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Analysis of the bioactive constituents of *ChanSu* in rat plasma by high performance liquid chromatography with mass spectrometric detection

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ABSTRACT

A plasma pharmacochemistry analysis of the bioactive constituents in rat plasma after oral administration of *ChanSu* was performed. High performance liquid chromatography coupled with the electrospray ionization mass spectrometry techniques of HPLC/ESI-IT-MS/MS and RRLC/ESI-Q-TOF-MS were used for the chemical profiling of samples of dosed plasma, control plasma and *ChanSu* extract. Comparative analysis of the resulting chemical profiles revealed 20 prototype compounds and 4 metabolites derived from *ChanSu* as potential bioactive components. By MS/MS analysis and accurate molecular weight assessments, the majority of these bioactive components (19 prototype compounds and 2 metabolites) were structurally identified. Moreover, seven were confirmed by comparing their retention behaviors using MS and MS/MS analysis. This study proposes a series of potential bioactive components of *ChanSu*, which we hope will lead to new drug discovery based on *ChanSu* and other traditional Chinese medicines.

1. Introduction

ChanSu, the skin secretions of the giant toad (containing *Bufo bufo gargarizans* Cantor and *Bufo melanostictus* Schneider), is an important traditional Chinese medicine (TCM) that is used in the treatment of a number of ailments such as sore throat, swells, pains, heart failure, skin problems and cancer [1]. *ChanSu* is often used as a raw medicinal material in many complicated formulas of TCMs such as the Liushen Pill [2], the Shexiang Baoxin Pill [3] and the Niuhuang Xiaoyan Tablet [4]. Although it has been applied in numerous clinical situations for over 2000 years, the bioactive compounds in *ChanSu* are not fully known. Until recently, only a few studies have reported its pharmacological effects, and bufadienolides are generally agreed upon to be the major bioactive components of *ChanSu* [5]. Bufalin is one of the most abundant bufadienolides, whose pharmacological activities have recognized anti-tumor effects, abilities to induce apoptosis, and the capabilities to induce inflammatory cytokines [6,7]. In recent years, cinobufagin, bufotalin, arenobufagin and resibufogenin have also been reported to have similar effects [8,9]. Other bioactive components of *ChanSu* are seldom reported, and, thus, we embarked on a comprehensive investigation of the bioactive compounds in *ChanSu*, which is of great significance to enable drug discovery based on *ChanSu*.

To date, the study of the bioactive components of *ChanSu* has mainly been based on *in vitro* assays, but this approach has unavoidable limitation. In general, the bioactive compounds in TCMs go through a complicated process of forming, absorbing and distributing in the body before displaying pharmacological effects after oral administration. Many bioactive compounds in TCMs, however, may not be absorbed into blood and should not be considered "real bioactive compounds" even though strong bioactivity is observed in *in vitro* assays. Thus, presently employed *in vitro* approaches may lead to unrealistic results. According to the theory of plasma pharmacochemistry [10], only compounds absorbed into the blood have the possibility of showing pharmacological bioactivities. Plasma pharmacochemistry techniques are, therefore, *in vivo* methodologies and are efficient tools for bioactive compounds screening in TCMs taken from animal plasma. To the best of our knowledge, plasma pharmacochemistry techniques have not been applied for the discovery of bioactive compounds in *ChanSu*.

In this study, plasma pharmacochemistry techniques were employed to screen for bioactive components in *ChanSu*. Potential bioactive components were proposed by comparatively analyzing the chemical profiles of dosed rat plasma, control rat plasma and *ChanSu* extract. Their chemical structures were characterized by MS spectrometric analysis. This study suggests many new potential bioactive components in *ChanSu* that will be helpful to subsequent drug discovery based on *ChanSu* and other TCMs.

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2. Experimental

2.1. Instruments and chemicals

Agilent-1100 HPLC/MSD Trap XCT and Agilent-1200 Rapid Resolution LC (RRLC)/6520 Q-TOF mass spectrometry instruments were used (Agilent Technologies, Palo Alto, CA, USA).

HPLC grade acetonitrile and formic acid were purchased from Merck (Merck, Darmstadt, Germany). Deionized water was purified by the Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA). *ChanSu* was purchased from Nanjing Medicine Nantong Kambridge Co. Ltd. (Nantong, PR China) and was initially identified by Professor Hanchen Zheng of the Department of Phytochemistry at the Second Military Medical University. A specimen has been deposited at the Department of Phytochemistry of Second Military Medical University. Reference chemicals of seven bufadienolides including bufalin, gamabufotalin, arenobufagin, telocinobufagin, cinobufagin, resibufogenin and cinobufagiol were prepared in our laboratory using a Varian PreStar preparative HPLC system (Varian Inc., Palo Alto, CA.) with over 95.0% purity as determined by LC/UV detection at 299 nm. Their chemical structures were identified by multiple spectral analyses including IR, NMR, MS and UV [11–15].

2.2. Animals and drug administration

Thirteen male Sprague–Dawley (SD) rats (180 ± 20 g) were purchased from Shanghai SLAC Lab. Animal Co., Ltd. (Shanghai, China) and were randomly separated into a control group (5 rats) and a dosed group (8 rats). All animals were acclimatized to the facilities for seven days. For 12 h prior to administration, the rats were fasted but had free access to water.

ChanSu was ground into 60-mesh powder and dissolved in a 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution. The prepared suspension was orally administered to rats in the dosed group at a dose of 0.15 g *ChanSu* per 1000 g of body weight, and the 0.5% CMC-Na aqueous solution was orally administered to rats in the control group. Two hours after administration, the animals were anaesthetized by intraperitoneal injection of 1% pentobarbital sodium (0.15 mL/100 g body weight). Blood was collected from the hepatic portal vein and then centrifuged at $1400 \times g$ for 10 min at 4 °C. The supernatant was stored at −80 °C. The plasma was processed prior to analysis by previously described methods [16]. All procedures were in accordance with the National Institute of Health's guidelines regarding the principles of animal care (2004).

