.87	2.46	2.50
20	0.14	0.08	0.16	1.66	3.10	4.18
21	0.11	0.04	0.09	2.37	2.16	2.22
22	0.00	0.00	0.00	0.00	0.00	0.00
23	0.05	0.11	0.06	2.08	1.89	1.28
24	0.05	0.20	0.15	3.52	1.47	2.71
25	0.08	0.17	0.11	2.35	2.13	2.32
26	0.11	0.28	0.23	2.52	3.57	4.42
27	0.14	0.33	0.26	1.87	2.65	1.49
28	0.15	0.32	0.26	2.05	1.12	2.33

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Table 2. Evaluation of the similarity of 10 samples derived with CASE software

Sample NO.	Correlation Coefficients		Congruence Coefficients	
	Median	Average	Median	Average
1	0.9962	0.9966	0.9974	0.9978
2	0.9955	0.9965	0.9964	0.9973
3	0.9961	0.9964	0.9973	0.9974
4	0.9974	0.9975	0.9977	0.9975
5	0.9931	0.9934	0.9953	0.9957
6	0.9989	0.9985	0.9993	0.9990
7	0.9881	0.9873	0.9920	0.9917
8	0.9986	0.9979	0.9990	0.9985
9	0.9909	0.9931	0.9936	0.9948
10	0.9932	0.9950	0.9953	0.9967

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Table 3. Retention times ± standard deviation (t_R ± SD) and maximum absorption wavelength of 16 identified peaks

Peak NO.	t_R ± SD (min)	λ_{max} (nm)	Identification
7	19.23 ± 0.18	324	ferulic acid
8	23.04 ± 0.16	300	gamabufotalin
9	23.60 ± 0.15	276	senkyunolide I
10	24.75 ± 0.15	276	senkyunolide H
12	27.64 ± 0.20	298	arenobufagin
14	36.44 ± 0.32	300	telocinobufagin
15	37.08 ± 0.39	295	desacetylcinobufotalin
17	38.01 ± 0.31	294	bufotalin
19	42.04 ± 0.36	294	cinobufotalin
21	48.34 ± 0.39	300	bufalin
22	53.30 ± 0.43	317	coniferylferulate
23	57.86 ± 0.44	294	cinobufagin
24	59.21 ± 0.40	279	senkyunolide A
25	60.36 ± 0.44	299	resibufogenin
27	71.91 ± 0.47	327	ligustilide
28	72.93 ± 0.49	313	n-butylidenephthalide

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Table 4. Regression equation, linear range, and LODs of the developed method (n=6)

Constituents	Regression equation [a]	Correlation coefficient (r)	Linearity range (μg mL^{-1})	LOD (μg mL^{-1})
ferulic acid	$y = 7.8222 \times 10^{-6} x - 0.6581$	0.9999	0.47-120	0.02
gamabufotalin	$y = 3.8314 \times 10^{-5} x - 0.4756$	0.9998	0.31-80	0.08
senkyunolide I	$y = 4.7093 \times 10^{-5} x - 0.8360$	0.9997	0.47-120	0.12
senkyunolide H	$y = 1.9638 \times 10^{-5} x - 0.2629$	0.9998	0.16-40	0.05
arenobufagin	$y = 4.6342 \times 10^{-5} x - 0.5905$	0.9999	0.43-110	0.11
telocinobufagin	$y = 3.7615 \times 10^{-5} x - 0.4643$	0.9998	0.31-80	0.10
desacetylcinobufotalin	$y = 5.9740 \times 10^{-5} x - 0.2746$	0.9999	0.20-50	0.10
bufotalin	$y = 4.6136 \times 10^{-5} x - 0.8232$	0.9998	0.55-140	0.11
cinobufotalin	$y = 5.2275 \times 10^{-5} x - 1.3812$	0.9998	0.94-240	0.12
bufalin	$y = 4.1298 \times 10^{-5} x - 1.5169$	0.9998	0.98-250	0.10
coniferylferulate	$y = 1.1265 \times 10^{-5} x - 0.4608$	1.0000	0.47-120	0.05
cinobufagin	$y = 4.2961 \times 10^{-5} x - 1.0978$	0.9999	0.78-200	0.10
senkyunolide A	$y = 3.6677 \times 10^{-4} x - 0.2775$	1.0000	0.78-200	0.20
resibufogenin	$y = 4.2848 \times 10^{-5} x - 0.5126$	0.9998	0.35-90	0.07
ligustilide	$y = 2.9985 \times 10^{-5} x - 1.3189$	0.9997	0.78-200	0.08
n-butylidenephthalide	$y = 4.0829 \times 10^{-5} x - 6.4239$	0.9997	3.52-900	0.12

