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Full Length Research Paper

The study of planting area on components of Baishouwu anti-tumor effective fraction by RP-HPLC

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Baishouwu glycosides (CGB) is an anti-tumor effective fraction that has been extracted and isolated from *Cynanchum auriculatum* Royle ex Wight.. To study the differences on components of CGB from 3 main planting areas, we extracted materia medica, isolated and purified CGB from the extract. Physical and chemical identification of CGB indicated that components in CGB had 2-deoxysugar steroids. The results of tin layer chromatography (TLC) proved that CGB of different planting areas had similar physical and chemical characteristics. However, the analytical results of Reversed-phase high-performance liquid chromatography (RP-HPLC) showed that the constituents of CGB from Gansu differed entirely from CGB produced Jiangsu and Shandong. *In vitro*, microculture tetrazolium (MTT) results showed that CGB from Gansu had a strong effect on cytotoxicity; however, CGB from Jiangsu and Shandong almost had no effect of cytotoxicity. Constitutes of CGB from Gansu differed entirely from CGB produced Jiangsu and Shandong, this might induce the effect of greatly varied cytotoxicity.

Key words: Baishouwu glycosides (CGB), *Cynanchum auriculatum* Royle ex Wight., anti-tumor effective fraction, tin layer chromatography (TLC), Reversed-phase high-performance liquid chromatography (RP-HPLC), microculture tetrazolium (MTT).

INTRODUCTION

Baishouwu is the dried root tuber of *Cynanchum auriculatum* Royle ex Wight., *Cynanchum wilfordii* Hemsl, and *Cynanchum bungei* Decne (family Asclepiadaceae). As a common traditional Chinese medicine (TCM), it has been widely used in clinics as a beneficial and tonic agent since ancient times. It functions in replenishing the liver and kidney, enriching vital essence and blood, astringing primordial energy, clearing away toxins and prolonging life (Kaibao Materia Medica, Song Dynasty, China). Modern pharmacological studies have shown that Baishouwu has a variety of pharmacological actions, including anti-tumor (Wang et al., 2007a; Wang et al., 2007b), elimination of free radicals (Song et al., 2001), and enhancement of immunity. CGB is an anti-tumor effective fraction that has been extracted and isolated from *C. auriculatum* Royle ex Wight. by the authors. In our previous study, CGB had a significant anti-tumor effect *in vivo* and *in vitro* (Zhang et al., 2000a); furthermore, we isolated and identified two new C-21 steroidal glycosides from CGB, they were cynanauriculosides A and B (Zhang et al., 2000b), as shown in Figure 1. The two new C-21 steroidal glycosides also had anti-tumor activity (Zhang et al., 2000c), moreover, auriculoside A was found to inhibit the cancer cells *in vitro* by activation of the apoptotic pathway (Zhang et al., 2007c). In our study, we found that, there existed significant differences of anti-tumor effect among CGB from different planting areas. The reason we supposed that CGB of the same source-base, might have had different chemical components due to their different planting areas.

In this study, under the premise of determining the source (*C. auriculatum* Royle ex Wight.) and the harvest period of Baishouwu, the differences on chemical components of CGB from 3 main planting areas was studied; meanwhile, cytotoxicity of CGB from 3 main planting areas was evaluated. To do this, a scientific basis was to be provided that CGB from different planting areas has significant differences of anti-tumor effect and