2.3. Sample preparation

All plasma samples from one group of rats were combined into one sample so as to eliminate the individual variability. An amount of 600 μL of methanol was added to 200 μL of plasma samples and vortexed for 60 s by a vortex-mixer. Then, the mixture was centrifuged at $15{,}000 \times g$ for 10 min. The supernatant was dried by a Speed Vacplus vacuum drier, and the dried residue was dissolved in 200 μL of methanol followed by centrifugation at $15{,}000 \times g$ for 10 min. The supernatant was stored at −20 °C until LC/MS analysis.

ChanSu (0.998 g) powder was dissolved in 20 mL of methanol and was ultrasonically extracted for 30 min. The methanol extraction was centrifuged for 10 min at $15{,}000 \times g$. The supernatant (5 μL) was added to 5 mL of methanol, centrifuged for 10 min at $15{,}000 \times g$ and then filtered through a 0.2-μm membrane. A portion of the reference chemicals was dissolved in methanol and stored in the refrigerator at 4 °C until analysis.

2.4. HPLC/MS

2.4.1. HPLC/IT-MS conditions

An Eclipse Plus C_{18} column (3.5 μm, 2.1 mm × 100 mm, Agilent, USA) with a column temperature maintained at 25 °C was employed. Solvent A (water with 0.1% formic acid, v/v) and solvent B (acetonitrile) were used for gradient elution with the following program: 0–6 min, 15–35% solvent B; 6–30 min, 35–57% solvent B; 30–40 min, 57–85% solvent B; 40–45 min, 85–100% solvent B; 45–55 min, 100% solvent B. The flow rate was set at 0.5 mL/min, while a 0.2-mL/min portion of the column effluent was delivered into the ion source of mass spectrometry. The injection volume was 5 μL. Operation conditions of mass spectrometer were as follows: the collision gas was helium of over 99.999% purity, the nebulizer gas was N_2 at 35 psi, the drying gas was N_2 at 10 L/min, the drying temperature was 350 °C, the HV voltage was 3.5 kV, the electrospray ionization mode was utilized and the mass range was m/z 50–1000. Both positive and negative ionization modes were applied, and the data were acquired and analyzed using Agilent Chemstation software (Agilent Technologies, Palo Alto, CA, USA).

2.4.2. RRLC/TOF-MS conditions

An Eclipse Plus C_{18} column (3.5 μm, 2.1 mm × 100 mm, Agilent, USA) with a column temperature maintained at 25 °C was used. Solvent A (water with 0.1% formic acid, v/v) and solvent B (acetonitrile) were used for gradient elution with the following program: 0–4 min, 10–50% solvent B; 4–9 min, 50–85% solvent B; 9–12 min, 85–98% solvent B; 12–14 min, 98% solvent B. The flow rate was set at 0.2 mL/min. The injection volume was 3 μL. The mass detection was operated with parameters set as follows: the electrospray ionization mode was utilized, the drying gas was N_2 at 10 L/min, the pressure of the nebulizer gas N_2 was 10 psig, the gas temperature was 330 °C, the scan range was 50–1000 m/z and the HV voltage was 3500 V in negative ion mode and 3800 V in positive ion mode. Data were acquired and analyzed using MassHunter Workstation Software (Agilent Technologies, Palo Alto, CA, USA).

3. Results and discussion

3.1. HPLC profiling

To acquire LC/MS profiles involving the highest-quality chemical information, the chromatographic conditions including chromatographic column composition, mobile phase, column temperature and flow rate were optimized.

Several C_{18} columns packed with 3.5 μm or 5 μm particles were investigated in this study. The results suggest that Zorbax Eclipse Plus C_{18} column was superior to Zorbax XDB-C_{18} and SB-C_{18} columns, and columns packed with 3.5 μm particles showed better separation abilities than those packed with 5.0 μm particles. Thus, a Zorbax Eclipse Plus C_{18} column packed with 3.5 μm particles was selected for LC/MS analysis. Acetonitrile and methanol are commonly used as the mobile phases in HPLC analysis. Additives such as formic acid, acetic acid and ammonia are often doped into the mobile phase. We compared the four mobile phases of acetonitrile/H_2O, methanol/H_2O, acetonitrile/0.1% formic acid and methanol/0.1% formic acid. We determined that acetonitrile/0.1% formic acid was best because it yielded the best separation ability and a good peak shape.

Using these optimized conditions, the chemical profiles of dosed plasma, control plasma and *ChanSu* extract were acquired. Comparing these three HPLC/IT-MS profiles (both in positive and negative modes), we found that the majority of the chromatographic peaks eluted in the first 35 min; many eluted between 10 min and 25 min. Therefore, only peaks with retention times smaller than 35 min in

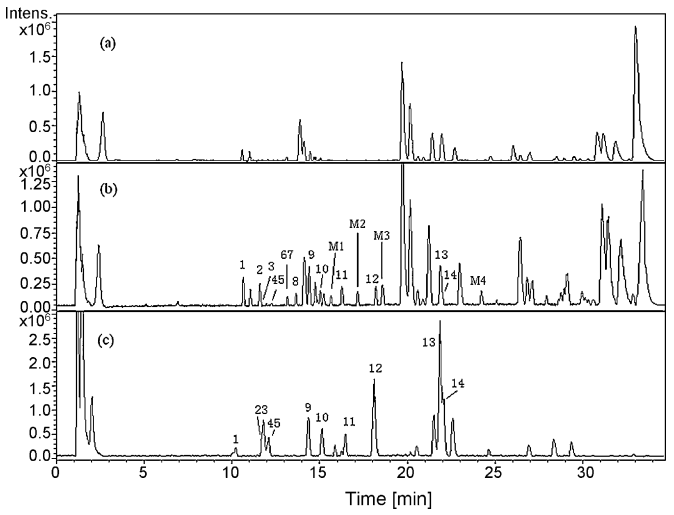

Fig. 1. Total ion chromatogram in the positive mode of control plasma (a), dosed plasma (b) and *ChanSu* extract (c) as determined by HPLC/IT-MS. Peaks 1–14 were prototype components of *ChanSu* as they were observed in dosed plasma and *ChanSu* extract but not in control plasma. Peaks M1–M4 were exogenous metabolites that were only observed in dosed plasma.

the LC/MS profiles needed to be investigated to discover potential bioactive components. Figs. 1 and 2 show HPLC/IT-MS profiles of these three samples in the positive and negative modes, respectively. In this study, conventional ESI was used, which is less sensitive (the LOD of conventional ESI is often at the μg/L level or the μg/kg level) [17–19] when compared with microfluid ESI [20] and nano-ESI [21,22]. Although some bioactive components with low concentrations may be missed by this method, conventional ESI still can reveal new bioactive compounds. With the application of microfluid ESI, nano-ESI and other advanced techniques, HPLC/MS is expected become an even more powerful tool in bioactive compound discovery.