[a] y: concentration of components; x: peak area of components.

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Table 5. Precision and accuracy of the developed method

Constituents	Nominal concentration ($\mu g\ mL^{-1}$)	Precision					
		Intra-day (n =6)			Inter-day (n =6)		
		Mean±SD ($\mu g\ mL^{-1}$)	Accuracy (%)	RSD (%)	Mean±SD ($\mu g\ mL^{-1}$)	Accuracy (%)	RSD (%)
	30.00	29.50±0.39	98.32	1.33	29.84±0.22	99.46	0.75
ferulic acid	7.50	7.33±0.14	97.72	1.86	7.57±0.09	100.99	1.25
	1.88	1.83±0.03	97.80	1.43	1.90±0.03	101.54	1.80
	20.00	20.12±0.35	100.62	1.75	19.90±0.31	99.49	1.58
gamabufotalin	5.00	4.92±0.08	98.32	1.70	4.90±0.06	98.02	1.26
	1.25	1.24±0.02	99.19	1.56	1.26±0.01	101.05	1.19
	30.00	29.09±0.42	96.97	1.43	30.25±0.21	100.85	0.70
senkyunolide I	7.50	7.25±0.14	96.64	1.98	7.42±0.09	98.88	1.21
	1.88	1.88±0.03	100.13	1.79	1.87±0.02	99.74	1.32
	10.00	9.70±0.17	96.96	1.75	9.65±0.16	96.47	1.65
senkyunolide H	2.50	2.45±0.04	97.89	1.71	2.47±0.02	98.73	0.91
	0.63	0.61±0.01	97.41	0.84	0.62±0.01	99.47	1.46
	27.50	27.66±0.52	100.57	1.88	27.97±0.49	101.72	1.77
arenobufagin	6.88	6.97±0.12	101.39	1.70	7.03±0.06	102.18	0.84
	1.72	1.75±0.03	101.73	1.71	1.77±0.02	102.98	1.11
	20.00	19.96±0.29	99.78	1.46	19.46±0.30	97.31	1.56
telocinobufagin	5.00	4.94±0.08	98.83	1.56	5.12±0.09	102.36	1.73
	1.25	1.27±0.02	101.22	1.72	1.25±0.02	99.82	1.63
	12.50	12.31±0.23	98.50	1.88	12.52±0.16	100.13	1.27
desacetylcinobufotalin	3.13	3.13±0.05	100.13	1.50	3.07±0.05	98.12	1.55
	0.78	0.77±0.01	98.35	1.73	0.81±0.00	103.24	0.57
	35.00	34.42±0.60	98.34	1.74	35.26±0.48	100.74	1.36
bufotalin	8.75	8.52±0.16	97.36	1.91	8.65±0.09	98.81	1.09
	2.19	2.20±0.04	100.58	1.80	2.20±0.04	100.63	1.71
	60.00	61.32±0.74	102.20	1.20	59.05 ±0.73	98.41	1.24
cinobufotalin	15.00	15.17±0.14	101.11	0.89	15.30±0.21	102.01	1.35
	3.75	3.68±0.06	98.12	1.51	3.76±0.02	100.30	0.51
	62.50	62.37±0.88	99.79	1.41	65.06±0.72	104.09	1.11
bufalin	15.63	15.26±0.26	97.69	1.67	15.92±0.20	101.88	1.26
	3.91	3.94±0.05	100.97	1.31	4.02±0.02	102.85	0.59
	30.00	30.48±0.52	101.60	1.72	28.94±0.52	96.47	1.78
coniferylferulate	7.50	7.55±0.08	100.66	1.00	7.48±0.10	99.69	1.35
	1.88	1.83±0.03	97.70	1.66	1.90±0.03	101.27	1.59
	50.00	50.76±0.59	101.51	1.17	50.80±0.79	101.60	1.56
cinobufagin	12.50	12.37±0.17	98.98	1.38	12.77±0.17	102.15	1.32
	3.13	3.04±0.05	97.44	1.49	3.18±0.04	101.73	1.39
	50.00	50.94±0.57	101.89	1.12	49.21±0.92	98.43	1.88
senkyunolide A	12.50	12.59±0.25	100.70	1.97	12.81±0.21	102.50	1.63
	3.13	3.08±0.05	98.42	1.72	2.99±0.05	95.61	1.76

