Chromatographic Fingerprint-Bioactivity Relationships of the Antitumor Properties of *Bufo bufo gargarizans* Skin Extracts

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Statistical analyses of high-performance liquid chromatography (HPLC)-based chemical fingerprints of *Bufo bufo gargarizans* toad skin extracts were integrated with antitumor evaluations as a means to more effectively identify bioactive constituents. Specifically, chemical fingerprints of *Bufo bufo gargarizans* skin extract samples obtained from ten different regions in China were generated by HPLC, and then subjected to similarity analysis and principal component analysis (PCA). Concurrently, a MCF-7 breast cancer cell model was adopted to obtain the antitumor activity of different toad skin extracts. The chromatographic fingerprint-bioactivity relationships of the *Bufo bufo gargarizans* extracts were then evaluated by Pearson correlation analysis. The results demonstrated that all skin extract samples inhibited the proliferation of MCF-7 cells to some extent, and that there was a correlation between the chemical fingerprints and the anti-proliferative activity. Bufotalin, bufalin, and cinobufagin, three known components of *Bufo bufo gargarizans* that were identified in the chemical fingerprints, were highlighted as constituents responsible for the observed bioactivity. The activity of cinobufagin was confirmed by cell viability and colony formation assays using MCF-7 cells. The approach documented here provides a more effective means to associate chemical information on medicinal extracts with the principle components responsible for their bioactivity, and to therefore expedite drug discovery.

Keywords *Bufo bufo gargarizans*, chemical fingerprint, principal component analysis, Pearson correlation analysis, MCF-7 breast cancer cells

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Introduction

Owing to its extraordinary therapeutic effects in the treatment of complicated diseases, Traditional Chinese Medicine (TCM) has attracted increasing attention in recent years. The skin of *Bufo bufo gargarizans*, also called toad skin, has been widely used in China for thousands years. Previous studies have demonstrated that the major pharmacological constituents derived from toad skin are lipo-soluble steroidal cardiac glycosides.¹ The bufadienolides that have Na⁺-K⁺-ATPase inhibitory activity, have been confirmed as the main active component of toad skin.² These compounds exhibit a variety of biological activities, including anti-arrhythmia, cardiotonic, antimicrobial, local anesthetic, antipyretic, diuresis activities.³⁻⁶ In recent years, they have been further shown to exhibit remarkable anticancer activities against a broad spectrum of cancer cell lines.⁷⁻¹⁰ The investigation of bufadienolides in toad skin has therefore been a hotspot in natural product research.

Moreover, cinobufacini, an aqueous extract of toad skin, which has been proved to have significant antitumor activity against liver, lung, colon, pancreatic, gastric, esophageal carcinomas¹¹⁻¹⁵ from *in vitro* and *in vivo* datasets, was approved by China Food and Drug Administration (CFDA) for use in

clinical treatment.

TCM has multi-target and multi-component characteristics, which make identification of bioactive components challenging. Owing to its high resolution, selectivity, and convenience, high-performance liquid chromatography (HPLC) is an important analytical tool in the study of food, herbal medicines, and other natural extracts.¹⁶⁻¹⁹ Chemical fingerprints based on chromatographic profiles have been recommended as a method for assessing natural products.²⁰ Similarity evaluation or pattern recognition algorithms can be applied to such chromatographic fingerprints to identify the chemical richness or novelty of complex mixtures, and many studies on chromatographic fingerprint methods have now been reported.^{21,22}

However, chromatographic fingerprints of traditional medicines cannot, by themselves, provide direct information on their pharmacodynamic properties or enable unequivocal identification of bioactive constituents. Thus, an efficient means to integrate pharmacological effects and chromatographic fingerprints could enable improved drug discovery from TCMs. This study is therefore aimed to develop HPLC-based chromatographic fingerprints of methanol extracts of *Bufo bufo gargarizans* skin collected from different regions, and to associate chromatographic features with *Bufo bufo gargarizans* activity in cell proliferations assays. Owing to its essential role in carcinogenesis, the inhibition of cell proliferation is crucial for preventing cancer, and therefore a cell proliferation assay based on MCF-7 breast cancer cells was used to assess the