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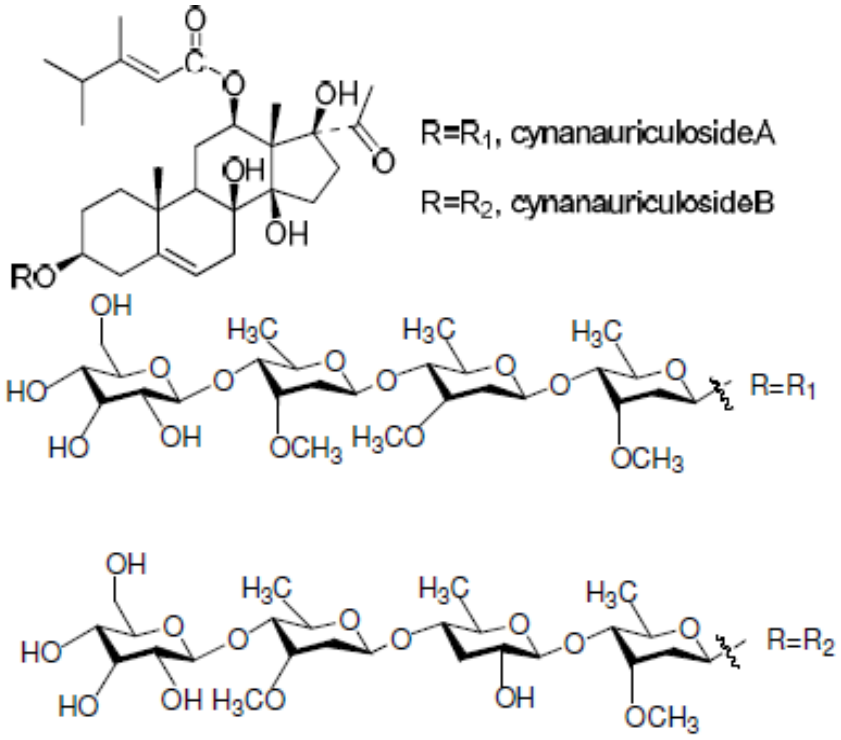

Figure 1. Structure of C-21 steroida glycosides from *Cynanchum auriculatum* Royle ex Wight.

Table 1. Collecting area and planting area of 10 batches of Baishouwu.

Batch number	Latin name	Collected areas	Planting origin
001	*Cynanchum auriculatum* Royle ex Wight.	Zhejiang Hangzhou	Jiangsu
002	*Cynanchum auriculatum* Royle ex Wight.	Jiangsu Binhai	Jiangsu
003	*Cynanchum auriculatum* Royle ex Wight.	Jiangsu Binhai	Jiangsu
004	*Cynanchum auriculatum* Royle ex Wight.	Jiangsu Binhai	Jiangsu
005	*Cynanchum auriculatum* Royle ex Wight.	Hebei Anguo	Gansu
006	*Cynanchum auriculatum* Royle ex Wight.	Gansu Tianshui	Gansu
007	*Cynanchum auriculatum* Royle ex Wight.	Zhejiang Ningbo	Shandong
008	*Cynanchum auriculatum* Royle ex Wight.	Anhui Bozhou	Shandong
009	*Cynanchum auriculatum* Royle ex Wight.	Anhui Bozhou	Shandong
010	*Cynanchum auriculatum* Royle ex Wight.	Shandong Linyi	Shandong

may relate to differences in their chemical compositions. On this basis, the chemical fingerprint and quality standards for CGB will be established in the future.

EXPERIMENTAL

Plant material

10 batches of Baishouwu were purchased from herbal medicine market, and were identified by Professor Rusong Zhang, College of Pharmaceutical Sciences, Zhejiang University of Chinese Medicine. The collecting areas and planting areas of the 10 batches of Baishouwu were showed as Table 1.

Chemicals and reagents

Cynanauriculosides A and B were isolated and purified from the dried root tuber of *C. auriculatum* Royle ex Wight. (Gansu, Tianshui) and identified according to their spectral data. The purity of these two components was about 98% determined by HPLC with the method of area normalization. CGB reference substance was isolated and purified by the authors from the dried root tuber of *C. auriculatum* Royle ex Wight. (Gansu, Tianshui). It was kept in the TCM resource engineering research center of Zhejiang Chinese medical university.Methanol and acetonitrile used as mobile phase were of chromatographic grade (Merck, Ger). Water was purified by a Millipore Milli-Q purification system (Milford, MA, USA), silica gel used for column chromatography was 200~300 mesh (Qingdao

Ocean Chemical Production, China), Thin-layer plate (Merck, Ger). Dichloromethane and methanol used for column chromatography were of chemical grade, and other regents used were of analytical grade, RPMI-1640 and DMEM medium (Invitrogen Life Technologies, USA), bovine serum (Hangzhou Sijiqing Biotechnology Co, China). Trypsin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Boehringer Mannheim (Mannheim, Germany).

Instruments

The experiment was carried out by using a high performance liquid chromatography system (Waters, USA) consisting of 1525 dual analytical pump, 717 automatic sampler and 2996 photodiode array detector (PAD). Rotary evaporator was purchased from Büchi Labortechnik AG (Büchi, Switzerland).

