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Chemical profiling and cytotoxicity assay of bufadienolides in toad venom and toad skin

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ABSTRACT

Ethnopharmacological relevance: Toad venom and toad skin have been widely used for treating various cancers in China. Bufadienolides are regarded as the main anticancer components of toad venom, but the difference on composition and anticancer activities of bufadienolides between toad venom and toad skin remains unclear.

Methods: Fractions enriched with free and conjugated bufadienolides were prepared from toad venom and toad skin. Bufadienolides in each fraction were comprehensively profiled by using a versatile UHPLC-TOF-MS method. Relative contents of major bufadienolides were determined by using three bufogenins and one bufotoxin as marker compounds with validated UHPLC-TOF-MS method. Furthermore, cytotoxicity of the fractions was examined by MTT assay.

Results: Two fractions, *i.e.*, bufogenin and bufotoxin fractions (TV-F and TV-C) were isolated from toad venom, and one bufotoxin fraction (TS-C) was isolated from toad skin. Totally 56 bufadienolides in these three fractions were identified, and 29 were quantified or semi-quantified. Bufotoxins were identified in both toad venom and toad skin, whereas bufogenins exist only in toad venom. Bufalin-3-conjugated bufotoxins are major components in toad venom, whereas cinobufotalin and cinobufagin-3-conjugated bufotoxins are main bufotoxins in toad skin. MTT assay revealed potent cytotoxicity of all the fractions in an order of TV-F > TV-C > TS-C.

Conclusions: Our study represents the most comprehensive investigation on the chemical profiles of toad venom and toad skin from both qualitative and quantitative aspects. Eight bufotoxins were identified in toad skin responsible for the cytotoxicity for the first time. Our research provides valuable chemical evidence for the appropriate processing method, quality control and rational exploration of toad skin and toad venom for the development of anticancer medicines.

1. Introduction

Toad venom (Bufonis Venenum, *"Chansu"* in Chinese, *"Senso"* in Japanese), the dried white secretion of the auricular glands of either *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider (Committee, 2010; Fei et al., 2009), is a famous traditional Chinese medicine. Since first recorded on Yao Xing Lun (Tang dynasty), it has been historically used for abscesses and cellulitis, deep-rooted boil and sore, swollen sore throat, loss of consciousness caused by summer heat stroke, vomiting and diarrhea with abdominal pain caused by Sha Zhang for several years (Committee, 2010; Wu and Song, 1998). Substantial studies have demonstrated its analgesia (Zhou et al., 2004), anti-inflammation (Qi et al., 2014), anesthesia (Okada and Suga, 1962), as well as significant cardiotonic (Chen et al., 1951; Liu et al., 2009) and anticancer activities (Chen and Kovaříková, 1967; Lee et al., 2014; Qi et al., 2014). The first record of anticancer activity of toad venom can be traced back to 1617 on Wai Ke Zheng Zong for Ru ai (breast cancer) (Jiang and Dan, 2011; Wu and Song, 1998). Toad skin (*"Chanpi"* in Chinese), the dried skin derived from either *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider (Wu and Song, 1998), is also a traditional Chinese medicine used for the treatment of swelling, pain, heart failure, various malignant sore and pyogenic infections since first recorded on Ben Jing Feng Yuan (Wang et al., 2009; Zuo, 2003). Recent clinical studies have suggested that toad skin had a profound effect on a number of cancers, such as gastrointestinal tract carcinomas (Qi et al., 2008; Zuo, 2003). Both toad venom and toad

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Table 1
Method validation of UHPLC-TOF-MS method for the quantification of bufodienolides.

Bufadienolides	Regression equation	r^2	Linearity (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)	Precision (RSD%, $n=6$)		Recovery ($n=6$)		Repeatability
						Intra-day	Inter-day	Mean (%)	RSD (%)	(RSD%, $n=6$)
bufalin	$y=5138.5x+16{,}405$	0.99	1.3–900	1.3	0.5	2.3	4.3	97.9	4.6	2.3
cinobufagin	$y=5175.5x+15{,}013$	0.99	1.0–900	1.0	0.3	2.7	3.8	94.7	4.3	3.4
resibufogenin	$y=3335.3x+11{,}588$	0.99	3.0–900	3.0	1.0	1.8	3.7	99.4	3.8	3.7
bufalitoxin	$y=3639.4x–15{,}854$	0.99	10.0–900	10.0	3.0	1.5	2.7	95.8	2.4	2.9