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Table 1 Sources of *Bufo bufo gargarizans* skin extracts

Sample	Source	Origins
S1	Wenshan city, Yunnan	<i>Bufo bufo gargarizans</i> Cantor
S2	Linyi city, Shandong	<i>Bufo bufo gargarizans</i> Cantor
S3	Nantong city, Jiangsu	<i>Bufo bufo gargarizans</i> Cantor
S4	Zhengzhou city, Henan	<i>Bufo bufo gargarizans</i> Cantor
S5	Anguo city, Hebei	<i>Bufo bufo gargarizans</i> Cantor
S6	Yulin city, Guangxi	<i>Bufo bufo gargarizans</i> Cantor
S7	Chengdu city, Sichuan	<i>Bufo bufo gargarizans</i> Cantor
S8	Shenyang city, Liaoning	<i>Bufo bufo gargarizans</i> Cantor
S9	Bozhou city, Anhui	<i>Bufo bufo gargarizans</i> Cantor
S10	Taiyuan city, Shanxi	<i>Bufo bufo gargarizans</i> Cantor

antitumor properties of the *Bufo bufo gargarizans* extracts. Similarity and principal-components analyses (PCA) were conducted on the HPLC-based chemical fingerprints, and Pearson correlation analysis was used to associate peaks in those fingerprints with the observed antitumor activity. The goal was to provide a chemical profile-bioactivity relationship model to screen for bioactive components in the skin of *Bufo bufo gargarizans* and a methodology that would be applicable to investigations of other traditional medicines.

Experimental

Reagents and materials

The dried skins of *Bufo bufo gargarizans* Cantor specimens from ten different regions were purchased from Chinese herbal medicine markets across several provinces and authenticated by Associate Chief Pharmacist Xiao-Dong Jiang of the First Affiliated Hospital of Bengbu Medical College. Samples were labeled according to their source and elaboration method (Table 1). Human breast cancer (MCF-7) cell lines were obtained from Shanghai Cell Bank (Shanghai, China). Bufadienolide standards (bufalin, resibufogenin, cinobufagin, cinobufotalin, bufotalin, telocinobufagin, arenobufagin, and gamabufotalin) were provided by Purifa Technology Development Co., Ltd. (Chengdu, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Acetic acid was analytical grade and purchased from China National Pharmaceutical Co., Ltd. (Beijing, China). HPLC-grade acetonitrile and methanol were purchased from Merck & Co., Inc. (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water system (Millipore, Bedford, USA).

Preparation of mixed standard solutions

Standard stock solutions of the bufadienolides were prepared by adding accurately weighed bufadienolide standards to a volumetric flask, and dissolving in 10 mL methanol. A mixed standard working solution containing 55.3 $\mu\text{g mL}^{-1}$ bufalin, 27.8 $\mu\text{g mL}^{-1}$ resibufogenin, 24.0 $\mu\text{g mL}^{-1}$ cinobufagin, 38.0 $\mu\text{g mL}^{-1}$ cinobufotalin, 39.4 $\mu\text{g mL}^{-1}$ bufotalin, 34.8 $\mu\text{g mL}^{-1}$ telocinobufagin, 17.0 $\mu\text{g mL}^{-1}$ arenobufagin, and 31.5 $\mu\text{g mL}^{-1}$ gamabufotalin, was obtained by sequential dilution of a mixture of the eight individual standard stock solutions using methanol.

Preparation of *Bufo bufo gargarizans* sample solutions

Dried *Bufo bufo gargarizans* skins obtained from the ten different regions were each milled into powder. Approximately 2.5 g of milled powder was weighed and refluxed with 50 mL

methanol for 1 h. This solution was centrifuged at 15000 rpm for 10 min, and then filtered through a 0.22- μm microporous membrane. The filtrate was used for HPLC fingerprint analysis.