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	22.50	22.95±0.33	101.98	1.45	21.74±0.24	96.64	1.13
resibufogenin	5.63	5.47±0.09	97.28	1.64	5.73±0.08	101.81	1.38
	1.41	1.37±0.02	97.68	1.12	1.44±0.02	102.72	1.25
	50.00	50.30±0.58	100.59	1.15	50.05±0.81	100.11	1.61
ligustilide	12.50	12.06±0.21	96.46	1.75	12.13±0.14	97.04	1.15
	3.13	3.10±0.04	99.20	1.22	3.09±0.06	98.75	1.78
	225.00	220.34±0.86	97.93	0.39	217.76±4.10	96.78	1.88
n-butylidenephthalide	56.25	54.83±0.90	97.47	1.64	55.32±0.66	98.35	1.18
	14.06	14.31±0.24	101.76	1.65	14.00±0.18	99.59	1.29

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Table 6. Recovery of the developed method (n=6)

Constituents	Sample Contents (μg)	Add Quantity (μg)	Measured Contents (μg, mean±SD)	Recovery (%)	RSD (%)
		852.00	1544.21±8.57	97.71	0.56
ferulic acid	711.71	710.00	1408.82±5.30	98.18	0.38
		568.00	1269.12±7.12	98.14	0.56
		84.00	153.90±1.67	99.37	1.09
gamabufotalin	70.43	70.00	139.28±1.40	98.36	1.00
		56.00	125.93±1.64	99.11	1.30
		990.00	1803.83±21.43	98.92	1.19
senkyunolide I	824.56	825.00	1637.92±8.42	98.59	0.51
		660.00	1477.20±9.95	98.88	0.67
		72.00	133.99±1.39	101.43	1.04
senkyunolide H	60.96	60.00	121.42±1.90	100.77	1.56
		48.00	109.86±1.36	101.87	1.24
		324.00	578.04±2.62	95.40	0.45
arenobufagin	268.93	270.00	528.93±8.73	96.29	1.65
		216.00	477.03±7.32	96.34	1.53
		120.00	216.51±3.65	96.54	1.69
telocinobufagin	100.67	100.00	196.84±0.95	96.17	0.48
		80.00	177.44±1.25	95.97	0.70
		102.00	188.36±1.48	101.18	0.79
desacetylcinobufotalin	85.15	85.00	171.19±2.74	101.22	1.60
		68.00	153.80±2.70	100.95	1.75
		258.00	472.36±1.68	99.47	0.36
bufotalin	215.72	215.00	430.46±3.53	99.88	0.82
		172.00	387.28±4.58	99.74	1.18
		492.00	911.21±3.67	101.58	0.40
cinobufotalin	411.46	410.00	829.39±9.83	101.93	1.18
		328.00	746.55±5.94	102.16	0.80
		354.00	635.44±5.41	96.11	0.85
bufalin	295.21	295.00	578.33±6.04	95.98	1.04
		236.00	523.25±6.09	96.63	1.16
		396.00	741.03±2.23	102.96	0.30
coniferylferulate	333.32	330.00	673.34±5.89	103.04	0.87
		264.00	605.52±4.39	103.10	0.73
		804.00	1474.11±13.00	99.93	0.88
cinobufagin	670.71	670.00	1349.24±7.25	101.27	0.54
		536.00	1209.68±19.96	100.55	1.65
		1548.00	2802.48±38.90	97.82	1.39
senkyunolide A	1288.23	1290.00	2568.99±14.19	99.28	0.55
		1032.00	2309.27±22.91	98.94	0.99
		144.00	257.95±2.42	95.23	0.94
resibufogenin	120.83	120.00	235.47±3.05	95.54	1.29