Cell cultures

Human liver cancer cells BEL-7402, human prostate adenocarcinoma PC-3, gastric cancer SGC-7901, were provided by the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SGC-7901 and BEL-7402 were cultured in RPMI 1640 medium, PC-3 was cultured in DMEM medium, all supplemented with 0.25% sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), and 2% penicillin-streptomycin. All the cell cultures were incubated at 95% relative humidity, 5% CO_2, and 37°C.

CGB preparation

10 batches of Baishouwu (1 kg/batch) were transferred into a round-bottom flask, respectively; 4 L of 90% ethanol was added. It was extracted for 1.5 h. The extraction was repeated twice in the same way with 4 L of new ethanol. The extracting solution was evaporated by rotary vaporization until there was no alcohol smell existed. Then, the liquid extract was suspended and extracted for four times by chloroform. The extracting solution was concentrated until dryness. The extract was total glycosides of Baishouwu (CG). Then, CG was isolated and purified by the silica gel column chromatography. The column was eluted with dichlormethane-methanol (30:1, 15:1, 5:1), using CGB reference substance as comparison, the fractions that containing CGB were collected, then the fractions were evaporated by rotary vaporization to dryness.

Preparation of CGB sample for HPLC analysis

10 batches (about 25 mg/batch) of CGB were weighted accurately, respectively; then, they were transferred into 10 ml measuring flask, and methanol was added to their scale. The samples for HPLC analysis were filtered through 0.45 μm nylon filters.

Physical and chemical identification of CGB

Lieberman-Burchard reaction and Keller-Kiliani reaction were carried out to determine the chemical types of CGB.

Analysis CGB by TLC

5 μl samples of 10 batches of CGB and CGB reference substance for HPLC analysis were measured, and, they were dotted at the same silica gel G thin-layer board, respectively. The thin-layer board was saturated by developing solvent for about 15 min; then, it was developed with chloroform-methanol-water (10:3:1), the developing distance was about 8 cm. After the developing solvent had been volatilized, 10% H_2SO_4-Ethanol was sprayed onto the thin-layer board; it was baked under 110°C until the color of flecks on the board appeared clearly.

Analytical method for CGB by RP-HPLC

A reversed-phase column (Kromasil ODS, 250×4.6 mm, 5 μm) was used in this study. Methanol-water (79:21) was used as a mobile phase. The flow-rate was 1.0 ml/min and the injection volume was 10 μl. The UV detection wavelength was set at 221 nm. The column temperature was maintained at 25°C. 10 μl samples of 10 batches of CGB for HPLC use was injected into HPLC respectively, samples were analyzed according to the earlier stated conditions.

Cytotoxicity effect of two C-21 steroidal glycosides and CGB from different planting area

Cytotoxicity was measured by microculture tetrazolium (MTT) assay (Mosmann et al., 1983). Three cancer cells, namely 1) human liver cancer cells BEL-7402, human gastric cancer cells SGC-7901, human prostate cancer cells PC-3, were seeded in 96-well flat-bottom plates at a cell density of $2.0\sim2.5 \times 10^3$ per well for 24 h. CGB of different planting area and cynanauriculoside A, B with various concentrations, 0.625 to 150 μg/ml were then added, for 72 h. After incubation, followed by adding 20 μl MTT solution (5 mg/ml, w/v, in phosphate-buffered saline (PBS) pH 7.4) in each well and being left for 3 h. The blue formazan precipitate was dissolved using 150 μl DMSO, absorbance was read on a Microplate Reader (Elx800, Bio-TEK instruments, USA) at 570 nm after plates were shaken for 5 min. Half-inhibitory concentration (IC_{50}) was used to evaluate the cytotoxicity activity of CGB from different planting area and two C-21 steroidal glycosides cynanauriculoside A, B.

RESULTS

Physical and chemical identification of CGB

The results of Lieberman-Burchard reaction and Keller-Kiliani reaction were positive, and it indicated that 10 batches of CGB were of the steroids and had 2-deoxysugar (Zhang et al., 2000a).

Analysis of CGB by TLC

As shown in Figure 2, having been isolated with TLC and colored with 10% H_2SO_4-Ethanol, 10 batches of CGB and CGB reference substance showed blue-violet stain points, and the Rf value of 10 batches of CGB was the same as CGB reference substance. It proved that 10 batches of CGB had extraordinarily similar physical and chemical properties.