HPLC conditions

Reverse-phase HPLC was performed on a Thermo ODS C₁₈ column (150 $\times$ 2.1 mm, 3 μm) at a column temperature of 30°C using a Thermo-Finnigan TSQ series HPLC (Waltham, MA, USA) equipped with a photo-diode array detector (PDA). Samples were eluted with a mobile phase composed of an acetic acid solution (0.1%, A) and acetonitrile (B) using a gradient program, as follows: 0 – 2 min, 90% solvent A, 2 – 15 min, 90% solvent A to 75% solvent A, 15 – 25 min, 75% solvent A to 70% solvent A, 25 – 60 min, 70% solvent A to 60% solvent A, 60 – 65 min, 60% solvent A to 90% solvent A, 65 – 80 min, 90% solvent A. The flow rate was 0.2 mL min⁻¹. Sample injection volumes were 3 μL and the eluent was monitored at 296 nm.

HPLC fingerprint analysis

A representative toad skin sample (S3, listed in Table 1) was selected to evaluate the reliability and repeatability of the HPLC system used in this study. Firstly, this sample was injected over an extended run time of 120 min where solvent A (see above) was run at 90% from 65 – 120 min, rather than 65 – 80 min, as indicated above. This demonstrated that no significant peaks were observed after 80 min, which was the basis of the selected run time for chromatographic analyses.

The repeatability and precision were evaluated by analyzing injections of five different samples prepared from S3 and five replicate injections of one individual S3 sample. In addition, the analysis of an individual sample at different times (0, 2, 4, 6, 8, 12 and 24 h) was used to investigate sample stability. The relative standard deviations (RSD) of the relative peak areas were calculated.

CFDA has suggested that similarities of chromatograms should be evaluated by calculating correlative coefficients and/or angle cosine values of the original chromatographic data.²³ Therefore, in this study, any common characteristic peak of every sample solution of methanol extract from toad skins and of the reference standard solution were obtained, and then chromatographic fingerprints were submitted to special software, named Similarity Evaluation System for Chromatographic Fingerprint of TCM program (Chinese Pharmacopoeia Committee, Ver. 2004 A) (Beijing, China), based on a median method to compare chromatograms of ten batch toad skin samples.

Principal component analysis (PCA)

Original inter-correlated variables were transformed by PCA into a fewer number of new independent uncorrelated variables (called principal components) without significant loss of the original information.^{24,25} In this study, the relative peak areas of chromatographic peaks common to each of ten different *Bufo bufo gargarizans* skin extracts were subjected to PCA by SPSS statistical software (SPSS 16.0, SPSS Inc., USA).

Antitumor activity experiment

MCF-7 cells in the logarithmic phase of growth were collected and seeded in 96-well plates at a density of 6×10^3 cells per well. After treatment with 0.8 mg mL⁻¹ toad skin extract for 24 h, 15 μL MTT was added. Four hours later, 150 μL DMSO was added to dissolve formazan crystals. The absorbance was read at 490 nm using an enzyme-linked immunosorbent assay reader (BioTek; Molecular Devices, Winooski, VT, USA). Data were collected from three separate experiments, and the percentage cell growth inhibition induced by each toad skin

Fig. 1 HPLC chromatographic fingerprints of *Bufo bufo gargarizans* skin extracts from ten different sources using the median method that was generated by Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Chinese Pharmacopoeia Committee, Ver. 2004 A).

Fig. 2 (A) Common peak fingerprint of *Bufo bufo gargarizans* skin extracts. (B) Reference standard fingerprint of *Bufo bufo gargarizans* skin extracts.

extract was determined by comparison to DMSO-treated controls. The inhibitory rate (IR%) was calculated according to the following formula and expressed as mean $\pm$ SD:

$$\text{IR}\% = 100 \times (1 - (\text{OD of test group}) / (\text{OD of control group}))$$

Chemical profile-bioactivity relationships for the common peaks from 10 samples

Pearson correlation analysis between the areas of the common

peaks and the cell growth inhibition rates of ten batch samples were performed using SPSS statistical software (SPSS 16.0).