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		96.00	213.44±3.10	96.47	1.45
		4428.00	8051.43±106.36	98.61	1.32
ligustilide	3684.99	3690.00	7301.80±48.68	98.02	0.67
		2952.00	6631.30±47.89	99.81	0.72
		492.00	880.78±9.83	95.25	1.12
n-butylidenephthalide	412.15	410.00	801.09±14.40	94.86	1.80
		328.00	723.55±6.17	94.94	0.85

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Table 7. Content of the 16 ingredients in ten batches of Antike capsule (n=3)

Constituents	Content (mean μg g^{-1})									
	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10
ferulic acid	324.84	355.86	321.00	359.37	326.43	334.61	336.30	320.72	388.05	335.39
gamabufotalin	29.61	35.22	27.13	15.72	36.78	18.28	27.15	24.65	20.67	34.10
senkyunolide I	385.32	412.28	370.16	414.65	396.36	377.22	415.43	376.64	400.79	371.12
senkyunolide H	30.75	30.48	30.14	30.48	32.69	25.70	33.44	29.13	27.33	29.06
arenobufagin	105.79	134.46	84.35	43.62	141.75	51.12	70.48	50.14	57.71	103.61
telocinobufagin	40.43	50.34	30.07	23.98	56.12	26.01	26.36	26.71	30.14	36.60
desacetylcinobufotalin	32.34	42.57	27.12	38.84	34.38	41.13	35.04	40.19	45.80	38.94
bufotalin	81.88	107.86	59.65	52.05	116.66	64.03	55.21	55.20	60.24	85.41
cinobufotalin	163.56	205.73	126.18	115.08	217.08	131.64	111.59	125.51	125.73	155.51
bufalin	99.06	147.60	72.07	77.62	123.70	97.99	96.82	102.36	93.19	93.00
coniferylferulate	169.77	166.67	153.65	179.06	180.83	174.26	189.81	178.02	104.66	110.64
cinobufagin	251.90	335.36	151.21	186.03	293.28	222.57	169.27	215.85	228.24	243.41
senkyunolide A	621.84	644.12	586.67	656.76	640.58	561.05	674.93	662.25	641.58	967.22
resibufogenin	41.65	60.41	25.16	32.88	48.46	35.53	29.44	33.51	36.72	47.39
ligustilide	1695.17	1842.50	1687.59	1894.14	1798.31	1694.13	1759.07	1775.10	1879.37	1654.19
n-butylidenephthalide	172.25	206.07	188.70	210.96	200.09	206.51	190.13	196.05	213.20	175.92

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Figure captions

Fig.1 Chemical structures of 16 reference compounds contained in Antike capsule

A. Nine ingredients derived from toad skin; B. Seven ingredients derived from angelica.

Fig. 2 Fingerprint of Antike capsule and its raw materials

A. The representative standard fingerprint obtained by CASE calculation.

B. The similarity of the fingerprint of 10 samples derived with CASE software.

C. The comparative chromatograms of Antike capsule (a), toad skin (b) and angelica (c).

Fig. 3 Typical chromatograms of Antike capsule samples (A) and reference compounds (B) at 296 nm.

