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The Pharmaceutical Research of *Bulbus Fritillariae*

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Review Article

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ABSTRACT

The *Bulbus Fritillariae* (BF) is a Traditional Chinese Medicine treating cough and asthma. This article summarized the present research on BFs and mainly focused on the alkaloids, which are the most effective components in BFs. The article contained the geographical distribution, identification of BFs, and the extraction, isolation of the alkaloids, as well as the pharmacological efficacy and bioavailability of BFs.

INTRODUCTION

The *Bulbus Fritillariae* (BF), known by the Chinese name "Bei-Mu", belongs to the family Liliaceae. There are five *Fritillaria* species documented as "Bei-Mu", which are *Bulbus Fritillariae Cirrhosae*, *Bulbus Ussuriensis Fritillariae*, *Bulbus Pallidiflorae Fritillariae*, *Bulbus Thunbergil Fritillariae* as well as *Bulbus Hupehensis Fritillariae*. Among these species, *Bulbus Fritillariae Cirrhosae* and *Bulbus Fritillariae Thunbergia*, which are called "Chuan-Bei-Mu" and "Zhe-Bei-Mu" in Chinese, are acknowledged as the two most effective species [1]. The former contains six species, including *Fritillaria cirrhosa* D.Don, *Fritillaria unibracteata* Hsiao et K.C.Hsia, *Fritillaria przewalskii Maxim, Fritillaria delavayi Franch, Fritillaria taipaiensis* P.Y.Li and *Fritillaria unibracteata Hsiao* et K.C.Hisa var.wabuenis [2,3].

Due to the BF's positive therapeutic effects, low toxicity and few side effects, the herb has been widely used as a traditional Chinese medicine (TCM) to treat cough and asthma for more than 2000 years [4]. And many species of *Fritillaria* have been traditionally used as herbal remedies in Japanese and Turkish folk medicines [5].

THE BOTANICAL CHARACTERISTIC AND DISTRIBUTION OF BFS

The bulbus is the medicinal part of BF; the following table shows the botanical characteristic of the bulbus of different species of *Fritillaria* [6] **(Table 1).**

Table 1. The botanical characteristic of different species of Fritillaria.

Species of Fritillaria	Shape	Size	Color
Fritillaria cirrhosa D.Don	Flat spherical or conical	0.4-1.4 cm in height, 0.4-1.6 cm in diameter	White or light yellow brown
Fritillaria unibracteata Hsiao et K.C. Hsia	Conical or subcordate, as small as pearl or bean	0.3-0.8 cm in height, 0.3-0.9 cm in diameter	white

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Fritillaria delavayi Franch	Conico acuminate	0.7-2.5 cm in height, 0.5-2.5 cm in diameter	Yellowish, with brown patches
Bulbus Ussuriensis Fritillariae	oblate	0.5-1 cm in height, 0.8-2 cm in diameter	Milky white, yellowish
Bulbus Pallidiflorae Fritillariae	Oval or conical	1-1.5 cm in height, 1-2 cm in diameter	Yellowish, a bit rough
Bulbus thunbergil Fritillariae	Crescent or oblate	1-2 cm in height, 1-3.5 cm in diameter	White or yellowish
Bulbus Hupehensis Fritillariae	Flat spherica	0.8-2.2 cm in height, 0.8-3.4 cm in diameter	White or light brown

The following table is about the geographical distribution of 5 *Fritillaria* species in China [6] **(Table 2 and Figure 1).**

Table 2. Geographical distribution of Fritillaria.

Fritillaria	**Producing area**	**Altitude/m**
Bulbus Fritillariae Cirrhosae	Yunnan, Szechwan, Qinghai, Tibet, Shanxi, Shaanxi, Gansu	2800-4700
Bulbus Ussuriensis Fritillariae	Heilongjiang, Jilin, Liaoning, Shanxi, Hebei, Shaanxi	500-1500
Bulbus Pallidiflorae Fritillariae	Yining Sinkiang, Suining	1300-2000
Bulbus Thunbergil Fritillariae	Zhejiang, Jiangsu, Anhui, Shanghai, Hunan	100-300
Bulbus Hupehensis Fritillariae	Hubei, Chongqing	1000-1700

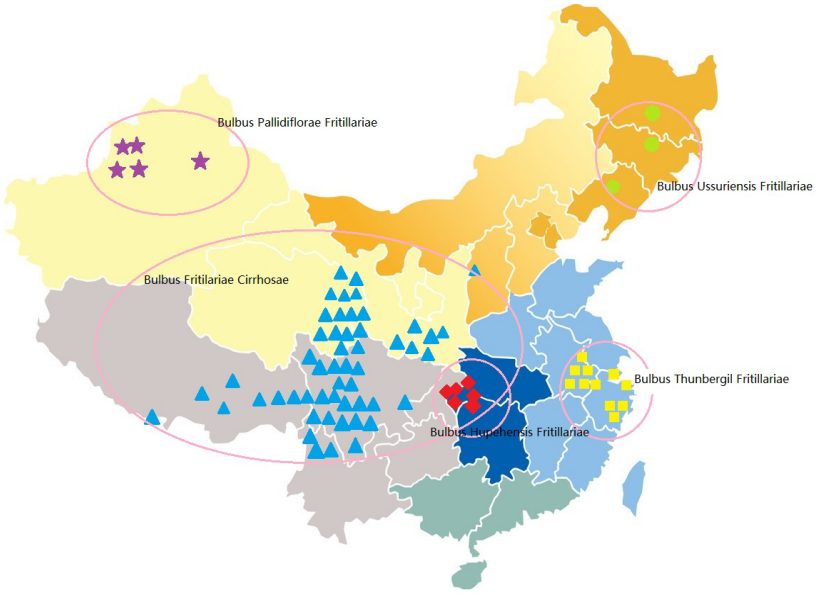

Figure 1. Geographical distribution of *Fritillaria.*

THE BIOACTIVE CONSTITUENTS IN BFS AND THE IDENTIFICATION OF THE ALKALOIDS IN BFS.