3.2. Potential bioactive compounds discovery

Using HPLC/IT-MS and RRLC/Q-TOF-MS, we acquired chromatographic profiles of dosed plasma, *ChanSu* extract and control plasma (see the profiles in Figs. 1 and 2). As shown in Figs. 1 and 2, the majority of the peaks in the profile of dosed plasma also appear in the control plasma profile. These peaks may be signals that there are large amount of endogenous metabolites in the plasma. On the other hand, there were also many peaks that appeared only in dosed plasma but have no presence in the spectrum of control plasma. These compounds were thus absorbed into the blood and might be potential bioactive compounds from *ChanSu*. Most of these potential bioactive compounds can be observed in the *ChanSu* extract profile, which may be considered as the reference for the bioactive components derived from *ChanSu*. Few of these potential bioactive compounds were not found in the *ChanSu* extract profile. These unique peaks may be derived from exogenous metabolites from *ChanSu* metabolism.

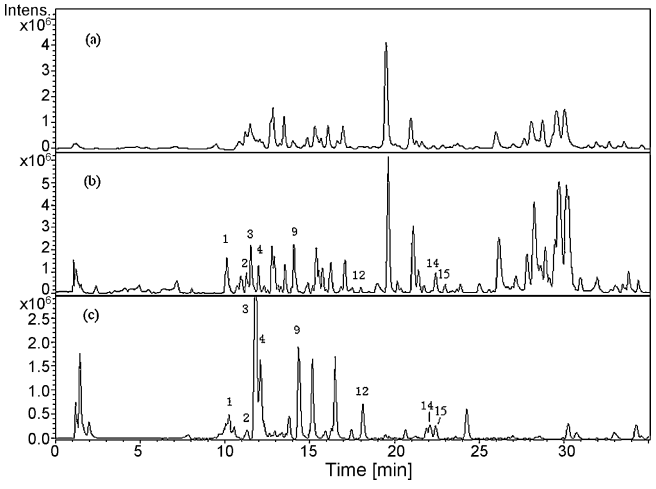

Fig. 2. Total ion chromatogram in the negative mode of control plasma (a), dosed plasma (b) and *ChanSu* extract (c) as determined by HPLC/IT-MS. Peaks 1–4, 9, 12, 14–15 are prototype components of *ChanSu* as they were observed in dosed plasma and *ChanSu* extract but not in the control plasma.

In Figs. 1 and 2, peaks 1–15 are eluted in the profiles of dosed plasma and *ChanSu* extract, whereas they have no equivalent peaks in the profile of the control plasma. These compounds were thus defined as prototype components. There were four peaks (marked as peaks M1–M4) that only appeared in dosed plasma and were assumed to be exogenous metabolites derived from *ChanSu* metabolism. In addition, RRLC/Q-TOF-MS analysis was performed on these samples, and 14 prototype potential bioactive components were discovered. Nine of these compounds were already detected in the HPLC/IT-MS profiles. Therefore, a total of 24 components (including 20 prototype components and 4 metabolites) were found to be in the blood of dosed rats and are likely the potential bioactive components belonging to *ChanSu*.

3.3. Structural illustration of proposed bioactive components

To determine the chemical structures of the proposed bioactive components of *ChanSu* in rat plasma, we first used HPLC/MS/MS. The structures of bioactive components were temporarily identified by MS/MS spectra and literature reported mass data. Then, RRLC/Q-TOF-MS was applied to validate the potential bioactive components by assessing their accurate molecular weights Finally, using LC/MS techniques and the reference chemicals that were available, the structures of the proposed bioactive compounds were partly confirmed by comparing their retention times (RTs), molecular weights (M.W.s) and MS/MS spectra with those of the reference chemicals. Of the 24 identified bioactive compounds, 21 were structurally identified (including 19 prototype compounds and 2 metabolites). Peak 15, M1 and M2 remained unidentified. The mass data from the HPLC/IT-MS and RRLC/Q-TOF-MS are listed in Tables 1 and 2 , respectively. Fig. 3 shows the chemical structures of these potential bioactive components.

3.3.1. Structure identification of prototype components

Whether in IT-MS or Q-TOF-MS spectra, the proposed prototypes are seen to involve many common mass behaviors. Ions of $[M+H]^+$, $[M+Na]^+$, $[2M+Na]^+$ or $[M+K]^+$ in the positive mode and $[M-H]^-$ or $[M+HCOO]^-$ in the negative mode can be easily observed for most components. Moreover, almost all of the base peaks tended to form both [M-18] and [M-28] ions in both positive and negative modes, which may be due to the neutral loss of H_2O and CO. Through analysis of the MS and MS/MS data, 19 prototype components were structurally characterized, as listed in Tables 1 and 2 (peak 15 again failed to be identified). Fig. 3 displays the chemical structures of these components, which are all bufadienolide structural compounds. So, it is also concluded that bufadienolide is the major bioactive components in *ChanSu*, which is in agreement with previously published results [5]. The structures of representative compounds are described below.

3.3.1.1. Peak 12: bufalin. The full-scan HPLC-IT/MS spectra of peak 12 contain strong $[M+H]^+$ ions at m/z 387 in both profiles of dosed plasma and *ChanSu* extract (shown in Table 1), which we temporarily assign as bufalin according to the reported mass data

Table 1
MS and MS/MS data (*m*/*z*) of the absorbed compounds and metabolites in rat plasma after oral administration of *ChanSu*.