7. ferulic acid, 8. gamabufotalin, 9. senkyunolide I, 10. senkyunolide H, 12. arenobufagin,14. telocinobufagin, 15. desacetylcinobufotalin, 17. bufotalin, 19. cinobufotalin, 21. bufalin, 22. coniferylferulate, 23. cinobufagin, 24. senkyunolide A, 25. resibufogenin, 27. ligustilide, 28. n-butylidenephthalide.

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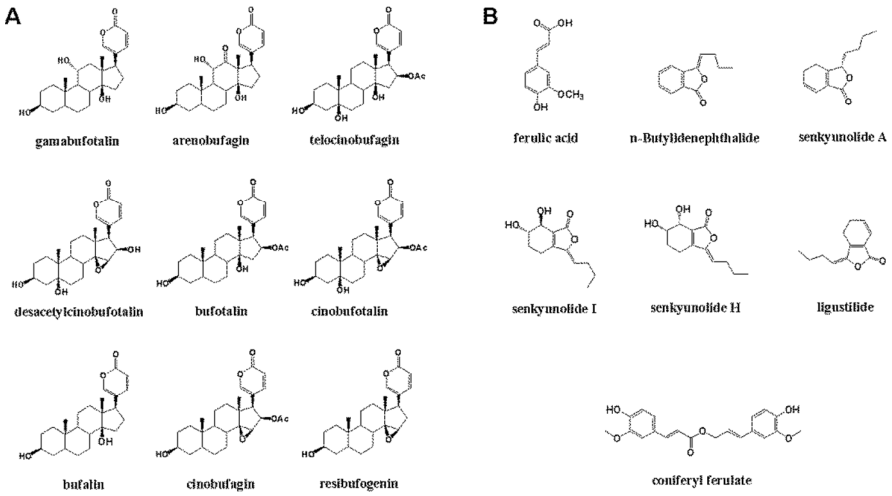

Fig. 1 Chemical structures of 16 reference compounds contained in Antike capsule A. Nine ingredients derived from toad skin; B. Seven ingredients derived from angelica.