The chemical constituents in BFs have been extensively investigated, which include alkaloids, steroidal saponins, diterpenes, polysaccharides and so forth. Steroidal saponins isolated from *Fritillaria* pallidiflora Schrenk (including PallidiflosideD, E, G, H, I) show cytotoxicity against C_6 and Hela cervix cancer cell lines [7]. Kaurane diterpenes isolated from the bulbs of *Fritillaria* ebeiensis, show neuroprotective effects against MPP+-induced neuronal cell death in human dopaminergic neuroblastoma SH-SY5Y cells [8]. Polysaccharides show antioxidant activity by scavenging capacity of DPPH and other radicals [9-11]. However, the isosteroid alkaloids, which includes verticine (peimine), verticinone(peiminine), peimisine, ebeidine, ebeiedinone, hupehenine, imperialine, puqietinone, isoverticine etc., have been demonstrated as the most bioactive constituents [12-19], and are researched most by scholars **(Figure 2).**

The identification of alkaloids is the basis of identification as well as the classification of the BFs. The identification method includes thin-layer chromatography technique, chromatographic techniques (GC and HPLC), mass spectrometry technique and polymerase chain reaction technique.

Thin-Layer Chromatography

TLC is the first analytical method which was developed for qualitative and quantitative determination of the is steroid alkaloids from the BFs. Compared with other detection methods, TLC determination is simple and effective, it was widely used from the 1980s to early 1990s. The TLC plate with spots of BFs extracts, was developed in solvent system of Ethyl acetate/methanol/strong ammonia/water (18:2:1:0.1, by volume), and visualized by spraying with dilute bismuth potassium iodide and 0.5% sodium hydroxide in 60% alcohol in turn. The result was that the alkaloids showed orange spots, the R_f value of peimisine, peiminine and

peimine were 0.528, 0.849 and 0.668, respectively [20,21]. This mobile phase system is one of the four commonly-used mobile phase systems, the other three are trichloromethane-methanol- ammonia [22,23], benzene or cyclohexane-ethyl acetate-diethyl amine [24-26] and diethyl ether-ethanol saturated with ammonia vapor [4] respectively, the last system is not as popular as the former three. However, due to the structural similarity of the major *Fritillaria* isosteroid alkaloids, most of the reported TLC scanning methods are not able to distinguish all of them. In spite of this disadvantage, TLC is still widely used to identify the species of *Fritillaria* considering its easy-operation and low-cost.