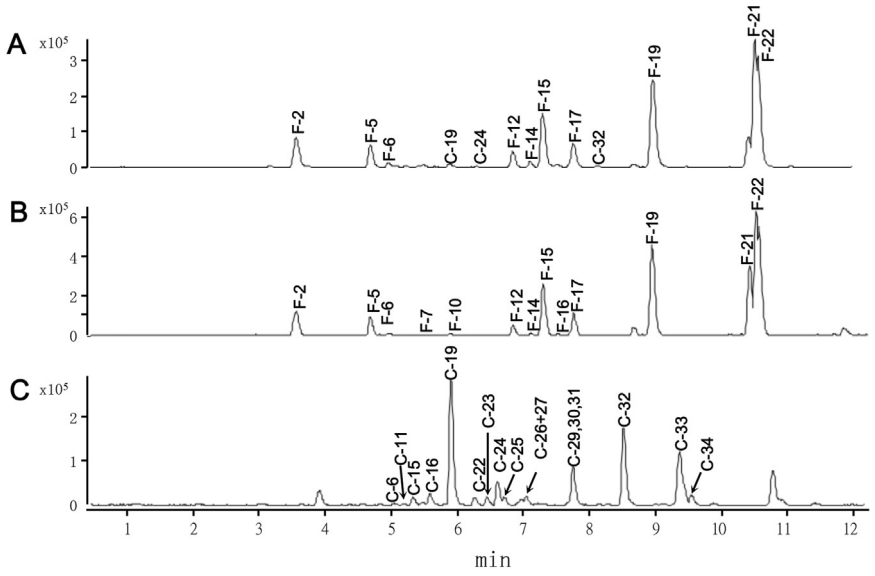

Fig. 1. Total ion chromatograms (TIC) of bufadienolides derived from toad venom. A: total extract (TV-T); B: bufogenins fraction (TV-F); C, bufotoxins fraction (TV-C).

skin are increasingly appreciated for their potent anticancer effects. *Chansu Injection*, a registered Chinese medicine derived from toad venom, has been clinically used as adjuvant therapy for various cancers (Hu et al., 2011; Takaya, 2000; Yang et al., 2005). Similarly, water extract preparations made from toad skin, have been used in clinic for treating cancers, especially middle to late-stage cancers in China (Kim et al., 2010; Qi et al., 2010, 2011). These clinical applications show promising therapeutic efficacy of these toad-derived medicinal materials.

Potent anticancer effects of toad venom are primarily attributable to bufadienolides, a group of cardiotonic steroids with a unique steroidal A/B *cis* and C/D *cis* skeleton and α-pyrone ring at C-17. Bufadienolides are generally classified into conjugated and free types according to the esterification of C-3 hydroxyl group. Conjugated-type bufadienolides, also termed as bufotoxins, are featured by the conjugation to C_3-OH to form various esters, such as sulfates, dicarboxylic, and amino acid-esters (arginine, glutamine, histidine, and methylhistidine with suberyl, succinyl, glutaryl, adipyl or pimelyl esters) (Wang et al., 2011). By contrast, free-type bufadienolides termed as bufogenins possesses a free hydroxyl group at C-3. Previous chemical studies have shown that bufogenins were major bioactive compounds in toad venom (Qi et al., 2010). Numerous studies on bufogenins have demonstrated that they could induce cell differentiation, cell cycle arrest and apoptosis of a variety of cancer cells (Qiu et al., 2013; Takai et al., 2012; Tsai et al., 2012) through multiple pathways (Emam et al., 2012; Li et al., 2012; Su et al., 2009; Wang et al., 2011b; Ye et al., 2005; Yin et al., 2013; Zhang et al., 2012, 2013), thereby exhibiting profound anticancer effects. Although extensive investigation on the anticancer activity of individual bufadienolide was reported, the integrated cytotoxicity of bufogenins and bufotoxins in toad venom were seldom studied. In addition, indole alkaloids were considered to be major anticancer chemical components of toad skin (Meng et al., 2009). Actually, bufadienolides also exist in toad skin, but their anticancer activity remains unclear. Therefore, we want to explore the difference on anticancer activities of bufadienolides both in toad venom and toad skin. Furthermore, the potency of traditional Chinese medicine is greatly related to its chemical components, so quality assessment is crucial for its bioactivity research (Qian et al., 2007). We herein carried out a comprehensive profiling and quantification study of bufadienolides obtained from toad venom and toad skin, respectively. Moreover, drug-induced cytotoxicity is useful in evaluation of anticancer activities of potent compounds and MTT assay has been widely used in traditional Chinese medicine (Dong et al., 2011). Cytotoxicity of bufogenin and bufotoxin fractions obtained from toad venom and toad skin towards several cancer cell lines were examined by using MTT assay.

2. Materials and methods

2.1. Reagents

Toad venom and toad skin derived from *Bufo bufo gargarizans* Cantor were purchased from Bolin Medicine Company (Shijiazhuang City, Hebei Province, China). The standards bufalin, resibufogenin, cinobufagin and bufalitoxin were prepared in our laboratory and identified based on UV, MS and NMR spectral data (purity > 97%). HPLC-grade acetonitrile and formic acid were purchased from Anaqua Chemicals Supply Inc., Ltd (Houston, Texas, U.S.A.). Ultra-pure water was prepared by using a Milli-Q plus system (Millipore, Billerica, MA, U.S.A.). All other solvents used for extraction were of analytical grade. The human colon