Determination of the UV detection wavelength

Photodiode array detector (PAD) was used for the

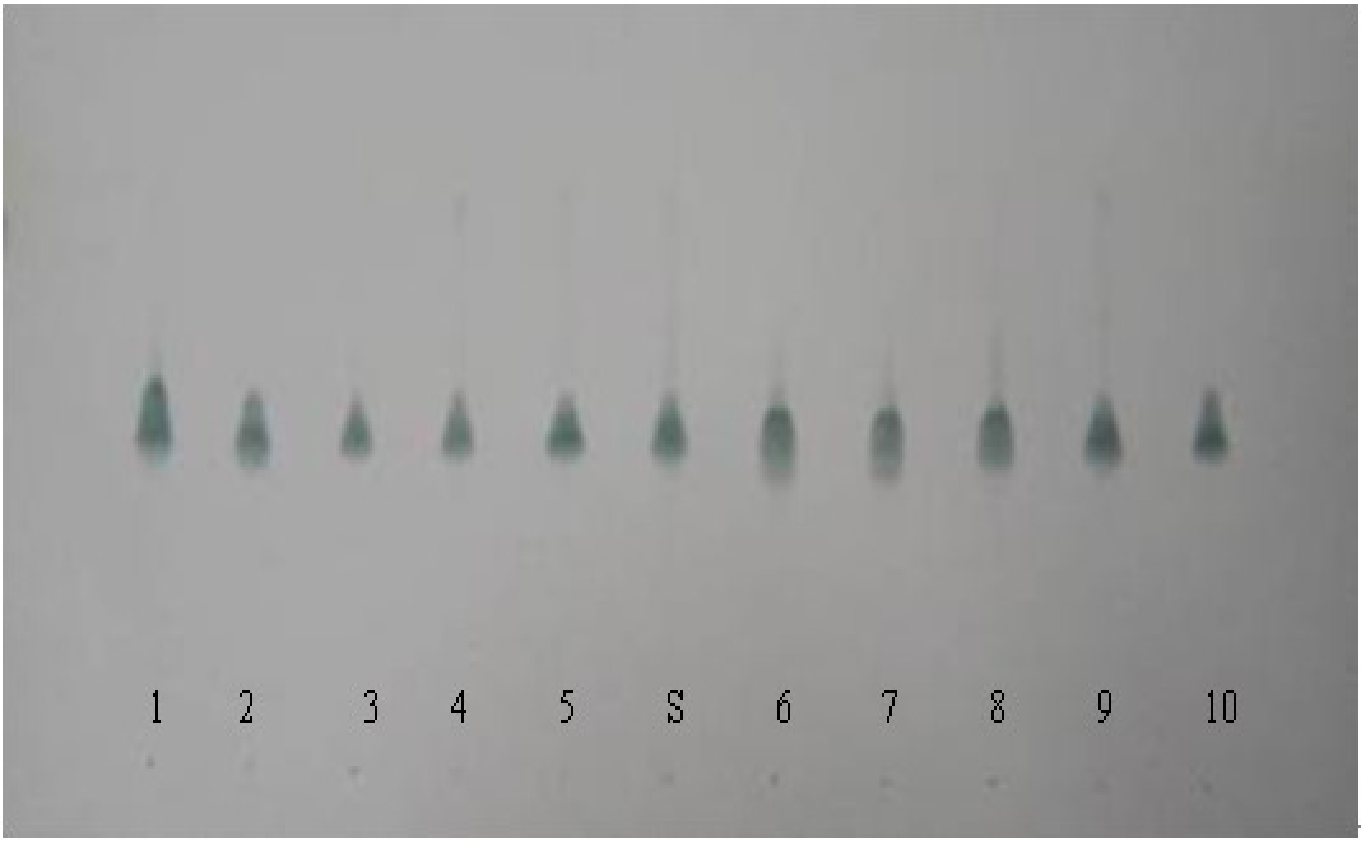

Figure 2. TLC chart of 10 batches of CGB and CGB reference substance. 1, 2, 3, 4 are CGB from Jiangsu; 5, 6 are CGB from Gansu; 7, 8, 9, 10 are CGB from Shandong; S is CGB reference substance. TLC conditions: stationary phasehe: panel of silica gel G; developer: chloroform-methanol-water (10:3:1); developing distance: 8 cm; chromogenic reagent: 10% H_2SO_4-Ethanol.

determination of UV detection wavelength of CGB. To determine the best wavelength, CGB reference substance was separated by RP-HPLC firstly, then, each chromatogram peak was scanned from 210 to 400 nm by PAD. The results suggested that the wavelength at 221 nm led to the highest intensities for the main peaks. Thus, 221 nm was chosen as the best detection wavelength in future studies.

