Journal of Ethnopharmacology 210 (2018) 39–46

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Investigation of association of chemical profiles with the tracheobronchial relaxant activity of Chinese medicinal herb Beimu derived from various *Fritillaria* species

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ARTICLE INFO

Keywords:
Beimu
Fritillaria
Isosteroidal alkaloid
Gas chromatography
Trachea
Bronchi

ABSTRACT

Ethnopharmacological relevance: Fritillariae Bulbus (Beimu in Chinese) is derived from the bulbus of many *Fritillaria* species (family *Liliaceae*), which has been used as an antitussive herb in traditional Chinese medicine for more than 2000 years. Due to the complexity of plant origins and significant variations in chemical profiles, the characterization of the profile of the major bioactive constituents and its association with pharmacological activity are important for the quality control of Beimu herbs from different origins.

Aim of the study: This study aims to investigate the distribution of major bioactive isosteroidal alkaloids in Beimu herbs of different origins and its correlation with the tracheobronchial relaxant activity.

Methods: Quantification of 7 main bioactive 5α-cevanine isosteroidal alkaloids, including ebeiedine, ebeiedinone, hupehenine, isoverticine, verticine, verticinone and imperialine, in 23 *Fritillaria* species was performed using gas chromatography. The relaxant effect of different extracts of 4 commonly used Beimu herbs, namely Zhe-Beimu (*F. thunbergii* Miq.), Chuan-Beimu (*F. cirrhosa* D. Don), Hubei-Beimu (*F. hupehensis* Hsiao et K. C. Hsia) and Yi-Beimu (*F. pallidiflora* Schrenk), was evaluated using rat isolated tracheal and bronchial preparations pre-contracted with carbachol, the well established in vitro antitussive model.

Results: Amongst 23 *Fritillaria* species detected, significant variations of the types and quantities of 7 major isosteroidal alkaloids were determined, which served as an important indicator for the classification of different Beimu herbs with distinct geographic distributions. Based on the type and quantity of these alkaloids, different origins of Beimu could be clearly clustered into several subgroups by principal component analysis. Furthermore, both crude alkaloid and water extracts of all 4 Beimu herbs showed a dose-dependent tracheobronchial relaxation with different potencies. The total content of alkaloids (weight adjusted based on the activity of individual alkaloids) in Beimu extracts significantly correlated with their tracheobronchial relaxation effects ($r^2 > 0.9$, $p < 0.001$).

Conclusions: The results demonstrated that the differences in chemical profile of major bioactive isosteroidal alkaloids and pharmacological activity of Beimu could be incorporated into a simple and unified method for quality control and potential prediction of activity of Beimu herbs from different origins.

1. Introduction

Beimu (Fritillariae Bulbus), which is derived from various *Fritillaria* plants, has been used as an antitussive herb in traditional Chinese medicine (TCM) for more than 2000 years (Chan et al., 2000; Ding et al., 1996). The extensive use of this cough remedy is due to

Abbreviations: FID, flame ionization detection; GC, gas chromatography; LC-MS, liquid chromatography mass spectrometry; LOQ, limit of quantification; PCA, principal component analysis; TCM, traditional Chinese medicine

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http://dx.doi.org/10.1016/j.jep.2017.08.027
Received 21 February 2017; Received in revised form 21 August 2017; Accepted 21 August 2017

their non-addictiveness and less side effects when compared with commercially available morphine-like anti-cough medicines containing codeine. *Fritillaria* species are widely distributed in China, and more than 40 species have been used as the plan sources for various Beimu herbs. Officially, Beimu herbs have been documented as the bulbs of 10 *Fritillaria* species in Pharmacopoeia of the People's Republic of China (2015 edition) (Commission, 2015). Amongst them, Chuan-Beimu (*F. cirrhosa* D. Don), Zhe-Beimu (*F. thunbergii* Miq.), Hubei-Beimu (*F. hupehensis* Hsiao et K. C. Hsia) and Yi-Beimu (*F. pallidiflora* Schrenk) are the four most commonly used Beimu herbs in clinic. In addition, many other Beimu herbs derived from over 30 species, such as Xin-Bei (*F. yuminensis* X. Z. Duan, *F. albidoflora* X. Z. Duan et X. J. Zheng, *F. tortifolia* X. Z. Duan et X. J. Zheng, *F. meleagroides* Patrin), Wan-Bei (*F. anhuiensis* S. C. Chen et S. P. Yin), Sui-Bei (*F. ebeiensis* G. D. Yu et G. Q. Ji, *F. ebeiensis* var. *purpurea* G. D. Yu et P. Li) and Dong-Beimu (*F. thunbergii* var. *chekiangensis* Hsiao et K. C. Hsia), are often used locally in different regions of China or as substituents of other officially documented ones (Shang and Liu, 1995). Although these Beimu herbs have similar clinical indications, due to the large number of varieties, complexity of botanical origins, and differences in clinical use in different regions, it is a challenging task for appropriate quality control of Beimu herbs derived from different *Fritillaria* species.

Extensive studies on the chemical ingredients in different *Fritillaria* species have been conducted by ours and several other research groups since 1950s (Hu et al., 1992; Jiang et al., 2005; Jin et al., 1985; Li et al., 1994; Zhang et al., 1993). Steroidal and isosteroidal alkaloids have been identified as the main ingredients in all *Fritillaria* species used for Beimu herbs (Li et al., 2006). Several studies have used liquid chromatography mass spectrometry (LC-MS) methods for characterization and quantitation of these alkaloids for quality control of different Beimu herbs (Li et al., 2009; Zhou et al., 2008). However, the chemical markers selected are generally not based on the pharmacological activities of Beimu herbs. Moreover, the currently adopted methods in Pharmacopoeia of China (2015 edition) for the quality control of different Beimu herbs are not unified. Some of them are using the ultraviolet-visible spectrophotometry for the determination of total alkaloids in Beimu, which are not specific and differentiable. Therefore, a simple but unified method using bioactivity-based markers is more practical and appropriate for the quality control of different Beimu herbs.

