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HPLC Fingerprint of Solid-State Fermentation of Fugui Gutong Compound Prescription

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Aspergillus niger (E6.22) was employed for solid state fermentation of Fu-Gui-Gu-Tong (FGGT, hereafter solid state fermentation FGGT), a notable Chinese compound prescription, to attenuate its toxicity. Results indicated that the optimal spore density was 1.8×10^4 mL^{-1}, incubation time was 7d, pH was 6.5, incubation temperature was 30 ºC. Under these conditions, paeoniflorin and total alkaloids were increased, but aconitine was decreased. Additionally, high-performance liquid chromatographic fingerprint of solid state fermentation Fu-Gui-Gu-Tong were determined and applied for monitoring the fermentation stability. The similarity index of 10 samples was 0.978, suggesting that the procedure of solid state fermentation Fu-Gui-Gu-Tong was stable in different batches. To investigate its acute toxicity, the mice was orally administered with graded doses of solid state fermentation Fu-Gui-Gu-Tong extract, the LD_{50} of the solid state fermentation Fu-Gui-Gu-Tong group was higher than Fu-Gui-Gu-Tong, meaning the less toxicity of solid state fermentation Fu-Gui-Gu-Tong. Hence, it is concluded that *A. niger* (E6.22) could alleviate the toxicity of Fu-Gui-Gu-Tong through solid state fermentation and the fermentation procedure was reliable and applicable.

Key Words: *Aspergillus niger*, Fu-Gui-Gu-Tong, Solid state fermentation, HPLC fingerprint.

INTRODUCTION

Fu-Gui-Gu-Tong (FGGT), a Chinese compound prescription used for curing arthritis and related disorders such as rheumatoid arthritis, hyperosteogeny and lumbago, is composed of eight traditional Chinese herbs (Table-1) and has been approved by the Ministry of Health of the People's Republic of China. Two kinds of herbs *Radix aconiti* and *Radix aconiti Lateralis preparata* in the formula were rich in alkaloids, such as aconitine, mesaconitine, hypaconitine and jesaconitine[1], all are active and toxic ingredients[2]. Of them, aconitine belongs to diester-diterpenoid aconitum alkaloid, which is also highly toxic. Our previous studies[3,4] showed that when the fungus *Aspergillus niger* (E6.22) was fermented with *Radix aconiti* or *Radix aconiti Lateralis preparata*, some diester-diterpenoid aconitum alkaloids were changed into hydrolysis monoester-diterpenoid alkaloids, which have less toxicity. Paeoniflorin from *Radix paeoniae alba* is another major active component showing anti-inflammatory and analgesic activity. In order to obtain better activity and less toxicity, myriads of methods were adopted to ensure the safety and curative effects, such as processing, compatibility of different herbs, decoction, *etc*.[5]. In recent years, solid-state fermentation has attracted great interest to produce fermented foods, enzymes, organic acids and to decrease the toxicity[6,7]. Therefore, in the present study, *A. niger* (E6.22) was used to ferment the prescription, the fermentation parameters were optimized and the fermentation stability was estimated by HPLC fingerprint, the half lethal dose (LD_{50}) was applied to evaluate the potential safety of Fu-Gui-Gu-Tong.

EXPERIMENTAL

Eight traditional Chinese herbs were purchased from TongRenTang Co. Ltd., (Beijing, China) and authenticated by Prof. Ge Xizhen in Biochemical Engineering College, Beijing Union University. A voucher was deposited at the College. Paeoniflorin and aconitine were purchased from Sigma, USA. *A. niger* (E6.22) was stored in this lab. All chemicals were of analytical or chromatography grade.

Solid state fermentation

Medium of solid-state fermentation Fu-Gui-Gu-Tong compound prescription (Table-1) containing eight traditional Chinese herbs were used as natural substrate for solid state fermentation according to the prescription ratio and no other constituent was added. The herbs were milled, sieved (10-20 mesh), mingled and heat-sterilized at 122 ºC for 0.5 h prior to inoculation.