Optimization of RP-HPLC mobile phase

To optimize the separation of CGB, water was used as the aqueous phase and either methanol or acetonitrile was used as the organic phase, respectively. The results displayed that using methanol as the organic phase was better than that of acetonitrile in better separation efficiency. Thus, methanol was selected as the optimum organic phase for further experiment.

Analysis of CGB by RP-HPLC

The HPLC chromatographic chart of 10 batches of CGB showed that different planting areas had a notable effect on the components of CGB. 2 batches of CGB from Gansu contained cynanauriculosides A and B; however, 4 batches of CGB from Jiangsu and 4 batches of CGB from Shandong contained none of the two components. Meanwhile, the main components contained in CGB from Jiangsu and Shandong did not exist in CGB from Gansu. HPLC chromatographic chart of CGB from three main planting areas were showed in Figure 3.

Method validation

Linear range, detection limit, and precision of standard

The repeatability of intra- and interday was assessed by analyzing the retention time and the peak area variations of six injections of cynanauriculoside A. The intraday RSD of the retention time was 1.05% and RSD of the peak area was 2.84%, interday RSD of the retention time was 1.92% and RSD of the peak area was 3.79%. The LOD for each analyte was defined as three times the noise level of matrix blank samples. The LOD and the linear

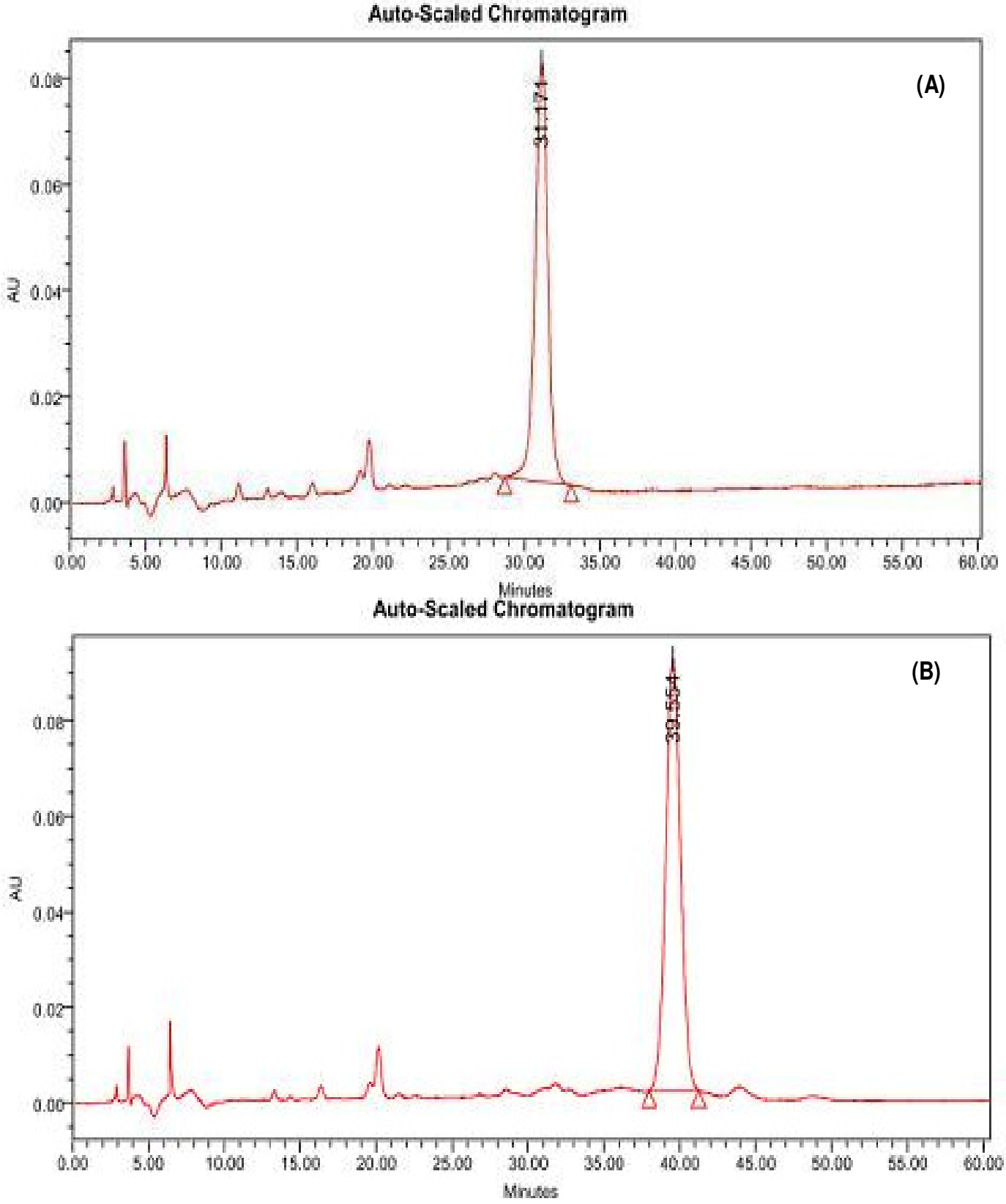

Figure 3. Chromatogram of Cynanauriculoside A, Cynanauriculoside B and CGB from *Cynanchum auriculatum* Royle ex Wight. by RP-HPLC. A is the chromatographic chart of cynanauriculoside A; B is cynanauriculoside B; C is CGB from Gansu; D is CGB from Jiangsu; E is CGB from Shandong; Mobile phase: methanol-water (79:21), flow rate: 1.0 ml/min, injection volume: 10 µl, detection wavelength: 221 nm, temperature: 25°C.