Reported in several studies conducted by ours and other research groups, 5*α*-cevanine isosteroidal alkaloids, such as ebeiedine, verticine, verticinone and imperialine, were demonstrated to be significantly more potent than that of steroidal alkaloids and the main bioactive constituents responsible for various antitussive related activities of different Beimu herbs (Chan et al., 2011; Wang et al., 2012, 2011). Thus 5*α*-cevanine isosteroidal alkaloids are generally considered to be ideal quality control markers. We have then developed several analytical methods based on LC-evaporative light scattering detection, pre-column derivatization gas chromatography (GC) and direct GC to simultaneously quantify these isosteroidal alkaloids in different Beimu samples (Li et al., 1999, 2000, 2001). However, till now, not a single attempt has been made to comprehensively characterize the profile of bioactive isosteroidal alkaloids in clinically used *Fritillaria* species and to investigate the association of such profile with their pharmacological activities.

Therefore, in the present work, we used our previously developed GC method to determine eight bioactive 5*α*-cevanine isosteroidal alkaloids in Beimu herbs derived from 23 *Fritillaria* species. The characteristics of the distribution of these alkaloids in different origins of Beimu herbs were revealed. By using principal component analysis, different Beimu herbs could be clearly clustered which is in accordance with their geographic distributions. On the other hand, the antitussive activity of Beimu has been well-established to be due to the inhibitory effect of various isosteroidal alkaloids on the muscarinic receptor activation-mediated tracheobronchial contraction (Chan et al., 2011; Lin et al., 2006; Zhou et al., 2006). Among various main bioactive isosteroidal alkaloids present in different Beimu herbs, imperialine was found about 10-fold potent of other isosteroidal alkaloids (Chan et al., 2011). Therefore, in order to find any correlation between the contents of bioactive isosteroidal alkaloids and the pharmacological activity in different Beimu herbs, the tracheobronchial relaxant effect, a key antitussive related activity which can be evaluated in vitro, of extracts (crude water and alkaloid extracts) of four commonly used Beimu herbs, including Zhe-Beimu (*F. thunbergii*), Chuan-Beimu (*F. cirrhosa*), Hubei-Beimu (*F. hupehensis*) and Yi-Beimu (*F. pallidiflora*), was evaluated and compared on rat isolated tracheal and bronchial preparations pre-contracted with carbachol, a muscarinic receptor agonist. The results obtained from the present study demonstrated that a simple but unified method for the quantitative measurement of the main bioactive isosteroidal alkaloids could be used for the quality control and the prediction of the antitussive efficacy of different Beimu herbs.

2. Materials and methods

2.1. Chemicals and reagents

All 7 isosteroidal alkaloids (ebeiedine, ebeiedinone, hupehenine, isoverticine, verticine, verticinone and imperialine) tested in the study were isolated and purified according to our previously developed methods (Lee et al., 1988; Li et al., 1990a, 1990b; Ping et al., 1995, 1992). The identities of individual alkaloids (purity higher than 99%) were determined by IR, ^{1}H, ^{13}C NMR, and MS analysis. According to the configuration of ring D and E in their structures (Fig. 1), they are classified into two types, namely type A (those with *trans*-D/E; e.g., ebeiedine, ebeiedinone, hupehenine, isoverticine, verticine, verticinone) and type B (those with *cis*-D/E; e.g., imperialine). Solanidine, carbamylcholine chloride (carbachol) and indomethacin were purchased from Sigma (St. Louis, MO, USA). Codeine phosphate was obtained from Research Biochemicals Inc. (Natick, MA, USA). HPLC grade methanol, dichloromethane and acetonitrile were purchased from Labscan Asia (Bangkok, Thailand). All other chemicals and reagents were of analytical grade.

2.2. Plant material

The bulbs of 23 *Fritillaria* species were collected from wild or cultivated sources in China (Table 1). As shown in Table 1, the investigated *Fritillaria* species are sources of various Beimu herbs with different trade names. All herbs were authenticated by one of the co-authors Prof. Ping Li in China Pharmaceutical University, and all the voucher specimens (Table 1) were deposited in State Key Laboratory of Natural Medicines, China Pharmaceutical University.

2.3. Determination of major isosteroidal alkaloids in Fritillaria species

2.3.1. Sample preparation

To extract the sample, individual grounded Beimu herb (0.1–0.2 g) was mixed with 5.0 mL diethyl ether (pre-alkalized with ammonium hydroxide) and 50 μL of 1 mg/mL solanidine solution (internal standard). The mixtures were shaken by vortex for 2 h and then centrifuged at 1780*g* for 10 min. The supernatants (2.5 mL) were transferred into vials and evaporated under reduced pressure to dryness. The obtained residues were resolved with 100 μL dichloromethane, and the resultant extracts were directly subjected to the GC analysis.

2.3.2. Gas chromatography

The quantification of 7 isosteroidal alkaloids: ebeiedine, ebeiedinone, hupehenine, isoverticine, verticine, verticinone and imperialine in different *Fritillaria* species was carried out based on our previously