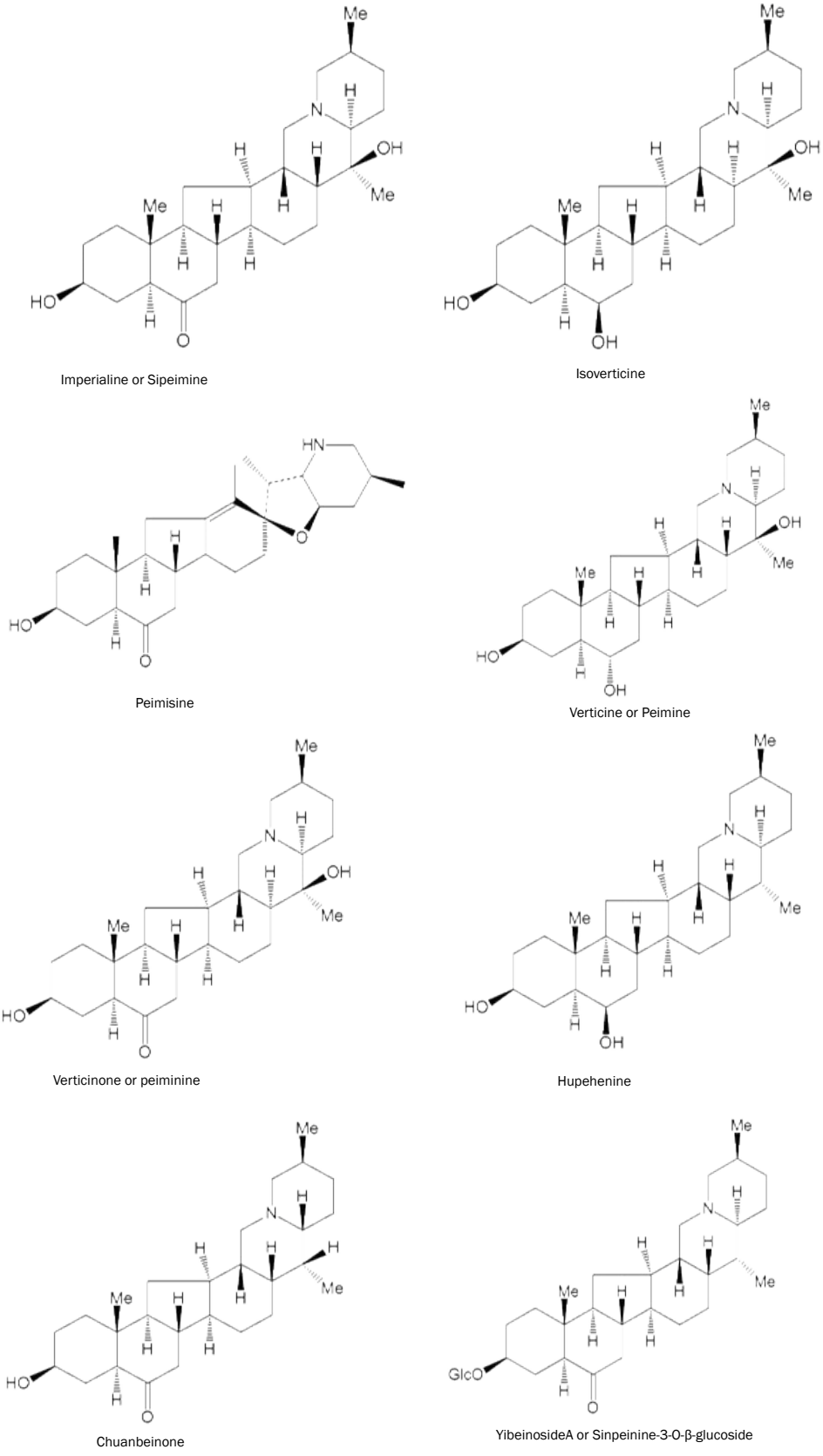

Figure 2. Structures of the main Fritillaria isosteroid alkaloids.

Chromatographic Techniques

Conventional GC (Gas Chromatograph) method cannot be directly utilized to analyze *Fritillaria* alkaloids owing to their high polarity and low volatility, and a pre-column derivatization process is required [12]. With trimethylsilylimidazole, these alkaloids were successfully separated on a certain GC condition [19].

As we can see, *Fritillaria* isosteroid alkaloids lack conjugated unsaturation and they do not display strong ultraviolet (UV) absorption, this structural property limits the selectivity and sensitivity of high-performance liquid chromatographic (HPLC) techniques coupled with UV detection. Pre-column derivatization with an UV-absorbing chromophore of these alkaloids can solve this problem. Li et al. [15] benzoylated the alkaloids with anhydrous pyridine and benzoyl chloride, then the benzoylized alkaloids could be detected at 235 nm.

Recently, reports about the HPLC coupled with evaporative light scattering detection (ELSD) have markedly increased. In comparison with UV detection, ELSD is a nonspecific method, in this detection; the signal intensity is related to the solute concentration in the effluent but not its optical characteristics [27]. ELSD is able to detect analytes without chromophore like *Fritillaria* alkaloids and it detects the solute molecules by light scattering after nebulization and evaporation of the mobile phase [28], so the flow-rate of nebulizing gas and the temperature of drift tube in ELSD chamber are the most important parameters, which should be appropriate to allow solvents in the mobile phase to be completely vaporized while the droplets reach the light scattering cell. The most two commonly-used mobile phase for *Fritillaria* alkaloids are acetonitrile-water-diethylamine (or triethylamine) [29,30] as well as acetonitrile and 10 mmol.L^{-1} NH_4HCO_3 (adjusted to PH10 by ammonia solution) [31,32]. The condition our laboratory built of HPLC-ELSD for determining *Fritillaria* alkaloids was, Ultimate Prime C18 column(250 mm×4. 6 mm, 5 μm), coupled with a Alltima C18 guard column(7. 5 mm×4. 6 mm, 5 μm), the column temperature was 25°C, the mobile phase was acetonitrile-water (containing 0.08% diethylamine) gradient elution, the flow-rate was 1 mL.min^{-1}, temperature of drift tube was 40°C, the pressure of carrier gas was 1.7 bar, the injection volume was 20 μL, the procedure was as below.

As the result **(Table 3)**, we found a common character in all species of *Bulbus Fritillariae* Cirrhosae (BFC), that was there were three to five specific peaks in the period of 20-30 min, and the total peak area of these peaks was over 60% of the total area before the peak of imperialine (the retention time of imperialine is 46 min under this chromatographic condition), and this character was one of the important identification features of BFC. And we also found that imperialine was one of the chief ingredients of BFC which can be used to identify it [33].

Table 3. Mobile phase A and B.

Time/min	Mobile phase A (water containing 0.08% diethylamine)/%	Mobile phase B(acetonitrile)/%
0-10	70	30
10-35	70-40	30-60
35-45	40	60
45-65	40-10	60-90
65-70	10	90

Mass Spectrometry

However, some alkaloids are too small amount to be detected by HPLC-ELSD, such as the peimine and peiminine in BFC [32,34]. To do more research, mass spectrometry (MS), a highly selective, sensitive and versatile analytical technique is in demand [35]. Zhang et al.[34] developed a HPLC-ESI/MS method for analysis of isosteroid alkaloids in *Bulbus Fritillariae* , the extracts were analyzed directly by ESI/MS, ESI was applied and operated in positive ion mode, the scanning range was m/z 210-800, the monitor ions were m/z 432(verticine) and m/z 430(verticinone), the HPLC/MS analysis was performed on a Sphrigel C18 column(200 mm × 4.6 mm, 5 μm), the mobile phase consisted of acetonitrile and 10 mmol/L ammonium formate(PH=8.0, 0.03% triethylamine by volume), eluted in gradient mode (0-6 min: acetonitrile 30%→45%; 6-22 min: acetonitrile 45%; 22-30 min: acetonitrile 45%→95%; 30-34 min: acetonitrile 30%), flow rate was 1 mL/min, the temperature of column was 25°C. The MS spectra of the extracts of *F. Cirrhosae D. Don* and *F. thunbergii* Miq showed that the major molecule ions were similar, but the relative abundance was different. Methodology validation showd that this method was sensitive, convenient, rapid, highly reproducible and suitable for the determination and analysis of isosteroid alkaloids in Bulbus of *Fritillariae*. Zhou et al. used liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (LC/ESI-QTOF-MS/MS) to study the fragmentation behaviors of alkaloids from *Fritillaria* species, and found that ring cleavage(hydrogen rearrangement and induction effect) of the basic skeletons occurred in the MS/MS process and produced characteristic fragment ions, which were useful for structural elucidation, and this method was finally used to investigate the primary steroidal alkaloids in the extracts of eight major *Fritillaria* species. As a result, 41 steroidal alkaloids were selectively identified in these *Fritillaria* species [13,36]