Results and Discussion

HPLC experiments and similarity of fingerprints

The repeatability of this experiment was in the range of 0.04 – 0.29% for retention times and 0.87 – 5.29% for the areas

Table 2 Variance contribution ratio and accumulated variance contribution rate of *Bufo bufo gargarizans* skin extracts

Principal component	Eigenvalue	Initial eigenvalue		Eigenvalue	Extracted eigenvalue	
		Variance contribution rate, %	Accumulated variance contribution rate, %		Variance contribution rate, %	Accumulated variance contribution rate, %
1	5.711	63.453	63.453	5.711	63.453	63.453
2	1.960	21.777	85.230	1.960	21.777	85.230

of nine common peaks of interest (see below), while the precision of the same sample solution was in the range of 0.06 – 0.22% for the retention times and 1.47 – 4.05% for the areas of the common peaks. The sample stability was 0.34 and 3.73% for the retention times and the areas of the common peaks, respectively. Thus, the study indicated that the validated method is accurate and precise enough to evaluate the bioactive components of toad skin samples. The HPLC fingerprints of *Bufo bufo gargarizans* skin extracts from ten different sources are shown in Fig. 1. Nine peaks with good separation that occurred in all samples was generated in Fig. 2A. Figure 2B shows the representative chromatographic peaks of standard substances from the skin of *Bufo bufo gargarizans*.

For each of the common peaks, the average peak area calculated across all samples from the ten different regions was determined as the reference peak area to subsequently calculate the relative peak area of each common peak for the individual samples.

The correlation coefficients between the fingerprints of reference standard and ten batch toad skin samples were 0.90, 0.88, 0.94, 0.91, 0.82, 0.84, 0.92, 0.89, 0.92 and 0.85, respectively, using vectorial angle cosine method based on the Similarity Evaluation System for Chromatographic Fingerprint of TCM software program.

Principal component analysis

PCA allows retention of the maximum possible variability within a data set using a much smaller number of principal components. Therefore, we utilized PCA to reduce the dimensionality of chromatographic data. As shown in Table 2, "Eigenvalue of the Correlation Matrix" PCA showed that two principal components include 63.45% (Z1) and 21.78% (Z2) information of the original indexes, respectively. Therefore, we could express the relations of two principal components according to Eigenvectors as follows:

$$Z1 = 0.384X1 + 0.41X2 + 0.364X3 + 0.368X4 + 0.228X5 + 0.4X6 + 0.28X7 - 0.097X8 + 0.342X9$$

$$Z2 = 0.066X1 - 0.121X2 - 0.276X3 - 0.181X4 + 0.259X5 - 0.161X6 + 0.492X7 + 0.641X8 + 0.354X9$$

where X corresponds to the average area of a given chromatographic peak.

A greater absolute value of the coefficient before X1 to X9 is indicative of a better correlation between the principal component and the original parameter. The above equations showed that the values of Z1 (the first principal component) were mainly associated with X2 and X6, which correspond to peak 2 (arenobufagin) and peak 6 (cinobufotalin), respectively. These findings suggest that these two components are associated with the greatest amount of variation in this study, and are therefore greatly influenced by the region from which the toad skin was obtained.

Table 3 Inhibition rate of *Bufo bufo gargarizans* skin extracts from ten different regions on MCF-7 breast cancer cells proliferation ($\bar{X} \pm S$, $n = 3$)

Sources	Inhibition rate, %
Wenshan city, Yunnan	48.14 $\pm$ 2.21
Linyi city, Shandong	66.48 $\pm$ 1.41
Nantong city, Jiangsu	55.02 $\pm$ 1.59
Zhengzhou city, Henan	60.90 $\pm$ 1.90
Anguo city, Hebei	68.69 $\pm$ 0.43
Yulin city, Guangxi	80.80 $\pm$ 0.78
Chengdu city, Sichuan	63.82 $\pm$ 1.77
Shenyang city, Liaoning	70.06 $\pm$ 2.54
Bozhou city, Anhui	69.40 $\pm$ 0.83
Taiyuan city, Shanxi	47.87 $\pm$ 1.59

Antitumor activity

As shown in Table 3, the toad skin extracts from the Guangxi province showed the strongest growth inhibitory effect on MCF-7 breast cancer cells at predetermined concentrations. However, the toad skins from different regions exhibited notable differences in the inhibitory effect, revealing that the biological activity of toad skin may be closely correlated with the producing area.