Spore suspension: *A. niger* (E6.22) was propagated on beef extract peptone medium (beef extracts 10 g, peptone 10 g,

TABLE-1
HERBS USED FOR PREPARING FU-GUI-GU-TONG DECOCTION OR MEDIUM

Family names	Botanical names	Chinese names	Ratio
Radix Aconiti Lateralis Preparata	*Aconitum carmichaeli Debx.*	Fuzi	4
Radix Aconiti	*Aconitum carmichaeli Debx.*	Chuanwu	2
Radix Paeoniae Alba	*Paeonia lactiflora Pall.*	Baishao	3
Radix Codonopsis Pilosulae	*Codonopsis pilosula(Franch.)Nannf.*	Dangshen	3
Radix Angelicae Sinensis	*Angelica sinensis(Oliv.)Diels*	Danggui	3
Herba Epimedii	*Epimedium brevicornum Maxim.*	Yingyanghuo	3
Olibanum	*Boswellia carterii Birdw.*	Ruxiang	2
Cortex Cinnamomi	*Cinnamomum cassia Presl.*	Rougui	1

dextrose 20 g, sodium chloride 5 g), 30º for 5-8 d, stored on slants at 4 ºC. The spores were suspended with sterile water. The density of spore suspension was adjusted to 1.8×10^2-1.8×10^5 spore/mL.

Fermentation procedure: Fermentations were carried out in a tray, 250 mL of spore suspension were added to 500 g of sterilized natural substrate and a exact amount of water to fit a moisture content of 60 % at last. The trays were incubated under static, constant temperature at a specific condition.

Fermentation parameters selected: Different fermentation conditions were studied in the present work, including fermentation temperature, fermentation time, pH and spore density.

Experiment temperatures were 25, 30, 35 and 38 ºC. Fermentation time was 5, 7, 9 and 11 d. Spore density was 1.8 × 102, 1.8 × 103, 1.8 × 104 and 1.8 × 105/mL. The pH was 5.5, 6.5, 7.5. All experiments were conducted in triplicate.

Preparation of crude extract of solid state fermentation of Fu-Gui-Gu-Tong: At the end of fermentation, the substrates in each experiment were extracted with water for 2 h, 90 ºC, 3 times, the suspension collected from every extraction was centrifuged (4000 rpm, 15 min) and the supernatant was filtered and concentrated to become a sticky glue-like substance in a rotary shaker under vacuum. After concentration, the residue was dissolved in a small volume of analytical pure methanol and this methanol solution was used as crude extract, stored at -20 ºC until needed for the later experiments. The fermentation method was compared with the traditional water extracting method.

Determination of the dry weight of extract: According to appendix X.A. the first section of Chinese Pharmacopoeia (2005), 10 mL of extract solution was transferred into a evaporating dish, dried by a water bath, then dried in oven at 105 ºC for 3 h, cooled for 30 min, weighted accurately, the weight of dry extract was calculated.

Detection of total alkaloids: The total alkaloids in the prescription was determined by previous method[8] based on acid dye colourimetry, in brief, the spectrometric features of the ion-pair compounds produced in the reaction between the aconitine-type alkaloids and bromcresol green.

Construction of HPLC of solid state fermentation of Fu-Gui-Gu-Tong: A mixture solution of reference substances containing paeoniflorin and aconitine were diluted to be the appropriate concentration for establishing calibration curves (Fig. 1). The appropriate HPLC conditions were as follows: waters analytical HPLC:

LC-10AT vp HPLC pump, CTO-10AS vp thermostated column compartment, SPD-10A vp detector and controller. Diamonsil C_{18} (250 × 4.6 mm, 5 μm) (DIKMA, American) chromatogram column, the flow rate was 1.0 mL/min, the column temperature was maintained at room temperature (25-30 ºC), detection at 230 nm was to analyze the product of solid state fermentation Fu-Gui-Gu-Tong, the mobile phase consisted of A (water) and B (acetonitrile), gradient elution was as follows: 0.01min, A:B = 96:4; 20 min, A:B = 90:10; 30 min, A:B = 85:15; 60 min, A:B = 60:40; 70 min, A:B = 10:90; 80 min, A:B = 4:96; 85 min, A:B = 96:4. The linearity of the peak area *versus* concentration curve for paeoniflorin and aconitine was used to calculate the contents of the biomarker substances in Fu-Gui-Gu-Tong.

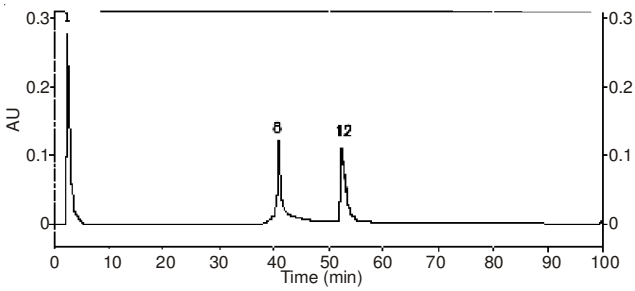

Fig. 1. HPLC analysis of standard solution. The peaks were identified as: 8 = Paeoniflorin; 12 = Aconitine.