LC/MS and LC/MS/MS methods are still good candidates for the pharmacokinetic study of alkaloids from BFs in vivo [37]. Wu et al. [38,39] developed and validated a sensitive and specific LC-MS method for the quantification of verticinone in biological samples of rats. The precision and accuracy of the method were acceptable for bio analytical assays, and the LLOQ was 0.1 ng/mL, which was sensitive enough to monitor the lowest amount of verticinone in rat plasma, the method has been successfully applied to the pharmacokinetic study of verticinone in rats.

Luo et al. [40] developed a liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for simultaneous

quantitation of four isosteroid alkaloids (peimine, peiminine, peimisine, and delavine) in rat plasma. Carbamazepine was selected as internal standard (IS). The LC-MS-MS system coupled with electrospray ionization (ESI) source was performed in the multiple reaction monitoring (MRM) modes. Blood sample was extracted with ethyl acetate after carbamazepine (IS) spiked. The separation was performed on a Welch C18 column (3.5 um, 2.1×100 mm), and a gradient elution of methanol and 5 mmol·L^{-1} ammonium acetate in 0.1 % formic acid aqueous solution was used. The retention time was less than 8.0 min. Linearity was obtained over the concentration range of 0.2-200 ng·mL^{-1} for peimine and peiminine, and 1.0-200 ng·mL^{-1} for peimisine and delavine. The method was linear for all analytes with correlation coefficients>0.995. The intra-day and inter-day accuracy and precision of the assay were acceptable. This method has been successfully applied to the pharmacokinetic study of *Bulbus Fritillariae Cirrhosae* extract after oral administration to rats.

Other Techniques

Chromatographic techniques are used to detect the alkaloids of BFs so that to differentiate the species of BFs. In addition to them, there are other techniques to identify BFs. Identification methods previously reported are based on chemical characteristics which are not as accurate as molecular identification. PCR techniques have been proved to be a good method to differentiate the species of BFs. Xu et al. [41] developed a simple and feasible polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) analysis of the nuclear ribosomal internal transcribed spacer 1(ITS1) region. The experimental procedure was described as follows: 20 mg dried bulbs of *Fritillaria* were pretreated with 75% ethanol and sterilized ultrapure water, and genomic DNA was extracted with the novel and universal plant genomic DNA extraction kit, then PCR amplification of ITS1 regions and restriction enzyme digestion reaction was carried out successively. The resultant electrophoresis spectrum showed that ITS1 regions of *Bulbus Fritillariae Cirrhosae* were recognized by restriction enzyme Sma Ⅰ with providing 2 distinct fragments between 100 bp and 200 bp, while other species could not be digested. Finally, 12 batches of commercial *Bulbus Fritillariae* were correctly differentiated through the above-mentioned method. This method is widely used now [42,43].

THE EXTRACTION METHODS OF ALKALOIDS FROM BFS

Conventional Methods

Water or acid water-organic solvent extraction

Alkalescence alkaloids and neutral alkaloids exist in the form of unstable salt or free base. Alkaloid salt is soluble in water and poorly soluble in organic solvents, while free base acts in the opposite way. Therefore, alkaloids are acidized to salt form with acid water, such as 0.5-1% acetic acid, muriatic acid and sulfuric acid, and then extract with acid water. After extracting and concentrating to a certain volume, base are used to alkalify the salts to free the alkaloids, then alkaloids are extracted with organic solvent like benzene and trichloromethane. Yin et al. [44] carried out an orthogonal experiment on extracting total alkaloids by acid percolate methods. The result showed that the highest extraction efficiency occurred when: the 1% HCl as solvent, the immersion time was 24 h and the percolate speed was 10 mL·min^{-1}·kg^{-1}.

Alcohols-acid water-organic solvent extraction

Alcohols can also be used to extract alkaloids. BFs are usually extracted with 60%-80% alcohol. As there are many non-alkaloid components in the extracts, further purification is needed. The process is that, after extracting with alcohols, concentrate the solvent to extractum, then redissolve it with acid water, filter away the insolubles, alkalify the acid water, and finally extract alkaloids with organic solvent **(Figure 3).**

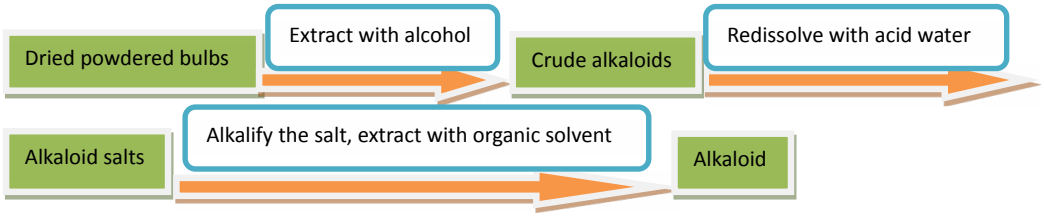

Figure 3. The process of alcohols-acid water-solvent extraction.