No	Rt min	M.W.	MS (pos.) (*m*/*z*)	MS/MS (pos.) (*m*/*z*)	MS (neg.) (*m*/*z*)	MS/MS (neg.) (*m*/*z*)	Identification
1	10.2	402	403 $[M+H]^+$	385 $[M+H-H_2O]^+$, 367 $[M+H-2H_2O]^+$	401 $[M-H]^-$	383 $[M-H-H_2O]^-$, 357 $[M-H-CO_2]^-$, 339 $[M-H-CO_2-H_2O]^-$	Gamabufotalin*
			425 $[M+Na]^+$	349 $[M+H-3H_2O]^+$, 331 $[M+H-4H_2O]^+$	447 $[M+HCOO]^-$		
			827 $[2M+Na]^+$	321 $[M+H-3H_2O-CO]^+$		322 $[M-H-2H_2O-CH_3-CO]^-$	
				253 $[M+H-3H_2O$-α-pyr$]^+$		307 $[M-H-2H_2O-2CH_3-CO]^-$	
2	11.6	402	403 $[M+H]^+$	385 $[M+H-H_2O]^+$, 367 $[M+H-2H_2O]^+$, 349 $[M+H-3H_2O]^+$, 331 $[M+H-4H_2O]^+$	401 $[M-H]^-$	383 $[M-H-H_2O]^-$	Desacetylbufotalin
				321 $[M+H-3H_2O-CO]^+$, 303 $[M+H-4H_2O-CO]^+$, 253 $[M+H-3H_2O$-α-pyr$]^+$		365 $[M-H-2H_2O]^-$	
				215 $[M+H-3H_2O-C_8H_6O_2]^+$		357 $[M-H-CO_2]^-$	
				185 $[M+H-3H_2O-C_{10}H_{12}O_2]^+$		347 $[M-H-3H_2O]^-$	
				159 $[M+H-3H_2O-C_{12}H_{12}O_2]^+$		329 $[M-H-4H_2O]^-$	
3	11.8	416	417 $[M+H]^+$	399 $[M+H-H_2O]^+$, 381 $[M+H-2H_2O]^+$, 363 $[M+H-3H_2O]^+$, 335 $[M+H-3H_2O-CO]^+$, 317 $[M+H-4H_2O-CO]^+$	415 $[M-H]^-$	397 $[M-H-H_2O]^-$	Arenobufagin*
				289 $[M+H-4H_2O-2CO]^+$	461 $[M+HCOO]^-$	371 $[M-H-CO_2]^-$	
						353 $[M-H-CO_2-H_2O]^-$	
4	12.1	402	403 $[M+H]^+$	385 $[M+H-H_2O]^+$, 367 $[M+H-2H_2O]^+$, 349 $[M+H-3H_2O]^+$, 321 $[M+H-3H_2O-CO]^+$, 337 $[M+H-2H_2O-CH_2O]^+$,	401 $[M-H]^-$	383 $[M-H-H_2O]^-$	19-Hydroxyl-bufalin
			425 $[M+Na]^+$	303 $[M+H-4H_2O-CO]^+$	447 $[M+HCOO]^-$	365 $[M-H-2H_2O]^-$	
				253 $[M+H-3H_2O$-α-pyr$]^+$		335 $[M-H-HCHO]^-$	
						305 $[M-H-$α-pyr$]^-$	
5	12.1	416	417 $[M+H]^+$	399 $[M+H-H_2O]^+$, 381 $[M+H-2H_2O]^+$, 363 $[M+H-3H_2O]^+$, 345 $[M+H-4H_2O]^+$	–	–	Hellebrigenin
			439 $[M+Na]^+$	335 $[M+H-3H_2O-CO]^+$			
			855 $[2M+Na]^+$	317 $[M+H-4H_2O-CO]^+$			
6	13.0	400	401 $[M+H]^+$	383 $[M+H-H_2O]^+$, 365 $[M+H-2H_2O]^+$, 347 $[M+H-3H_2O]^+$, 329 $[M+H-4H_2O]^+$	–	–	Marinobufagin
			801 $[2M+H]^+$				
			823 $[2M+Na]^+$				
7	13.3	414	415 $[M+H]^+$	397 $[M+H-H_2O]^+$, 379 $[M+H-2H_2O]^+$, 369 $[M+H-H_2O-CO]^+$, 361 $[M+H-3H_2O]^+$, 333 $[M+H-3H_2O-CO]^+$	–	–	19-*oxo*-Desacetylcinobufagin
			437 $[M+Na]^+$				
8	13.8	400	401 $[M+H]^+$	383 $[M+H-H_2O]^+$, 365 $[M+H-2H_2O]^+$, 347 $[M+H-3H_2O]^+$, 337 $[M+H-2H_2O-CO]^+$, 319 $[M+H-3H_2O-CO]^+$	–	–	19-*oxo*-Bufalin
			823 $[2M+Na]^+$	301 $[M+H-4H_2O-CO]^+$			
9	14.4	402	403 $[M+H]^+$	385 $[M+H-H_2O]^+$, 367 $[M+H-2H_2O]^+$, 349 $[M+H-3H_2O]^+$, 321 $[M+H-3H_2O-CO]^+$, 303 $[M+H-4H_2O-CO]^+$	401 $[M-H]^-$	383 $[M-H-H_2O]^-$	Telocinobufagin*
			827 $[2M+Na]^+$		447 $[M+HCOO]^-$	373 $[M-H-CO]^-$	
						357 $[M-H-CO_2]^-$	
10	15.4	400	401 $[M+H]^+$	383 $[M+H-H_2O]^+$, 365 $[M+H-2H_2O]^+$, 353 $[M+H-H_2O-CH_2O]^+$, 347 $[M+H-3H_2O]^+$, 337 $[M+H-2H_2O-CO]^+$	–	–	Resibufaginol
			423 $[M+Na]^+$				
M1	15.6	372	373 $[M+H]^+$	355, 337, 319, 255, 159	–	–	Unidentified
11	16.6	458	459 $[M+H]^+$	441 $[M+H-H_2O]^+$, 417 $[M+H-CH_2CO]^+$	–	–	Cinobufaginol*
			481 $[M+Na]^+$	413 $[M+H-H_2O-CO]^+$, 381 $[M+H-CH_2CO-2H_2O]^+$, 363 $[M+H-CH_2CO-3H_2O]^+$, 345 $[M+H-CH_2CO-4H_2O]^+$			
M2	17.1	372	373 $[M+H]^+$	355 $[M+H-H_2O]^+$, 337 $[M+H-2H_2O]^+$, 319 $[M+H-3H_2O]^+$, 255, 159	–	–	Unidentified
12	18.2	386	387 $[M+H]^+$	369 $[M+H-H_2O]^+$, 351 $[M+H-2H_2O]^+$, 333 $[M+H-3H_2O]^+$, 323 $[M+H-2H_2O-CO]^+$, 305 $[M+H-3H_2O-CO]^+$,	385 $[M-H]^-$	367 $[M-H-H_2O]^-$	Bufalin*