Establishment of fingerprint for solid state fermentation of Fu-Gui-Gu-Tong: Through optimization of solid state fermentation condition, the process was repeated for 10 batches, 100 min of chromatogram was recorded, meanwhile, the superposition chart (Fig. 6) was obtained using similarity evaluation system of traditional Chinese medicine chromatographic fingerprints provided by state pharmacopoeia committee (Version 2004A). The software was employed to calculate the correlative coefficient and evaluate the similarities of different chromatograms[9].

Study on acute toxicity (oral): According to the method of Bliss[10] and our reported toxicity through preliminary study, the lethal dose (100) of Fu-Gui-Gu-Tong was 190.0 g/kg and the survival dose (0) was 57.8 g/kg. Fifty mice were divided into five groups according to a random number table. The mice were orally administered with graded doses of solid state fermentation Fu-Gui-Gu-Tong 176.0, 140.9, 112.8, 90.2 and 72.2 g/kg (the ratio is 1: 0.8) to five different groups of mice, all groups were orally administered twice a day and then closely observed for any abnormal or toxic manifestation and mortality up to 14 d. The half lethal dose (LD_{50}) was calculated. Meanwhile, another 50 mice with Fu-Gui-Gu-Tong was designed and operated as the control group.

RESULTS AND DISCUSSION

Optimization of fermentation process parameters: Solid state fermentation was used for production of Fu-Gui-Gu-Tong, according to preliminary experiments, four important parameters (incubation temperature, time, pH and spore density) were considered as the independent variables and their effects on active ingredients were given in Figs. 2-5.

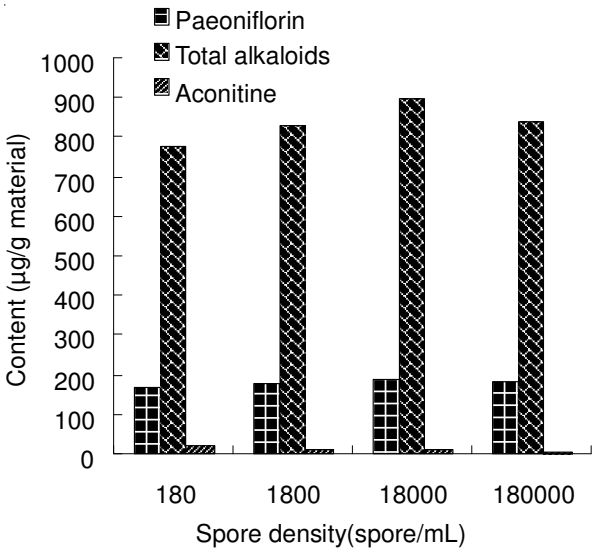

Fig. 2. Effect of spore density on the contents of paeoniflorin, total alkaloids and aconitine in fermentation extracts

The contents of paeoniflorin and total alkaloids at 1.8×10^4 spores/mL were higher, but the aconitine was lower.

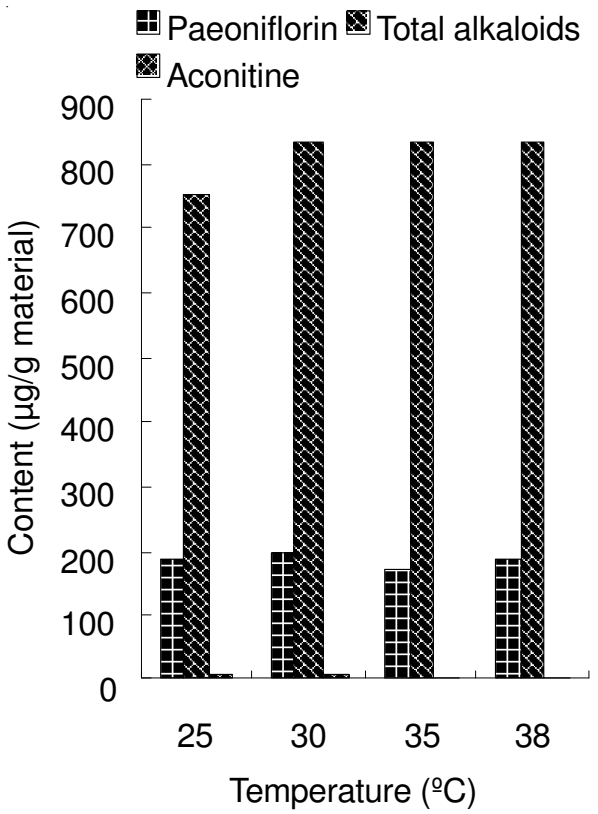

Fig. 3. Effect of temperature on the contents of paeoniflorin, total alkaloids and aconitine in fermentation extracts

The content of paeoniflorin at 30 ºC was higher. In contrast, aconitine became lower when the temperature rose.