Guo et al. [45] developed the method of extracting alkaloids from *Bulbus Pallidiflorae Fritillariae*; they used ethanol as solvent, heated by water-bath, refluxed 2 h twice, collected the filtrate and concentrated to extractum. Then they redissolved the extractum with acid water (HCl, pH2-3), filtrated and adjusted the pH of the filtrate to 10-11 with 1 mol/L NaOH. At last, they extracted the acid water thrice with trichloromethane. The total alkaloids are collected after vacuum drying. Wang et al. and Zhao et al. used the similar method as Guo's [46,47].

Lipophilic organic solvent extraction

Before extracting with lipophilic organic solvent, the alkaloids should be transformed from salts to free alkaloids. The common method is after wetting the powder of BFs in alkaline water, such as lime-milk, sodium carbonate solution or ammonia water for a certain time, extracting alkaloids by solvent like benzene and trichloromethane etc... Dongdong Wang used this method to get crude alkaloids [4]. Dried powdered bulbs (10 kg) of the BFC were percolated in ammonia water (1000 ml) for 12 h, and then

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extracted thrice with100 L $CHCl_3$-MeOH (4:1). The solvent was concentrated in vacuo to obtain crude extracts (27 g). This method is popular, and is recorded by Chinese pharmacopoeia [2,22,48] **(Figure 4).**

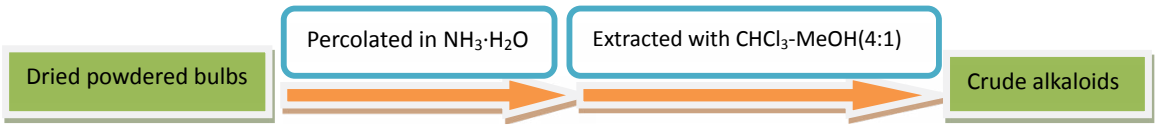

Figure 4. The process of lipophilic organic solvent extraction.

Percolation method

The powder of BFs is put into the percolator and added solvent from top constantly and the solvent take away the alkaloids when it flows through powder. Alcohols are the common solvent of this method. The rate of percolation, the concentration of the solvent, the volume of solvent etc. are the important factors of this method. Yang et al. [49] optimized extraction conditions of total alkaloids in BFCs by percolation. The alcohol concentration, soak time, velocity and volume were investigated by orthogonal experiment and HPLC was used to measure peimisine. And Yang obtained the optimum condition: soaked with 70% alcohol for 24 h, added 10 folds of 70% alcohol at speed of 3 $ml \cdot kg^{-1} \cdot min^{-1}$. Extraction yield of peimisine was 0. 016%.

Heat-reflux method

This method is usually conducted in Soxhlet extractor. The drugs are soaked in the flask with the volatile solvent. The solvent is heated and volatilize, then condensate in the condenser pipe and flow back to the flask, and the cycle repeats. The extraction temperature, duration, ratio of solvent to material, ethanol concentration, mean particle size, and number of extraction cycles are the influence factors. This method has distinct drawbacks, such as the large consumption of solvent and amounts of energy [50]. However, it is widely used to extract alkaloids form BFs because of its convenience. Han et al. [51] optimized the extraction process of total alkaloids from F.unibracteata Hsiao et K.C.Hsia. and found the optimum extraction process was as follows: particle size of 200 mesh, 75% alcohol, extraction duration was 30 min, and only once extraction. Xu et al. [52] optimized the extraction of alkaloids in *Bulbus Thunbergii Fritillariae*. And they found the optimum extraction condition was: 70% alcohol as solvent, the ratio of solvent to material was 6, extracted twice, and 2 h each time.

Other methods

Other methods like impregnation, decocting method are used because of its convenience; however, these methods are not as popular as the methods mentioned above.

New Techniques

Ultra sonication-assisted extraction (UAE)

Comparing with the conventional methods, ultra-sonication assisted extraction consumes less solvent, saves more time and increases extraction efficiency. Li et al. [53] compared the impregnation method with ultra-sonication assisted extraction method , and found that the later harvested double amount of alkaloids than the former, and they also found the optimum condition of ultra-sonication assisted extraction, which was: 75% alcohol, the extraction duration was 2 h, the ultra-sonication power was 200 W, and extracted 8 times.

Microwave-assisted extraction (MAE)

This technology induces rapid heating primarily within polar constituents due to dipole rotation and ionic drifting [54]. Wang et al. [55] compared oscillatory shaker extraction with microwave-assisted extraction methods of alkaloids from F.thunbergii, and confirmed the optimum extracting conditions of microwave-assisted method by single factor analysis and orthogonal test. The best conditions for microwave-assisted extraction were as follows: 80% ethanol as the solvent, solid to solvent ratio was 1:30 (g:mL), extraction time was150 s, and microwave power was 338 W.

Supercritical fluid extraction technique (SPE)

The extraction of nature products by supercritical carbon dioxide is widely applied in the food and pharmaceutical industries; it can prevent the use of organic solvents and reserve the bioactivity for extraction at low temperatures [56]. Li et al. [57] found the optimum conditions of extracting alkaloids from *Bulbus Pallidiflorae Fritillariae* of the SPE method by single factor analysis and orthogonal test. The condition was: extraction pressure was 20 Mpa, extraction temperature was 45°C, extraction duration was 2 h, and the flow rate of CO_2 was 2.5 mL/min. Under this condition, the yield of extraction was 0.198%.

Enzymatic mechanism extraction

The use of enzyme technology is becoming increasingly substantial for the extraction of TCM. This technology is environmentally friendly. The catalyzing reactions are also very specific. Cellulose enzyme is widely used in TCM extraction. This enzyme degrades and destructs the fiber organization of plants, and then promotes the dissolution of effective components and thus enhancing the yield of extraction. Wei et al. [58] used this method and found the yield of extraction increased from 0.0765% to 0.1065%.