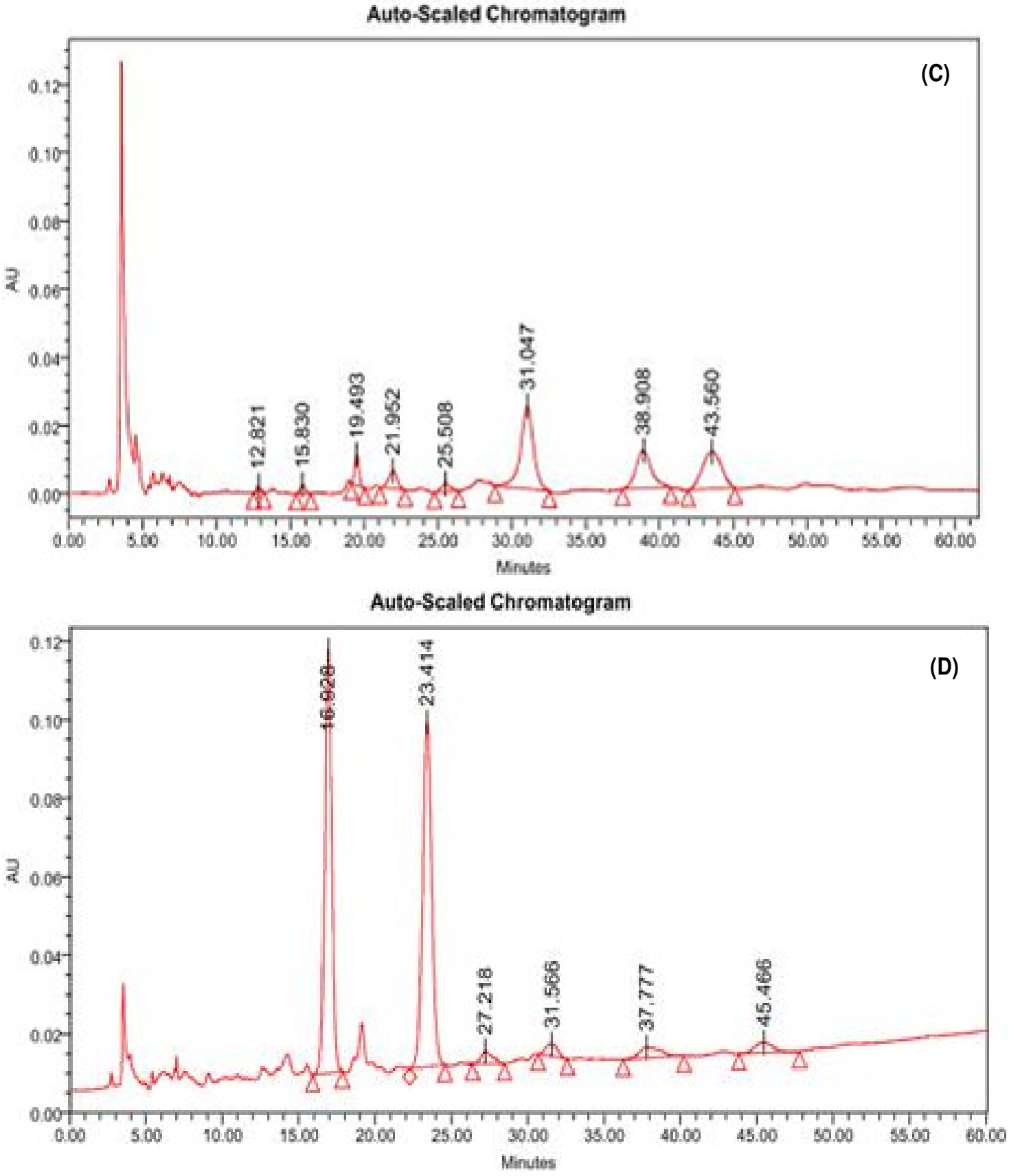

Figure 3. Contd.

