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Full Length Research Paper

Fungal risk in commonly used traditional Chinese patent medicines

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Fungal contamination of 27 commonly used Traditional Chinese Patent Medicines (TCPM) was investigated. Six (6) forms of prepared medicines including watered pills, honeyed pills, condensed pills, tablets, capsules and granules were chosen for fungal observation. Three (3) medicines, namely Trollius chinensis bunge capsules (TCBC), Yinqiao Jiedu watered pills (YJWP) and Fufang Danshen tablets (FDT) were contaminated, the mean CFU/g was 12, 12 and 0.7, respectively. According to morphology, as well as molecular methods, obtained fungal species were identified as belonging to the genera of *Aspergillus*, *Eurotium*, *Mucor*, *Paecilomyces* and *Penicillium*. The genus *Aspergillus* was predominant, consisting of 3 species isolated namely, *Aspergillus foetidus*, *Aspergillus flavus* and *Aspergillus niger*. Raw herbal materials especially fermented materials were supposed to be the main source of fungal contamination. To insure the medicinal safety, standard operation such as pure inoculation should be applied in fermentation. The finding of this study will help to provide necessary information for the safety assessment as well as quality improvement of TCPM.

Key words: *Aspergillus*, fermented material, fungal contamination, *Penicillium*, traditional Chinese patent medicines (TCPM).

INTRODUCTION

The history of using traditional Chinese herbal medicine could be back to the Eastern Han dynasty of China (AD 25-220). With thousands of years of experience in treating diseases with natural materials, traditional Chinese herbal medicines still play an important role in the healthcare system of modern China and are officially recognized not only in China, but also in Japan and some other eastern and south-eastern Asian countries which have the similar cultural traditions (Zhu and Woerdenbag, 1995). The characteristic application of Chinese herbal medicine is the combined uses of drugs with the approaches to increase or promote therapeutic effectiveness, to minimize toxicity or side effects, to accommodate complex clinical situations and to alternate the actions of the drugs, which generated various types of prescriptions.

According to prescriptions provided in "The Chinese Pharmacopeia" and "Standards of the Chinese patent medicine", traditional Chinese herbal medicine were made into different formulas of preparations namely, Traditional Chinese Patent Medicines (TCPM), including honeyed pills, watered pills, condensed pills, capsules, tablets, granules and so forth. Pills are made of fine powder of pulverized medical substances with sticky excipients. They can disintegrated slowly in stomach and intestine, thus, they are mostly used to treat chronic diseases and for health improvement. There are a variety of pills made by different methods. Honeyed pills are made of fine powder of pulverized medicinal substances with honey as binder. Each honeyed pill weighs more than 0.5 g. Watered pills are smaller and commonly use cold boiled water as binder. Condensed pill, also called as extracted medicinal pills, are made of condensed extracts with water or honey as binder. In modern pharmacy, new forms of prepared medicine were applied;

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pulverized medical substances were made into tablets, soluble granules or filled into capsules. Tablets are convenient to take orally, granules were absorbable and capsules have prolonged potency due to slow breaking down of external coating (Beijing Digital Museum of Traditional Chinese Medicines, 2007).

In recent years, the use of TCPM has gained popularity over the world. So far, more than 9,000 kinds of TCPM have been approved by the state to be sold in the markets. In 2007, the industrial output value of TCPM reached 177.2 billion yuan (approximate $ 27.7 billion), accounting for 26.53% of the total pharmaceutical industrial output value (Information Office of the State Council of the People's Republic of China, 2008).

The quality of crude herbs relates to the quality of TCPM. Due to the traditional methods of collection, processing and storage, crude herbal drugs were prone to fungal infestations (Yang et al., 2010; Chen et al., 2010, 2011). The fungal deterioration adversely affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs (Dutta and Roy, 1987). More importantly, even small amount of fungal spores may cause candidiasis, aspergillosis, zygomycosis, and other invasive filamentous infections in certain groups of immunosuppressed patients. Besides, some fungal species of *Aspergillus* and *Penicillium* may produce poisonous metabolites (Roy et al., 1988; Chen et al., 2011) which could cause mycotoxicoses in patients after oral administration. The mycotoxins detected from crude herbs as well as TCPM include aflatoxin and ochratoxin A (Ren et al., 1997; Liu et al., 1999; Tang et al., 2000; Li and Chen, 2000). However, few data are available concerning contaminated fungal species. Accordingly, the objective of this study was to determine the contaminated fungi present in commonly used TCPM.