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THE ISOLATION METHODS OF ALKALOIDS FROM BFS

Silica Gel Column Chromatography

Zhai et al. [59] used silica gel column chromatography to separate and purify total alkaloids from *Fritillaria Ussuriensis Maxim.* The solid phase was thin layer chromatography silica gel G and the mobile phase was ethyl acetate- methanol- ammonia (17:2:1), the purities of the separated products, peimine and peiminine were determined by HPLC-ELSD, respectively, each were 93.19% and 92.09%. The extracts (crude alkaloid, 8.5 g) extracted with $CHCl_3$ by Dongdong Wang [4,60] were purified repeatedly by silica gel(200-300 mesh) column chromatography with petroleum ether- acetone- diethyl amine (6:1:1-1:1:1) of increasing polarity as eluent.

High-Speed Counter Current Chromatography (HSCCC)

High-speed counter current chromatography (HSCCC) is an all-liquid method, in which separation is achieved by partitioning of the sample components between two immiscible liquids [61]. HSCCC has been widely used in the preparative isolation and purification of natural products with high recovery and loading capacity, low solvent consumption, acceptable efficiency, and the ease of scaling-up [62]. HSCCC coupled with ELSD was successfully applied to preparative separation and purification of verticine and verticinone from crude extracts of *Bulbus Fritillariae Thunbergii*. Chloroform-ethanol-0.2 mol·L^{-1} hydrochloric acid (3:2:2, v/v/v) was used as the solvent system, and HPLC analysis of the crude extracts(200 mg) collected on the preparative HSCCC showed that the purity of verticine (25.6 mg) was 96.8% and that of verticinone (10.3 mg) was 95.4% [63].

Macroporous Resins

The conventional methods like polyamide chromatography, gel chromatography and silica gel column have several disadvantages, including long time consuming, poisonous residual solvents and low recoveries. Recently, growing attention has been taken to macroporous resins for their convenience, low operating costs, low solvent consumption, high stability, and easy regeneration [64] **(Figure 5).**

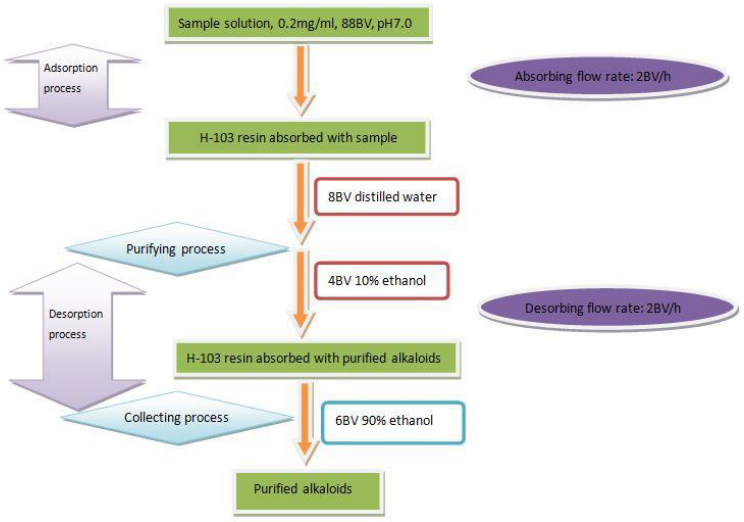

Figure 5. The purification process of BFs by Macroporous resins.

In Dongdong Wang's study [65], H-103 resin was chosen among 16 tested resins because of its higher adsorption capacity and desorption ratio. Dynamic adsorption and desorption tests were carried out in order to optimize the parameters for purifying alkaloids. The best enrichment conditions for alkaloids on H-103 resin were as below, for adsorption, 0.20 mg/mL as the initial concentrations of total alkaloids in sample solution; 1/10 as the diameter-to-height ratio of resin column; 2 bed volume (BV)/h as the loading flow rate; 88 BV as the feed volume; and pH 7.0; 40°C as the temperature and for desorption, 8 BV distilled water, 4 BV 10% ethanol, 6 BV 90% ethanol as a successive gradient elution; 2 BV/h as the flow rate **(Figure 5).** After one treatment with H-103 resin, the recovery yields of total alkaloids, imperialine, and peimisine in the product were 94.43%, 90.57%, and 96.16%, respectively.

Dong et al. [66] optimized purification process of total alkaloids from *Fritillaria thunbergii* by macroporous resin. Optimum macroporous resin and its purification process were selected by static and dynamic adsorption and desorption kinetics test. Optimal purification process conditions were: the optimal macroporous resin type was HPD100, the diameter-height ratio was 1:9, the concentration of sample solution was 5 g·mL^{-1} with pH8.0, the maximum sample volume was 13 BV, the flow rate of adsorption and elution was 1 mL·min^{-1}, the eluent was 8 BV water and 10 BV 80% ethanol, and collected eluent of 80% ethanol. Under this optimized condition, the content of total alkaloids was more than 65% by UV detector, HPD100 type macroporous resin showed good comprehensive adsorption property for enrichment of total alkaloids from *F.Thunbergii*, and suitable for separation and purification of them.

Zhang et al. [67] studied technological parameters of enriching and purifying total alkaloids from *Fritillariae Cirrhosae Bulbus* by macroporous resins. The adsorptive and desorptive ratio of the total alkaloids was determined by UV spectra. HPD-

100 macroporous resins were selected as the best material among 7 candidates. The optimized technological parameters they screened out were 0.293 g/mL concentration, pH11.6, eluted by 6 BV 80% alcohol, the elution rate was 2 BV/h. They found it was easy and reliable to enrich total alkaloids in *Fritillariae Cirrhosa Bulbus* by HPD-100 macroporous resins, which could also provide a reference for other *Fritillariae* species.