Table 1 (*Continued*)

No	Rt min	M.W.	MS (pos.) (*m*/*z*)	MS/MS (pos.) (*m*/*z*)	MS (neg.) (*m*/*z*)	MS/MS (neg.) (*m*/*z*)	Identification
			409 $[M+Na]^+$ 795 $[2M+Na]^+$	255 $[M+H-2H_2O-\alpha\text{-pyr}]^+$		357 $[M-H-CO]^-$ 349 $[M-H-2H_2O]^-$ 296 $[M-H-H_2O-C_4H_7O]^-$	
M3	18.6	386	387 $[M+H]^+$	369 $[M+H-H_2O]^+$, 351 $[M+H-2H_2O]^+$, 333 $[M+H-3H_2O]^+$, 323 $[M+H-2H_2O-CO]^+$, 305 $[M+H-3H_2O-CO]^+$ 255 $[M+H-2H_2O-\alpha\text{-pyr}]^+$	–	–	3-*epi*-Bufalin
13	22.0	384	385 $[M+H]^+$ 791 $[2M+Na]^+$	367 $[M+H-H_2O]^+$, 349 $[M+H-2H_2O]^+$, 339 $[M+H-H_2O-CO]^+$, 331 $[M+H-3H_2O]^+$, 321 $[M+H-2H_2O-CO]^+$ 253 $[M+H-2H_2O-\alpha\text{-pyr}]^+$	–	–	Resibufogenin*
14	22.1	442	443 $[M+H]^+$ 907 $[2M+Na]^+$	401 $[M+H-CH_2CO]^+$, 383 $[M+H-HOAc]^+$, 365 $[M+H-HOAc-H_2O]^+$ 347 $[M+H-HOAc-2H_2O]^+$, 337 $[M+H-HOAc-H_2O-CO]^+$, 319 $[347-CO]^+$	441 $[M-H]^-$ 487 $[M+HCOO]^-$	399 $[M-H-CH_2CO]^-$ 383 $[M-H-2CH_3-CO]^-$	Cinobufagin*
15	22.9	478	–	–	477 $[M-H]^-$	435 $[M-H-CH_2CO]^-$ 417 $[M-H-HOAc]^-$ 389 $[M-H-HOAc-CO]^-$	Unidentified
M4	24.2	384	385 $[M+H]^+$ 791 $[2M+Na]^+$	367 $[M+H-H_2O]^+$, 349 $[M+H-2H_2O]^+$, 339 $[M+H-H_2O-CO]^+$, 331 $[M+H-3H_2O]^+$, 321 $[M+H-2H_2O-CO]^+$ 253 $[M+H-2H_2O-\alpha\text{-pyr}]^+$	–	–	3-*epi*-Resibufogenin

* Structurally confirmed by comparison with reference chemicals.

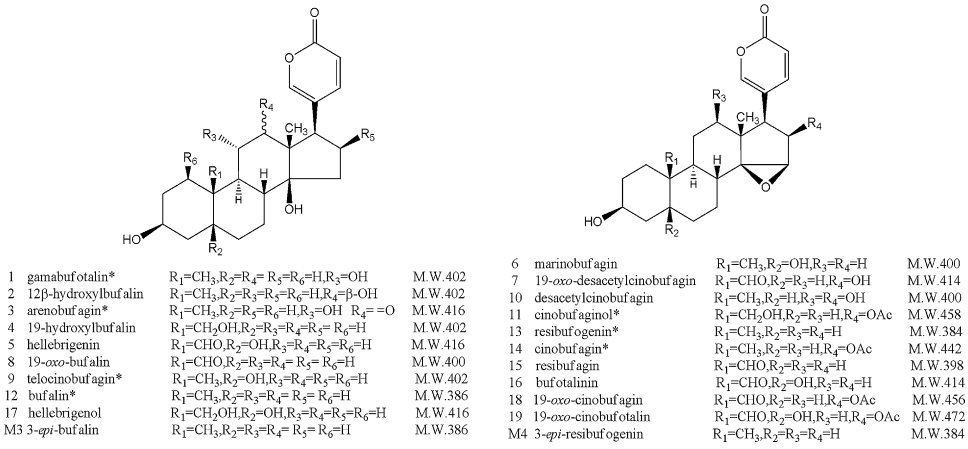

Fig. 3. Structures of identified compounds in rat plasma after oral administration of *ChanSu*. Those that were structurally confirmed by comparison with reference chemicals are marked with asterisks.

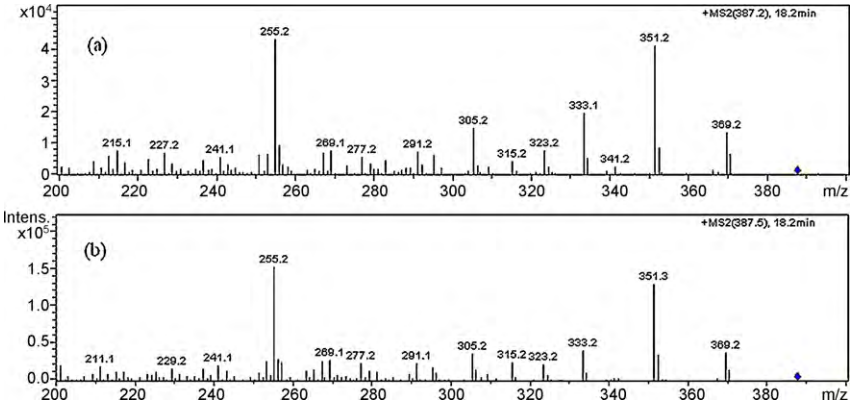

Fig. 4. MS/MS spectra of bufalin in dosed rat plasma (a) and in the reference chemicals (b) as determined by HPLC/IT-MS/MS in the positive mode.