Chemical profile-bioactivity between HPLC fingerprints and antitumor activities

In this study, we employed the Pearson correlation analysis to associate chromatographic peak areas with observed bioactivity. The results showed that peak 5 (bufotalin), peak 7 (bufalin), and peak 8 (cinobufagin) of the HPLC fingerprints were the most strongly associated with an inhibitory effect on MCF-7 cells when compared to the other peaks (Table 4).

Subsequently, cinobufagin, which was associated with bioactivity, was chosen for confirmatory analysis in proliferation and colony formation assays using MCF-7 breast cancer cells. The results demonstrated that cell viability decreased in a concentration-dependent manner after the treatment with cinobufagin (Fig. 3A). Colony formation was also inhibited by increasing concentrations of cinobufagin, confirming that cinobufagin could effectively inhibit MCF-7 cell proliferation and validating our chromatographic profile-bioactivity profiling approach (Fig. 3B).

This paper is the first to integrate HPLC fingerprinting and observations of antitumor activities associated with extracts of *Bufo bufo gargarizans* toad skin of wide geographical diversity making it possible to efficiently target chromatographic peaks most likely to be responsible for bioactivity. Our finding showed that peaks 5, 7, and 8 associated with bufotalin, bufalin and cinobufagin respectively are most likely to be the main effective components of antitumor activities. These results are consistent with previous reports.^{26,27}

Table 4 Relative peak areas of common peaks and correlation coefficients of *Bufo bufo gargarizans* skin extracts from different producing regions

Sample	1	2	3	4	5	6	7	8	9
S1	1.29	1.71	1.92	1.54	0.79	1.32	0.88	0.33	0.84
S2	1.67	2.55	2.39	2.10	1.47	1.83	1.52	0.60	1.38
S3	0.72	0.15	0.22	0.50	1.38	0.48	0.87	1.42	1.05
S4	0.42	0.19	0.43	0.86	0.84	0.74	1.68	2.71	1.16
S5	1.93	2.57	1.45	1.69	1.27	1.81	1.68	0.77	1.93
S6	1.13	0.20	0.42	0.48	0.79	0.47	1.09	2.60	0.94
S7	1.53	1.74	1.30	0.91	1.00	1.33	1.08	0.53	1.39
S8	0.44	0.33	0.74	0.39	1.00	0.65	0.31	0.51	0.45
S9	0.54	0.30	0.45	0.45	1.07	0.50	0.78	0.48	0.49
S10	0.33	0.25	0.68	1.09	0.38	0.86	0.12	0.04	0.37
Correlation coefficient	0.233	0.008	-0.138	-0.26	0.321	-0.106	0.318	0.411	0.215

Fig. 3 Cell growth inhibition by cinobufagin. (A) MCF-7 breast cancer cells were treated with medium, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 $\mu\text{g mL}^{-1}$ cinobufagin for 24 and 48 h, respectively. The MTT assay was adopted to measure cell viability. (B) Inhibition of colony formation in MCF-7 breast cancer cells by cinobufagin. MCF-7 breast cancer cells were treated with medium, 0.01, 0.02 and 0.04 $\mu\text{g mL}^{-1}$ cinobufagin for 5 days.

Conclusions

The results from this study demonstrated that the antitumor activities of toad skin were associated with key compounds whose levels were also affected by conditions associated with the producing regions. Bufotalin, bufalin, and cinobufagin were identified, based on our chromatographic profile-bioactivity relationship approach, as the components most associated with activity. This was validated in confirmatory studies on the effect of cinobufagin on cell proliferation and colony formation in MCF-7 breast cancer cells.

Our experimental study provides a general model for integrating chromatographic and bioactivity data to evaluate the antitumor activities of skin of *Bufo bufo gargarizans*. We believe that our chemical profile-bioactivity relationship model can be extended further as a methodology for screening for bioactive components in investigations of other traditional Chinese medicines.

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