MATERIALS AND METHODS

Sample collection

Twenty-seven (27) TCPM was purchased from different drugstores during 2009 to 2010. Different forms of prepared medicines including watered pills, honeyed pills, condensed pills, tablets, capsules and granules were chosen for fungal investigation. For each medicine, 3 batches were selected (Table 1) and processed within 3 days after collection.

Isolation of fungi

Rose Bengal chloramphenicol agar (RBCA) was prepared in sterile plastic plates (9 cm) for fungal isolation. For watered pills and tablets, approximately 1 g (10 pills or 3 tablets) were directly placed on plates (in triplicate). For honeyed pills, each pill (approximately 1 g) was cut into 4 pieces with sterile scalpel and placed on plates (in triplicate). For the capsules and granules, the contents (approximately 1 g) were spread directly on plates (in triplicate). All plates were incubated at 25°C for 5 to 7 days. The representative fungi from each plate were sub-cultured and identified preliminarily according to morphology; the isolates of *Aspergillus*, *Penicillium* and *Eurotium* were cultured on malt extract agar (MEA) and Czapek yeast extract agar (CYA), isolates of other species on potato dextrose agar (PDA). All of them were cultured at 25°C for 7 to 10 days, and stored at 4°C for subsequent characterization.

Identification of fungi

Each species was identified using specific media and proper references (Pitt, 1979; Carmichael et al., 1980; Klich and Pitt, 1988; Samson and Frisvad, 2004). For those confusing species, β-tubulin gene and the internal transcribed spacer (ITS) of ribosomal deoxyribonucleic acid (DNA) were amplified and sequenced. Similar taxon retrieved by basic local alignment search tool (BLAST) in GenBank/NCBI was used as reference for further morphological examination and identification. β-Tubulin gene was amplified using the primers Bt2a and Bt2b (Glass and Donaldson, 1995) with the following conditions: denaturing at 94°C for 5 min, followed by 35 cycles at 94°C for 40 s, 54°C for 40 s and 72°C for 1 min, then a final extension at 72°C for 10 min after cycling. ITS gene was amplified using the primers ITS4 and ITS5 (White et al., 1990) with the same polymerase chain reaction (PCR) condition.

RESULTS

Contaminated fungal species were isolated from 7 batches of 3 TCPM, namely, 3 batches of Trollius Chinensis bunge capsules (TCBC), 2 batches of Yinqiao Jiedu watered pills (YJWP) and 2 batches of Fufang Danshen tablets (FDT). In the other 24 medicines, no fungal contamination was observed (Figure 1). The mean CFU/g for the fungal isolates of the 3 medicines was 12, 12 and 0.7 in TCBC, YJWP and FDT, respectively (Table 2).

On the basis of morphology as well as molecular evidence, *Aspergillus* and *Penicillium* were found to be commonly present in most samples examined. *Aspergillus*, the most common genus, including *Aspergillus foetidus*, *Aspergillus flavus* and *Aspergillus niger*, was isolated in all the 3 medicines. The genus ranked second in terms of incidence was *Penicillium*, which was found in two of the medicines. This genus was represented by 3 species as *Penicillium variable*, *Penicillium corylophilum* and *Penicillium chrysogenum*. Isolates of *Paecilomyces varioti* were detected on TCBC with high frequency of 84%. While species of *Eurotium rubrum* emerged in YJWP only (Figure 2 and Table 2).

DISCUSSION

According to drug hygienic standard in China, fungal colony counts should not exceed 500 CFU/g in pills and tablets. Yuan et al. (2003) investigated 20 TCPM, among them 10% were contaminated with excessive fungi (colony counts > 500 CFU/g). This study showed that 3 TCPM (11%) were contaminated by fungi, however, the fungal colony counts were less than 50 CFU/g. On the whole, TCPM investigated in this study were qualified.

Table 1. Traditional Chinese patent medicines for fungal determination.