THE PHARMACOLOGICAL EFFICACY OF *FRITILLARIA* ALKALOIDS

The Pharmacological Activity of Respiratory System

Tracheobronchial relaxation effects

Chan et al. [18] investigated and compared the relaxant effect of five major *Fritillaria* alkaloids (imperialine, verticine, verticinone, ebeiedine and puqietinone) using rat isolated tracheal and bronchial preparation pre-contracted with carbachol. And all five alkaloids caused a concentration-dependent relaxation of both tracheal and bronchial preparations. The imperialine was found to be the most potent while puqietinone was the least potent. And they speculated the mechanisms of relaxant effect that alkaloids acted as competitive antagonism of muscarinic pathway and also the inhibition of influx of calcium ions.

Asthma relieving effect

Xu et al. [68] investigated the anti-asthmatic effect and its mechanism of the total alkaloids from *Fritillaria Hupehensis* on guinea pigs by observing the latent period of asthma induced by acetylcholine-histamine. They observed the influence of the total alkaloids from *Fritillaria Hupehensis* both on the basal tension and the contraction induced by acetylcholine, histamine and 5-HT by measuring the tension of isolated guinea pig tracheal strips in vitro. As the result, 4 mg/kg (i.g.) of the total alkaloids could significantly prolong the latent period of asthma. However, the total alkaloids could not change the basal tension and the contraction induced by histamine and 5-HT, while it could antagonize the contraction induced by acetylcholine. That meaned the antiasthma effect of of *Fritillaria Hupehensis* may be correlated with the competitive antagonism of muscarinic receptor (M-receptor) on guinea pig tracheal smooth muscle. Zhou et al. [69] did the further study and found 5 kinds of alkaloids in BFs (including verticine, verticinone, imperialine, imperialine glycosides and puqietinone) could inhibit the contraction of isolated guinea pig tracheal strips induced by carbachol.

Antitussive effect

Dongdong Wang et al. [4,60] researched 7 alkaloids in BFs, including imperialine, chuanbeinone, verticinone, and verticine, imperialine--N-oxide, isoverticine, and isoverticine--N-oxide, the result was that, positive control drug(codeine phosphate) and test compounds(3.0 mg/kg) significantly enhanced the latent period of cough, and reduced the cough frequency of mice compared with that of Control. At dose of 1.5 mg/kg (low dose), only isoverticine and isoverticine--N-oxide markedly enhanced the latent period of cough. In addition, imperialine and isoverticine inhibited cough frequency in a dose-dependent manner.

Shen et al. [70] studied the effect of relieving cough between *Fritillaria taipaiensis and F.unibracteata*. The mice cough models were established by strong aqua ammonia. And they found the low dose (1 g/kg) of F. taipaiensis could reduce the frequency of cough ($P<0.05$); The high dose(2 g/kg) of *F.unibracteata* had remarkable effection of expectorant ($P<0.05$). And they found *F.unibracteata* and *F. taipaiensis* had no remarkable difference on expectorant effection, however the *F.unibracteata* was better on reducing the cough of mice.

Expectorant effect

In Dongdong Wang's study [4,60], each mouse was treated with a single dose of 7 alkaloids of BFs for 30 minutes before intraperitoneal injection of phenol red solution (5% in saline solution, w/v, and 0.2 ml/20 g body weight). Mice were sacrificed after 30 minutes by cervical dislocation without damaging the tracheas. The trachea was disparted free from adjacent organs and removed from the thyroid cartilage to the main stem bronchi, then put into 1 ml normal saline immediately. After ultrasonic for 15 min, 1 ml of 5% NaHCO3 solution was added into the normal saline, and optical density was measured at 558.5 nm using Alpha-1900PC UV-Vis spectrophotometer. At dose of 3.0 mg/kg, imperialine, imperialine--N-oxide, isoverticine--N-oxide, verticinone and verticine significantly enhanced tracheal phenol red output, compared with control group. Moreover, effect of isoverticine--N-oxide on increasing phenol red output was better than that of the positive control (ammonium chloride). However, chuanbeinone showed no obvious phenol red output promoting effect.

Liang et al. [71] studied the expelling phlegm effect (by phenol red method) of *Fritillaria taipaiensis*, and contrasted it to *Fritillaria unibracteata Hsiao* et K.C.Hsia and *Fritillaria delavayi Franch*. All of the three Tendrilleaf Fritillary Bulb had the expelling phlegm effect and could facilitate the extraction of phenol red, *Fritillaria unibracteata* Hsiao et K.C.Hsia had the strongest effect, while *Fritillaria taipaiensis* and *Fritillaria delavay* had the same effect with each other.

Anti-Inflammatory

Dongdong Wang also conducted the experiment about the anti-inflammatory effect of 7 alkaloids in BFCs [4,18]. In the experiment, the anterior and posterior surfaces of rat's right ears were applied with 0.05 mL xylene thirty minutes after oral administration of the isolated alkaloids or dexamethasone. 30 minutes later, mice were sacrificed and both ears were removed. Ear disks (diameter of 6.0 mm) were punched out and weighed. The difference between the weight of right and left ear (as control) was used to calculate the extent of ear edema and inhibition of the extent of ear edema. At dose of 3.0 mg/kg (medium dose), the

5(including imperialine, chuanbeinone, imperialine--N-oxide, isoverticine, and isoverticine--N-oxide) alkaloids significantly inhibited the xylene-induced mice ear edema, but verticinone and verticine showed no significant anti-inflammatory effect. However, at dose of 1.5 mg/kg, only the isoverticine presented remarkable inhibition of the xylene induced mice ear edema. All 7 alkaloids inhibited the xylene-induced mice ear edema in a dose-dependent manner.