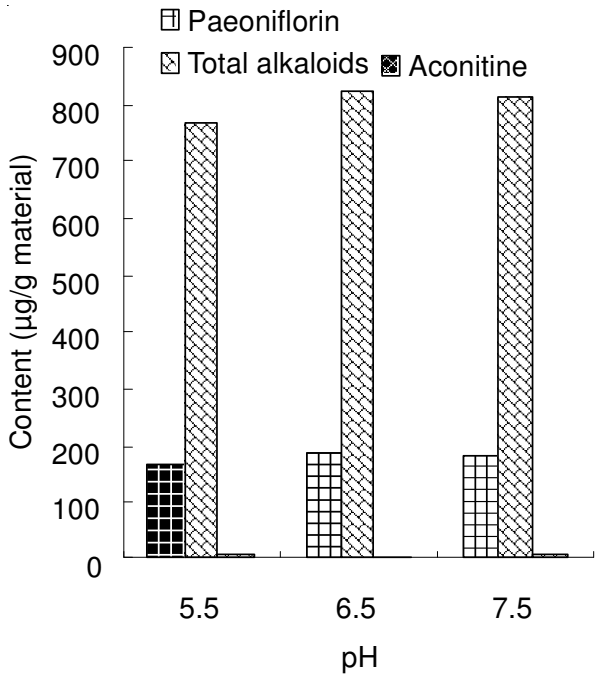

Fig. 4. Effect of pH on the contents of paeoniflorin, total alkaloids and aconitine in fermentation extracts

The contents of paeoniflorin and total alkaloids at pH 6.5 were higher, while the aconitine was lower. The *A. niger* grow well, suggesting that pH 6.5 is appropriate.

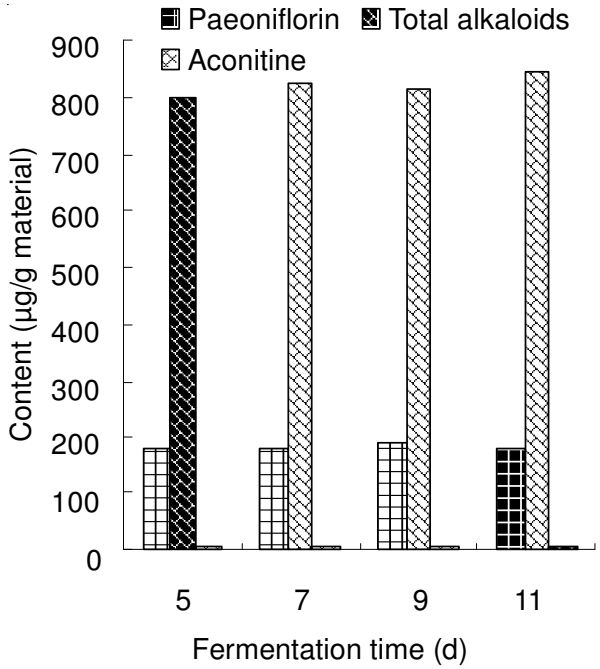

Fig. 5. Effect of fermentation time on the contents of paeoniflorin, total alkaloids and aconitine in fermentation extracts.

The contents of paeoniflorin and total alkaloids at 9 or 11 d were higher. In contrast, aconitine became lower with the increase of fermentation time.

The total alkaloids varied in different fermentation process, in general, the total alkaloids rose up with the increase

TABLE-2
COMPARISON OF FERMENTATION WITH TRADITIONAL METHOD

Methods	Paeoniflorin μg/g material	Total alkaloids μg/g material	Aconitine μg/g material	Weight of dry extract(g)/100 g herbal material
Tradition	181.4	421.9	33.14	32.31
Fermentation	245.2	853.7	4.01	30.56

TABLE-3
SIMILARITY FOR 10 BATCHES OF SOLID STATE FERMENTATION FU-GUI-GU-TONG

Sample No.	1	2	3	4	5	6	7	8	9	10	Control
Similarity	0.978	0.982	0.995	1.000	0.978	0.992	0.998	0.978	0.984	0.994	1.000