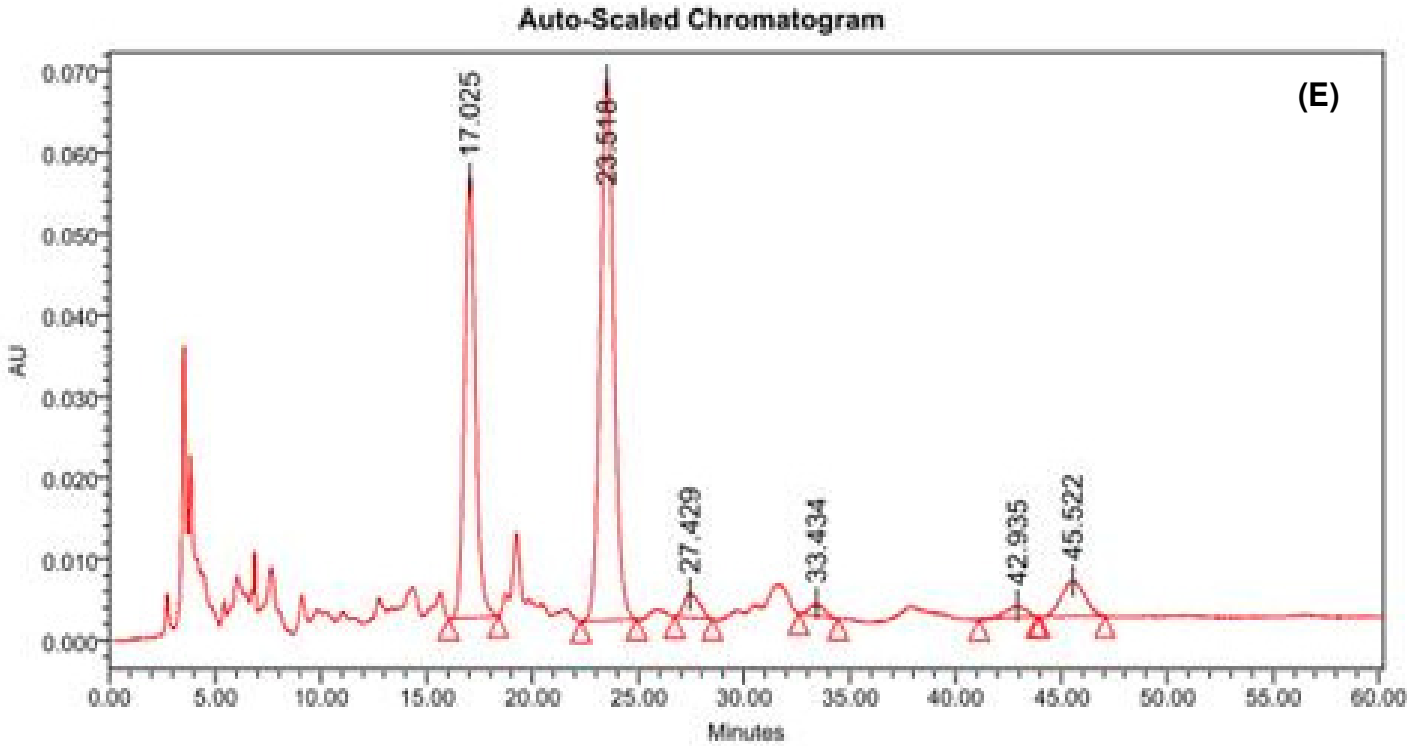

Figure 3. Contd.