Li et al. [72] investigated anti-inflammatory effects and mechanisms of aqueous extract of *Fritillaria* ussuriensis Maxim. Animal models of auricular edema induced by xylene in mice, capillary permeability induced by acetic acid in mice and paw edema induced by albumen in rats were established to observe anti-inflammatory effects of aqueous extract of *Fritillaria* ussuriensis Maxim. The contents of PGE_2 and MDA in inflammatory exudates were measured to explore anti-inflammatory mechanisms of three samples. As the result, aqueous extract of *Fritillaria* ussuriensis Maxim exhibited good attenuation effects on the three animal models.

Antitumor Effects

Imperialine, Peimisine, Chuanbeinone are the three main alkaloids monomers in the total alkaloids of Bulbus *Fritillaria* Cirrhosae, when each tested on LLC cells, they showed significant inhibition of proliferation of the cells. Chuanbeinone and peimisine showed markedly higher inhibitory effects against LLC cells growth than imperialine [73]. Another study indicated imperialine and peimisine showed effect on A2780, HepG2 and A549 with IC50 values 39.18, 84.26, 117.84 and 17.43, 92.07, 36.11 μg/mL [74].

Antihypertensive Effect

Dae Gill Kang et al. [75] investigated the antihypertensive effect of Bulbus *Fritillaria* water extract (BFWE) on the models induced by N^G-nitro-l-arginine methylester (L-NAME). Treatment of rats with L-NAME (60 mg/l drinking water, 4 weeks) caused a sustained increase in systolic blood pressure (SBP). The NO concentration in plasma and NO productions in the vascular tissues of the L-NAME-treated group were significantly reduced compared with control group, whereas the expressions of nitric oxide synthase (NOS) proteins were not altered. The results suggested that BFWE attenuated the increase of SBP in the L-NAME-induced hypertension via enhancing the generation of vascular NO and amelioration of renal functions.

THE BIOAVAILABILITY OF ALKALOIDS FROM *FRITILLARIA*

The BFs pharmacological effects have been lucubrated for many years and there are plenty of document information about it, while its bioavailability were researched by few people. In the limited references, we know that its oral bioavailability is a little poor, however the reason of it still remain a mystery. There are some hypotheses about it, including first-pass metabolism, intestinal metabolism etc. The most popular method of quantification of alkaloids is liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) due to its high sensitivity and selectivity.

Wu et al. [39] did a systemic preclinical pharmacokinetic study of verticinone, which is the major alkaloid in *Fritillaria* hupehensis. First, they developed and validated a sensitive and specific LC-MS method for the quantification of verticinone in biological samples of rats. The verticinone were orally dosed to rats and the blood was collected at the certain time through oculi chorioideae vein. Verticinone and the interal strandard (IS), hupehenine, were extracted from plasma sample by a simple liquid-liquid extraction with ethyl acetate after being alkalified by ammonia hydroxide. They discovered that there was gender difference of absorption pharmacokinetics of verticinone in rats, The C_{max} and AUC_{0-t} in male rats were significantly higher than that in female rats, but the CL and Vd in male rats were lower than that in female rats, which indicated that the elimination of verticinone was faster in female rats than that in male. The absolute oral bioavailabilities of verticinone in male and female rats were 44.8% and 2.7%, respectively. The tissue distribution of verticinone in rats was conducted and the result was the concentrations of verticinone in most tissues were higher than in plasma, that meaned verticinone had good tissue penetrability and a high tissue affinity. And they also researched the excretion of verticinone, the verticinone in bile, urine and feces were quantified and the result showed unchanged form was not the main excretion path of verticinone in rats. This scholar conjectured it was the sulfation of verticinone in intestines that resulted in the low oral bioavailability.

Lin et al. [76] used chromatography-electrospray ionization-tandem mass spectrometric to quantify imperialine in rat plasma, a major active constituent extracted from *Bulbus Fritillariae Cirrhosae*. Before analysis, plasma samples were precipitated with acetonitrile to remove protein and extract imperialine and the internal standard, carbamazepine. Three doses imperialine were administered in single doses orally or through the caudal vena cava, and pharmacokinetic parameters were evaluated. Oral bioavailability with dose of 1 mg/kg was 31.2%; 5 mg/kg, 53.6%; and 10 mg/kg, 47.4%.

Liu et al. [77] quantified peimine in rabbit plasma with LC-MS/MS method. And they found that the oral bioavailability of peimine in rabbits was only 10.65%, the poor bioavailability may result from the low water-solubility, gastrointestinal enzymes metabolism or efflux pump mechanisms.

CONCLUSION

Bulbus of *Fritillaria*, as one of the most important and commonly used traditional Chinese herbal medicines, has been effectively used for respiratory disease in oriental clinical practices. It also has been used in combinations with various other herbal ingredients for the purpose of prevention and treatment of cough, asthma, etc. And Bulbus of *Fritillaria* has also been used in many health-related products.

The following three aspects are important for future research into Bulbus of *Fritillaria*. First of all, alkaloids are the most active constituents, which are acknowledged, however the other constituents like polysaccharide and saponin etc. may have synergistic effect with alkaloids in curing respiratory disease, and the interaction between alkaloids and others is still a mystery. Secondly, the pharmacological mechanisms of Bulbus of *Fritillaria* lack depth, the molecular mechanism and the structure-function relationship of alkaloids in Bulbus has been little researched [78,79]. Finally, though the Bulbus of *Fritillaria* is considered hypotoxicity, the toxicity of it has been scarcely researched.

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