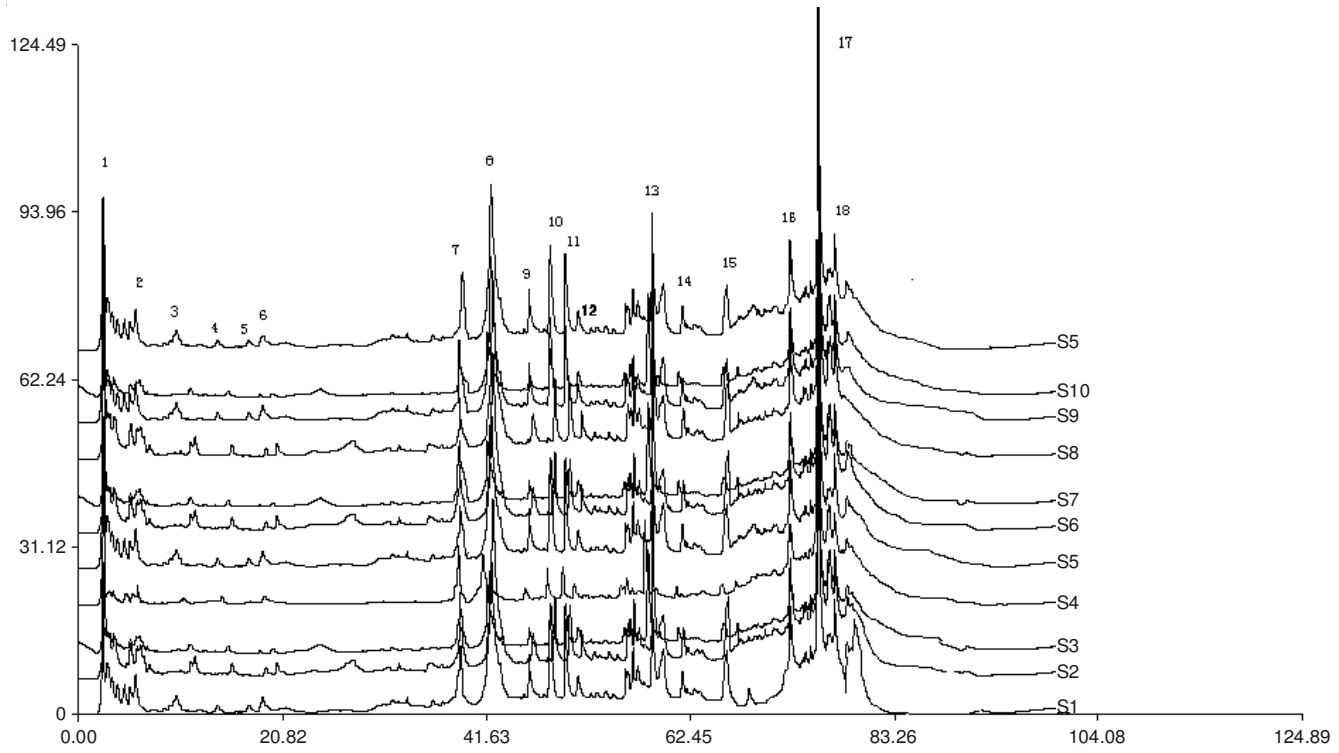

Fig. 6. Matched chromatographys of 10 patches of solid state fermentation Fu-Gui-Gu-Tong

of the spore number, incubation time, or the incubation temperature. Conversely, the aconitine decreased, which is one of the major aconitum alkaloid typically symbolizing the prescription's toxicity[2]. Considering the labor cost, material, time cost and the growth status, the optimal setting of experimental parameters for solid state fermentation Fu-Gui-Gu-Tong were as follows: spore density, 1.8×10^4 spore/mL; incubation time, 7d; pH, 6.5; incubation temperature, 30 ºC.

Comparison of this fermentation with conventional methods: Solid state fermentation (Table-2) was showed to be better for optimum production of paeoniflorin (245.2 μg/g) and total alkaloids (853.7 μg/g). Both of them were higher than traditional method (181.4, 421.9 μg/g), though the dry extract (30.56 g/g) in solid state fermentation was lower, the extraction liquid was clear with low viscosity. It was thus deduced that the microbes most possibly the *A. niger* (E6.22) decomposed the polysaccharide in the extract[11]. The data revealed that the amount of aconitine in solid state fermentation Fu-Gui-Gu extract was very low (4.01 μg/g), because the oral lethal dose 50 % (LD_{50}) of aconitine for mice is 1.8 mg/kg[12] and the lethal dose of these alkaloids for humans is 1-2 mg[13]. It could be deduced that the solid state fermentation Fu-Gui-Gu extract was safe.