Table 2
MS/MS data of (+) ESI-MS spectra and (−) ESI-MS spectra, and the identification results of the constituents of *ChanSu*.

No	Identification	Rt (min)	Negative ion (*m*/*z*)	Calculated (*m*/*z*)	ppm error	Positive ion (*m*/*z*)	ppm error	Elemental composition
1	19-*oxo*-Cinobufagin	2.834	–			457.2211 $[M+H]^+$ 341.1639 $[M+H-C_5H_8O_3]^+$ 201.0892 $[M+H-C_{12}H_{16}O_6]^+$ 163.1334 $[M+H-C_{15}H_{18}O_6]^+$ 107.0701 $[M+H-C_{18}H_{22}O_7]^+$	−3.28	$C_{26}H_{32}O_7$
2	Gamabufotalin[a]	3.091	447.2409 $[M+HCOO]^-$ 401.2357 $[M-H]^-$ 381.1648 $[M+HCOO-CH_6O_3]^-$ 353.2053 $[M+HCOO-C_3H_{10}O_3]^-$ 339.2011 $[M+HCOO-C_4H_{12}O_3]^-$	447.2383 401.2328	5.81 7.23	441.2082 $[M+K]^+$ 425.2345 $[M+Na]^+$ 403.2486 $[M+H]^+$ 379.2294 $[M+Na-CH_2O_2]^+$ 185.1156 $[M+H-C_{10}H_{18}O_5]^+$	8.84 9.64 0.496	$C_{24}H_{34}O_5$
3	Hellebrigenol	3.458	463.2374 $[M+HCOO]^-$ 417.2298 $[M-H]^-$ 381.1605 $[M-H-2H_2O]^-$	463.2332 417.2277	9.07 5.03	441.2079 $[M+Na]^+$ 419.2429 $[M+H]^+$ 383.2219 $[M+H-2H_2O]^+$ 353.2112 $[M+H-2H_2O-CH_2O]^+$ 223.1482 $[M+H-C_7H_{16}O_6]^+$	−39.4 −0.954	$C_{24}H_{34}O_6$
4	Arenobufagin[a]	3.539	461.2219 $[M+HCOO]^-$ 415.2164 $[M-H]^-$ 381.1749 $[M-H-H_2O-CH_4]^-$ 325.1875 $[M-H-H_2O-CH_4-2CO]^-$	461.2276 415.2121	−12.4 10.4	455.1908 $[M+K]^+$ 417.2271 $[M+H]^+$ 363.1935 $[M+H-3H_2O]^+$ 335.2004 $[M+H-3H_2O-CO]^+$ 327.1579 $[M+H-C_4H_{10}O_2]^+$ 281.1482 $[M+H-C_4H_{10}O_2-H_2O-CO]^+$ 139.0184 $[M+H-C_4H_{10}O_2-H_2O-CO-C_{11}H_{10}]^+$	15.8 −1.44	$C_{24}H_{32}O_6$
5	19-*oxo*-Cinobufotalin	3.965	471.2024 $[M-H]^-$ 417.1055 $[M-H-3H_2O]^-$ 401.1616 $[M-H-CO-CH_2CO]^-$ 325.1862 $[M-H-AcOH-2CO-2CH_3]^-$	471.2019	1.06	–	–	$C_{26}H_{32}O_8$
6	Telocinobufagin[a]	4.104	447.2398 $[M+HCOO]^-$ 401.2366 $[M-H]^-$ 343.0632 $[M-H-CO-2CH_3]^-$ 325.1899 $[M-H-CO-2CH_3-H_2O]^-$	447.2383 401.2328	3.35 9.47	441.2067 $[M+K]^+$ 425.2326 $[M+Na]^+$ 403.2479 $[M+H]^+$ 385.2375 $[M+H-H_2O]^+$ 349.2154 $[M+H-3H_2O]^+$ 321.2215 $[M+H-3H_2O-CO]^+$ 289.2155 $[M+H-H_2O$-α-pyr$]^+$ 253.0143 $[M+H-3H_2O$-α-pyr$]^+$	5.44 5.17 −1.24	$C_{24}H_{34}O_5$
7	19-*oxo*-Bufalin	4.330	445.2271 $[M+HCOO]^-$ 399.2191 $[M-H]^-$ 383.1876 $[M-H-CH_3-H]^-$ 347.1661 $[M-H-CH_3-H-2H_2O]^-$ 319.1712 $[M-H-CH_3-H-2H_2O-CO]^-$	445.2227 399.2172	9.88 4.76	439.1869 $[M+K]^+$ 423.2180 $[M+Na]^+$ 401.2335 $[M+H]^+$ 373.2388 $[M+H-CO]^+$ 345.2468 $[M+H-2CO]^+$ 337.2178 $[M+H-2H2O-CO]^+$ 277.2173 $[M+H-CO$-α-pyr$]^+$ 241.1955 $[M+H-2H_2O-CO$-α-pyr$]^+$ 225.1306 $[M+H-3H_2O$-α-pyr-$CH_3-H]^+$	−4.10 7.56 1.74	$C_{24}H_{32}O_5$
8	Resibufagin	4.529	–	–	–	437.1735 $[M+K]^+$ 399.2153 $[M+H]^+$ 285.6665 $[M+H-H_2O$-α-pyr$]^+$ 243.6370 $[M+H-H_2O$-α-pyr-$CO-CH_3]^+$	1.14 4.51	$C_{24}H_{30}O_5$

Table 2 (*Continued*)