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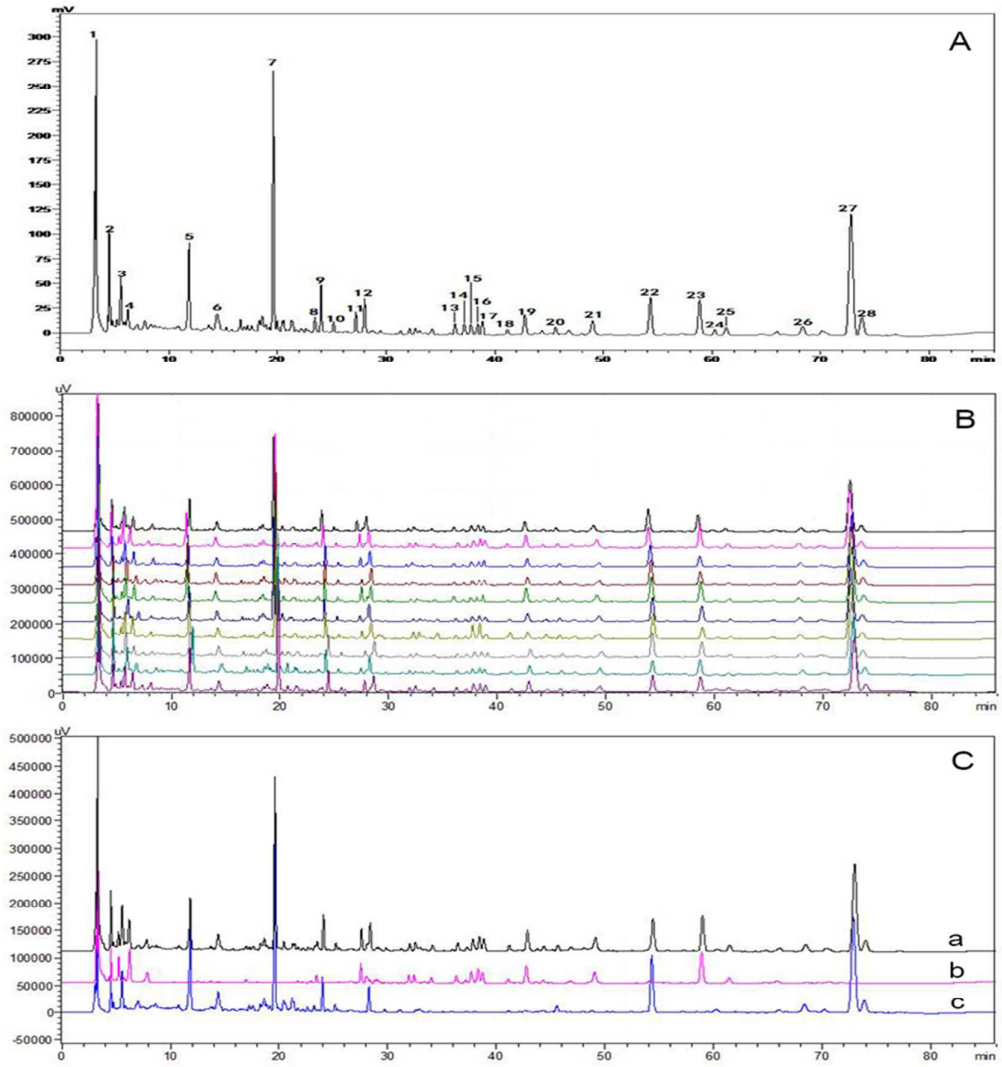

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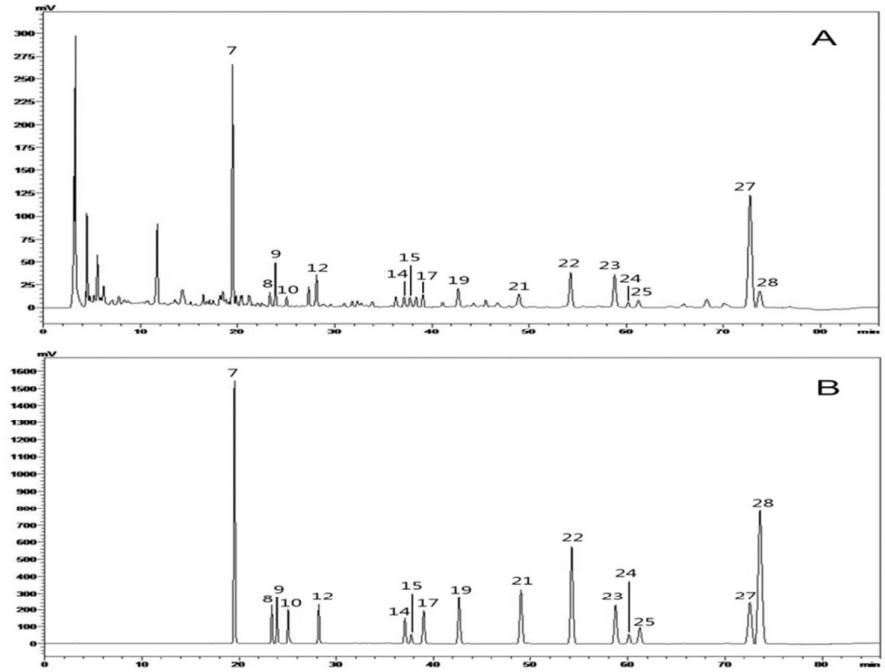

Fig. 3 Typical chromatograms of Antike capsule samples (A) and reference compounds (B) at 296 nm.

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