Fingerprinting and similarity index of solid state fermentation Fu-Gui-Gu-Tong: Fu-Gui-Gu-Tong was a compound prescription containing diverse active compounds, while the paeoniflorin in *Radix paeoniae alba* was the main constituent with high concentration, the peak height is moderate with good separation from the adjacent peaks (Fig. 6). Hence, the chromatography of paeoniflorin was regarded as reference peak. The retention times of this biomarker substance and aconitine were 41.242, 52.275 min, respectively. Among all peaks observed, 18 peaks of them (denoted from 1 to 18) were defined as common peaks because they showed up in all samples. The relative retention time and relative retention area of these 18 peaks were not shown. The similarity index of 10 samples was higher than 0.978 (Table-3), indicated that the samples from different batches in the same solid state fermentation condition shared the similar chromatographic pattern, thereby suggesting the stability of the constituents of solid state fermentation of Fu-Gui-Gu-Tong in different batches, as well as the reliability and applicability of this fermentation procedure.

Acute toxicity results: The mice became dispirited, some moved slowly or died after being orally administered with

Fu-Gui-Gu-Tong or solid state fermentation of Fu-Gui-Gu-Tong[14] days later. Before dying, the mice manifested toxic symptoms with a low respiratory rate, gasping for breath and flapping of nose wing. The death numbers could be clearly seen from Table-4, in control group, the LD_{50} was 118.16 g/kg, its 95 confident limit was 101.77-139.53 g/kg. For the solid state fermentation Fu-Gui-Gu-Tong group, the LD_{50} was 133.70 g/kg, which was higher (118.16 g/kg), its 95 confident limit was 114.73-166.67g/kg. The above results indicated that when Fu-Gui-Gu-Tong treated by solid state fermentation, its toxicity became less and safer than the original prescription Fu-Gui-Gu-Tong. Therefore the solid state fermentation Fu-Gui-Gu-Tong is helpful for the goals *i.e.*, low toxicity and safety[10,14].

TABLE-4
RESULTS OF DIFFERENT DOSES OF SOLID STATE FERMENTATION (SSF) FU-GUI-GU-TONG (FGGT) ORALLY GIVEN TO MICE

Dose (g/kg)	Dose (g/kg)	n	Death number	Percentage mortality	LD_{50} (g/kg)
SSF FGGT	176.0	10	8	80.0	133.70*
	140.9	10	6	60.0	
	112.8	10	3	30.0	
	90.2	10	1	10.0	
	72.2	10	1	10.0	
FGGT	176.0	10	9	90.0	118.16**
	140.9	10	7	70.0	
	112.8	10	4	40.0	
	90.2	10	2	20.0	
	72.2	10	1	10.0	

*LD_{50}95 % was 133.70 g/kg, 95 confident limit was 114.73-166.67 g/kg; **LD_{50} 95 % was 118.16 g/kg, 95 confident limit was 101.77-139.53 g/kg; SSF FGGT: Solid state fermentation of Fu-Gui-Gu-Tong.

Conclusion

Three conclusions have been drawn from this study.

(i) the optimal parameters for fermenting Fu-Gui-Gu-Tong were as follows: *A. niger* (E6.22) spore density, 1.8×10^4 spores/mL; incubation time, 7d; pH, 6.5; incubation temperature, 30 ºC. In this condition, paeoniflorin and total alkaloids could be increased, whereas, the composition of aconitine was decreased.

(ii) the appropriate HPLC conditions for solid state fermentation of Fu-Gui-Gu-Tong were as follows: diamonsil C_{18} (250 × 4.6 mm, 5 μm) chromatogram column, the flow rate was 1.0 mL/min, the column temperature was 25-30 ºC, detection was at 230 nm, the mobile phase was gradient elution consisting of A (water) and B (acetonitrile). The similarity index of samples was 0.978. HPLC fingerprinting was stable and feasibile to test the fermentation procedure of Fu-Gui-Gu-Tong.

(iii) when Fu-Gui-Gu-Tong was treated by solid state fermentation, its toxicity became less and was safer than the original prescription Fu-Gui-Gu-Tong. It is concluded that the procedure of solid state fermentation was stable and reliable and the fermented Fu-Gui-Gu-Tong was safe.

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