No	Identification	Rt (min)	Negative ion (*m*/*z*)	Calculated (*m*/*z*)	ppm error	Positive ion (*m*/*z*)	ppm error	Elemental composition
9	Resibufaginol	4.812	445.2263 $[M+HCOO]^-$ 399.2212 $[M-H]^-$ 369.2089 $[M-H-CH_2O]^-$ 353.2389 $[M-H-CH_2O-CH_3-H]^-$ 317.0279 $[M-H-CH_2O-CH_3-H-2H_2O]^-$	445.2227 399.2172	8.09 10.0	439.1882 $[M+K]^+$ 423.2191 $[M+Na]^+$ 401.2337 $[M+H]^+$ 383.2235 $[M+H-H_2O]^+$ 353.2133 $[M+H-H_2O-CH_2O]^+$ 337.1815 $[M+H-H_2O-CH_2O-CH_4]^+$	−1.14 10.2 2.24	$C_{24}H_{32}O_5$
10	Bufalin[a]	4.894	431.2460 $[M+HCOO]^-$ 385.2378 $[M-H]^-$ 341.2367 $[M-H-CO_2]^-$	431.2434 385.2379	6.03 −0.26	425.2098 $[M+K]^+$ 409.2373 $[M+Na]^+$ 387.2538 $[M+H]^+$ 373.2786 $[M+Na-2H_2O]^+$ 355.2588 $[M+Na-3H_2O]^+$ 327.2639 $[M+Na-3H_2O-CO]^+$	0.941 4.40 0.775	$C_{24}H_{34}O_4$
11	Cinobufagin[a]	5.388	487.2332 $[M+HCOO]^-$ 429.2030 $[M+HCOO-CH_4-CH_2CO]^-$	487.2339	−1.44	443.2433 $[M+H]^+$ 407.2245 $[M+H-2H_2O]^+$ 385.2404 $[M+H-CH_2CO-CH_3-H]^+$ 273.6350 $[M+H-CH_2CO-CH_3-OH-\alpha\text{-pyr}]^+$	−0.451	$C_{26}H_{34}O_6$
12	19-*oxo*-Desacetylcinobufagin	5.694	–	–	–	453.1689 $[M+K]^+$ 437.1947 $[M+Na]^+$ 415.2133 $[M+H]^+$ 363.1986 $[M+H-2H_2O-O]^+$ 319.2097 $[M+H-2H_2O-O-CO_2]^+$ 275.1815 $[M+H-2H_2O-O-CO_2-CO-CH_4]^+$ 255.1790 $[M+H-2H_2O-CO-\alpha\text{-pyr}]^+$ 178.1228 $[M+H-2H_2O-CO-\alpha\text{-pyr-}C_6H_4]^+$	2.21 1.60 3.13	$C_{24}H_{30}O_6$
13	Resibufogenin[a]	5.741	–	–	–	423.1942 $[M+K]^+$ 407.2201 $[M+Na]^+$ 385.2383 $[M+H]^+$ 334.2902 $[M+H-2CH_4-H_2O]^+$ 253.6302 $[M+H-\alpha\text{-pyr-}H_2O-(O+2H)]^+$	0.945 0.491 1.04	$C_{24}H_{32}O_4$
14	Bufotalinin	6.002	–	–	–	453.1694 $[M+K]^+$ 437.1967 $[M+Na]^+$ 415.2143 $[M+H]^+$ 363.1986 $[M+H-2H_2O-O]^+$ 319.2089 $[M+H-2H_2O-O-CO_2]^+$ 275.1902 $[M+H-2H_2O-O-CO_2-CO-CH_4]^+$ 237.1679 $[M+H-3H_2O-CO-\alpha\text{-pyr}]^+$	3.31 6.18 5.54	$C_{24}H_{30}O_6$

α-pyr refers to α-pyrone ring.

[a] Structure confirmed by comparison with reference standards.

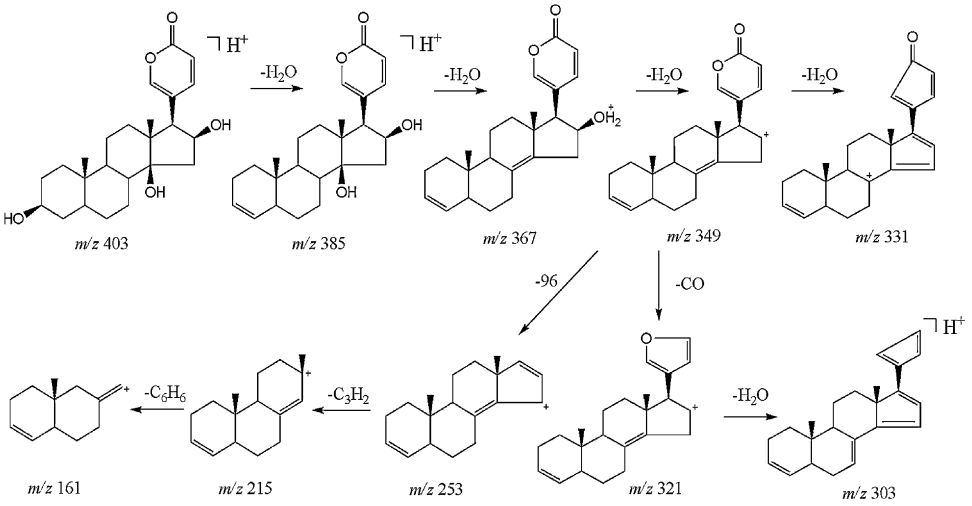

Fig. 5. Proposed MS/MS fragmentation mechanism of protonated desacetylbufotalin.

[23]. In the MS/MS spectra, a series of fragmented ions were observed including *m/z* 369 $[M+H-H_2O]^+$, 351 $[M+H-2H_2O]^+$, 333 $[M+H-3H_2O]^+$, 323 $[M+H-2H_2O-CO]^+$, 305 $[M+H-3H_2O-CO]^+$ and 255 $[M+H-2H_2O$-α-pyr$]^+$, which can all be explained by the possible MS fragmentation mechanisms of bufalin. In addition, there was a very strong ion of *m/z* 255 that originated from a neutral loss of 96 Da from the ion of *m/z* 351. According to the published mass data [23], this fragment may result from the elimination of the α-pyrone ring at C-17. In the Q-TOF MS spectrum, there are several characteristic fragmentation patterns of *m/z* 387.2538 $[M+H]^+$, 409.2373 $[M+Na]^+$, 425.2098 $[M+K]^+$, 385.2379 $[M-H]^+$ and 431.2460 $[M+HCOO]^-$. The M.W. values of these peaks are very close to the calculated value (the error ranges from −0.26 ppm to 6.03 ppm) as shown in Table 2. The MS/MS fragmentation pattern, the literature reported MS data and the accurate M.W. values strongly support our temporary identification of peak 12. To confirm these results, we comparatively analyzed dosed plasma with the reference chemicals, and we found that peak 12 and bufalin have the same retention times and MS/MS fragmentation (as shown in Fig. 4).