Sample	Preparation form	Production area	Batch number	Period of validity
Liuwei Dihuang Pills	Watered pills	Beijing, China	9030400	2009-02 to 2013-01
			9031550	2009-04 to 2013-03
			9032110	2009-04 to 2013-03
Diyu Huaijiao Pills	Watered pills	Beijing, China	8035188	2008-07 to 2012-06
			9035002	2009-01 to 2012-12
			9035013	2009-01 to 2012-12
Yinqiao Jiedu Pills	Watered pills	Beijing, China	8033975	2008-09 to 2012-08
			9030143	2009-02 to 2013-01
			9033215	2009-06 to 2013-05
Yangyin Qingfei pills	Watered pills	Beijing, China	8030019	2008-03 to 2012-02
			9035084	2009-03 to 2013-02
			9035091	2009-03 to 2013-02
Jinkui Shenqi Pills	Watered pills	Beijing, China	8034814	2008-11 to 2012-10
			9030108	2009-01 to 2012-12
			9030111	2009-01 to 2012-12
Liuwei Dihuang Pills	Honeyed pills	Beijing, China	9010288	2009-03 to 2014-02
			9010296	2009-05 to 2014-04
			9010298	2009-05 to 2014-04
Wuji Baifeng Pills	Honeyed pills	Beijing, China	8015472	2008-11 to 2013-10
			9015054	2009-01 to 2013-12
			9015064	2009-01 to 2013-12
Jiawei Huoxiang Zhengqi pills	Honeyed pills	Beijing, China	9010358	2009-01 to 2013-12
			9010359	2009-01 to 2013-12
			9010363	2009-01 to 2013-12
Diyu Huaijiao Pills	Honeyed pills	Beijing, China	9010172	2009-03 to 2014-02
			9010267	2009-05 to 2014-04
			9010274	2009-05 to 2014-04
Niuhuang Jiedu Pills	Honeyed pills	Beijing, China	8013871	2008-11 to 2013-10
			9010028	2009-04 to 2014-03
			9010031	2009-04 to 2014-03
Liuwei Dihuang Pills	Condensed pills	Beijing, China	9073718	2009-01 to 2012-12
			9073748	2009-04 to 2013-03
			9073768	2009-06 to 2013-05
Qiju Dihuang pills	Condensed pills	Beijing, China	9072677	2009-05 to 2013-04
			9072839	2009-05 to 2013-04
			9072845	2009-05 to 2013-04
Fufang Caoshanhu Buccal tablets	Tablets	Jiangxi, China	09050828	2009-05 to 2011-04
			09060803	2009-06 to 2011-05
			09060805	2009-06 to 2011-05

Table 1. Contd.

Xiguashuang Runhou tablets	Tablets	Guilin, China	0807007 0905017 0907024	2008-07 to 2011-07 2009-05 to 2012-05 2009-07 to 2012-07
Jianwei Xiaoshi Tablets	Tablets	Jiangxi, China	09060085 09090013 09110223	2009-06 to 2011-05 2009-09 to 2011-08 2009-11 to 2011-11
Shujin Huoxue Tablets	Tablets	Beijing, China	8125209 8125211 8125213	2008-01 to 2010-12 2008-01 to 2010-12 2008-01 to 2010-12
Fufang Danshen Tablets	Tablets	Beijing, China	081024 090807 090808	2008-10 to 2011-09 2009-08 to 2012-07 2009-08 to 2012-07
Liuweidihuang Capsula Mollis	Capsules	Beijing, China	8171422 8171421 8171424	2008-12 to 2010-11 2008-12 to 2010-11 2008-12 to 2010-11
Yangxue Shengfa Capsules	Capsules	Tianjing, China	101013 101051 101053	2009-01 to 2011-12 2009-03 to 2012-02 2009-03 to 2012-02
Trollius chinensis Bunge capsules	Capsules	Jilin, China	20080601 20080702 20100101	2008-06 to 2011-05 2008-07 to 2011-06 2010-01 to 2012-12
Ganmao soft Capsules	Capsules	Beijing, China	8173141 9172347 9172350	2008-06 to 2010-05 2009-05 to 2011-04 2009-05 to 2011-04
Maren Runchang Soft capsules	Capsules	Beijing, China	8175307 8175309 8175311	2008-01 to 2009-12 2008-01 to 2009-12 2008-01 to 2009-12
Xiaoer Ganmao Granules	Granules	Beijing, China	8112409 8112408 8112410	2008-04 to 2011-03 2008-04 to 2011-03 2008-04 to 2011-03
Xiangsha yangwei granules	Granules	Beijing, China Guangzhou, China	9115006 9115008 9115010	2009-01 to 2011-12 2009-01 to 2011-12 2009-01 to 2011-12
Guangdong Liangcha granules	Granules	Guangzhou, China	0904020 0904032 0904036	2009-04 to 2011-03 2009-04 to 2011-03 2009-04 to 2011-03
Herba Leonuri Granules	Granules	Jilin, China	090608 090808 091008	2009-06 to 2012-05 2009-08 to 2011-01 2009-10 to 2011-03
Indigowoad root Granules	Granules	Beijing, China	9114989 811529 9114994	2009-04 to 2012-03 2008-02 to 2011-03 2009-04 to 2012-03