Table 2. Results of regression analysis on calibration curves.

Steroids	Regression equation y = bx+a [a)]	Correlation coefficient	Linear range (mg/ml)	Detection limit [b)] (µg/ml)
Cynanauriculoside a	$y = 5\times10^6 x-20950$	0.9996	0.103-2.06	2.05
Cynanauriculoside b	$y = 5\times10^6 x+2179$	0.9997	0.113-2.26	2.35

a) y and x stand for the peak area and the concentration (mg/ml) of the analytes, respectively. b) The detection limit was defined as the concentration at the S/N of 3.

range for cynanauriculosides A and B were shown in Table 2.

Precision and recovery of samples

To evaluate the repeatability of the analytical method, CGB (Gansu) sample for HPLC analysis was prepared and injected for RP-HPLC analysis. Triplicate performances were made to evaluate the intraday variations. The RSD of the retention time and peak area of two steroids in the three sample were determined. The interday variations of the retention time and peak area of cynanauriculosides A and B in the daily preparation of samples were also determined for 3 consecutive days. The precision of samples was shown in Table 3.

Cytotoxicity effect of two C-21 steroidal glycosides and CGB from different planting areas

In vitro, the anti-tumor results of CGB from different planting area showed that CGB from Gansu had strong effect of cytotoxicity; however, CGB from Jiangsu and Shandong almost had no effect of cytotoxicity. The two new C-21 steroidal glycosides cynanauriculoside A, B isolated from CGB (Gansu) had a stronger cytotoxicity than CGB. The results were showed in Table 4.

DISCUSSION

Baishouwu from Gansu, Jiangsu and Shandong were all the dried root tuber of *C. auriculatum* Royle ex Wight., the results of physical and chemical identification indicated

Table 3. Precision of CGB sample (n = 3).

Steroids	Intraday		Interday	
	RSD for retention time (%)	RSD for area (%)	RSD for retention time (%)	RSD for area (%)
Cynanauriculoside A	1.12	2.49	2.19	5.12
Cynanauriculoside B	1.36	2.78	2.32	5.29

Table 4. IC_{50} of CGB from different planting areas and Cynanauriculoside A, B on three human cancer cells (mean ± SD, n =3).

Drugs	IC_{50} (μg/ml)		
	BEL-7402	SGC-7901	PC-3
CGB (Gansu)	46.2±0.68	37.2±0. 45	33.6±0.22
CGB (Jiangsu)	82.3±0.34	74.7±0.86	100.3±0.85
CGB (Shandong)	92.6±0.79	84.3±0.97	95.4±0.71
cynanauriculoside A	30.3±0.48	29.6±0.23	28.7±0.39
cynanauriculoside B	35.8±0.58	32.9±0.38	34.1±0.21

that CGB from the 3 main planting areas all had 2-deoxysugar steroids, the results of TLC also proved that CGB from 3 main planting areas had similar physical and chemical characteristics.

However, the analytical results of RP-HPLC showed that constituents of CGB from Gansu differed entirely from CGB from Jiangsu and Shandong. The reasons might be that chemical constituents of CGB are the secondary metabolites, the main chemical components are closely related to the environmental factors, Gansu is located in northwest of China, whereas Jiangsu and Shandong are located in the east of China, the climate in these 3 areas are quite different. Therefore, the chemical components of CGB from Gansu differed greatly from Jiangsu and Shandong. Components of CGB from Jiangsu and Shandong need further study in the future. The significant differences on the components possibly induce the notable differences of cytotoxicity effect.

From the aspect of developing CGB into a new anti-tumor drug, it was necessary to study the chemical fingerprint of CGB from Gansu. According to the technical details of fingerprint research, the mutual fingerprint peaks and non mutual fingerprint peaks should be determined, and the relative content of main components contained in CGB should be determined, when the effect of anti-tumor activity is the most strong. Since the components of CGB from Gansu differed greatly from Jiangsu and Shandong, in consideration of plant taxonomy, there might be variation in what has happened in species of *C. auriculatum* Royle ex Wight.. We supposed that if we could collect some proofs about variation in morphology and in microscopic characteristics of *C. auriculatum* Royle ex Wight., we could find a variety in *C. auriculatum* Royle ex Wight., this is of great significance in identification of germplasm resources.

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