According to the data described above, peak 12 was identified as bufalin, which has significant bioactivity and can induce apoptosis in human leukemia cells and differentiation and apoptosis in human monocytic cells [7,8].

3.3.1.2. Peaks 2, 1, 4, and 9: desacetylbufotalin and its isomers, respectively. In profiles of dosed plasma and *ChanSu* extract, peak 2 contains a base peak at *m/z* 403 $[M+H]^+$ in the positive mode, and its MS/MS spectra showed fragmentation ions at *m/z* 385 $[M+H-H_2O]^+$, *m/z* 367 $[M+H-2H_2O]^+$, *m/z* 349 $[M+H-3H_2O]^+$, *m/z* 331 $[M+H-4H_2O]^+$, *m/z* 321 $[M+H-3H_2O-CO]^+$, *m/z* 303 $[M+H-4H_2O-CO]^+$ and *m/z* 253 $[M+H-3H_2O$-α-pyr$]^+$ (as shown in Table 1). According to these fragmentation patterns and all possible cleavage mechanisms, this peak was likely composed of desacetylbufotalin. These fragments successively lose 18 Da and 28 Da, which may be caused by loss of a hydroxyl and a carbonyl group, respectively. A possible fragment pathway of desacetylbufotalin is proposed in Fig. 5.

In the extracted ion chromatogram for the peak at *m/z* 403, three additional peaks (peaks 1, 4 and 9) were observed. These four isomers show the same ion fragments of *m/z* 403 $[M+H]^+$ and *m/z* 827 $[2M+Na]^+$, but their retention behavior is quite different; peaks 1, 2, 4 and 9 have retention times of 10.2 min, 11.6 min, 12.1 min and 14.4 min, respectively (as shown in Fig. 1). In their MS/MS spectra, four compounds within the peaks of 1, 2, 4 and 9 also have common fragmentation patterns including the peaks of *m/z* 385 $[M+H-H_2O]^+$, 367 $[M+H-2H_2O]^+$, 349 $[M+H-3H_2O]^+$ and 321 $[M+H-3H_2O-CO]^+$. Using only this mass data, it is difficult to identify the absolute structures. Fortunately, reference chemicals are partly available for peaks 1, 2 and 9, which were confirmed to be gamabufotalin (peak 1), desacetylbufotalin (peak 2) and telocinobufagin (peak 9) via a comparative analysis with the reference chemicals. However, no reference chemicals had the same retention time and MS fragmentations as the compound of peak 4, and its structure, therefore, has not yet been determined.

We investigated the MS/MS fragmentation of these isomers in Table 1, and we found that peak 4 is the only peak that has an ion at *m/z* 337 in the MS/MS spectrum, which might originate from the loss of formaldehyde (HCHO, 30 Da) from the fragment of *m/z* 367 $[M+H-2H_2O]^+$. This suggests the presence of a 19-hydroxyl group, which is a common substitution for natural bufadienolides according to the literature [23]. Therefore, this compound was temporarily assumed to be 19-hydroxylbufalin.

3.3.1.3. Peak 13 and 14: resibufogenin and cinobufagin. Peaks 13 and 14 were identified as resibufogenin and cinobufagin, respectively, by comparing their retention times with reference chemicals. They have many common mass fragmentation patters such as $[M+H]^+$, $[M+Na]^+$ and $[2M+Na]^+$ in the positive mode and ions of $[M-H]^-$ or $[M+HCOO]^-$ in the negative mode. These two chemicals have been previously reported as important active bufadienolides of *ChanSu* [24], and, obviously, they are capable of being absorbed into blood as prototype bioactive compounds.

3.3.2. Identification of metabolites derived from ChanSu

Beyond the 20 prototype compounds, 4 metabolites were also proposed as potential bioactive components in *ChanSu*. These metabolites were detected only in dosed plasma. As shown in Table 1, we found many compounds that had fragmentation ions of *m/z* 253 and 255, which are characteristic ions of bufadienolides. Considering that peaks M1–M4 also have strong ions of *m/z* 253 and 255, we suggest that these 4 compounds are structurally similar to bufadienolides and may be metabolites derived from *ChanSu*.

Peak M3 was eluted at 18.6 min in the LC/IT-MS profile of dosed plasma. Its MS/MS fragmentation ions are shown in Table 1. They display the same MS and MS/MS fragmentations as bufalin. Min Ye and De-an Guo reported the structure of 3-*epi*-bufalin, an isomer of bufalin, and its mass behavior in 2005 [23]. According to our results and the literature reported data, we temporarily identified peak M3 as 3-*epi*-bufalin. Peak M4 had similar fragmentation ions as

resibufogenin including those with m/z 367 $[M+H-H_2O]^+$, m/z 349 $[M+H-2H_2O]^+$, m/z 339 $[M+H-H_2O-CO]^+$, m/z 331 $[M+H-3H_2O]^+$, m/z 321 $[M+H-2H_2O-CO]^+$ and m/z 253 $[M-H-2H_2O$-α-pyr$]^+$. It was tentatively presumed that this compound was a chiral isomer of resibufogenin, 3-*epi*-resibufogenin, which has not previously been reported to exist in *ChanSu*. The details of this identification are still in progress.

In the MS spectra, peaks M1 and M2 were eluted at 15.6 min and 17.1 min, respectively. Their MS behaviors were very similar. Both had the same base peak of m/z 373 $[M+H]^+$ in the positive mode and common fragmentation ions of m/z 355, 337, 319, 255 and 159 in the MS/MS spectra. Based only on the mass data and presently reported data, the structures of these compounds cannot yet be identified. Further study toward this end is currently underway.

4. Conclusion

A plasma pharmacochemistry-based method was applied to analyze the bioactive components in *ChanSu*. A total of 24 components were proposed as potential bioactive compounds. From these, 19 prototype compounds and 2 metabolites were structurally identified using liquid chromatography tandem mass spectrometry. This approach yielded a series of potential bioactive components, which is both useful for the drug discovery of compounds from *ChanSu* and provides a strategy for characterizing and identifying bioactive compounds in a high-throughput manner.

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