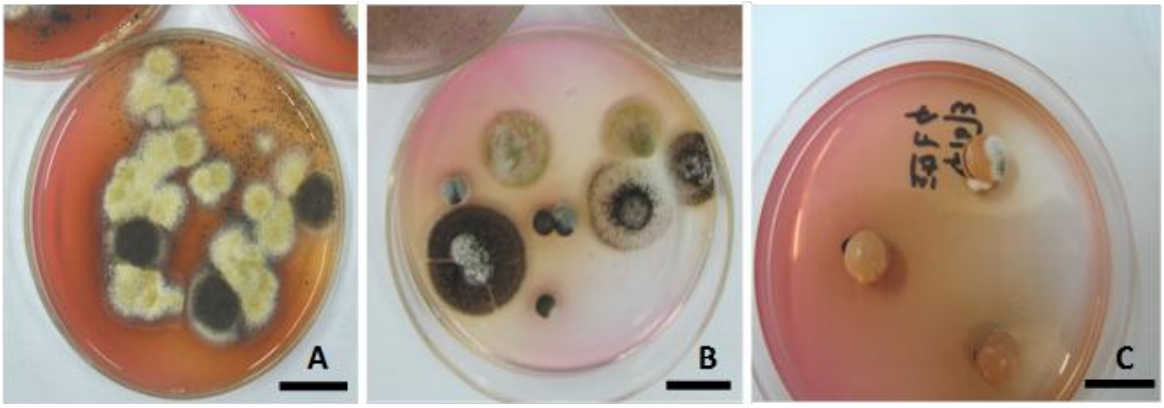

Figure 1. Fungal contamination of 3 Traditional Chinese Patent Medicines. A, *Trollius chinensis* bunge capsules; B, Yinqiao Jiedu watered pills; C, Fufang Danshen tablets; Bars A-C, 2 cm.

Table 2. Contaminated fungi isolated in 3 traditional Chinese patent medicines.

Sample	Total fungal counts (CFU/g)	Fungal species	Frequency (%)
Trollius chinensis	5 to19	*A. foetidus*	16
Bunge capsules		*P. varioti*	84
Yinqiao Jiedu watered	1 to 23	*A. flavus*	9
Pills		*A. niger*	18
		E. rubrum	23
		M. racemosus	45
		P. variable	5
Fufang Danshen tablets	0.3 to 1	*A. foetidus*	25
		P. chrysogenum	25
		P. corylophilum	50

These results indicated that along with the modernization of TCPM, sanitary condition is better than ever before. However, in order to promote the internationalization of TCPM, strict hygienic standard should be performed. Information concerning contaminated fungal count and composition in TCPM will help to improve TCPM's quality and reduce such kind of contamination.

The fungal contamination may be derived from raw herbal materials, production staff and production environment. According to TCPM, raw herbal materials were supposed to be the main source of fungal contamination. Due to the difference from western medicines, TCPM are characteristic of combined use of two or more substances with the idea of increasing or promoting therapeutic effectiveness (Zhu and Woerdenbag, 1995). The substances made into TCPM were preliminarily processed, pulverized medicinal substances or crude extracts instead of chemically defined single compound. The pulverized medicinal substances and crude extracts may contain ingredients that favor fungal invasion. In addition, some extracts could not be sterilized under high temperature to keep potency. The commonly employed sterilization process such as γ-irradiation was effective; however, this method may not be able to kill all fungi in case of improper operation.

Fungal species in YJWP were the most compare to other TCPM examined. Five (5) species including *A. flavus*, *A. niger*, *Eurotium rubrum*, *Mucor racemosus* and *P. variable* were observed. Possible reason is that it contains semen sojae preparatum (SSP), which was made from fermented soybean. According to traditional production method of SSP, soybeans were mixed with extracts of sweet wormwood herb and mulberry leaves, than they were fermented naturally until full of fungal mycelia (The Chinese Pharmacopeia, 2005). Others' research showed that fungal counts in YJWP were 90 CFU/g (Yuan et al., 2003). This problem is made worse by the fact that aflatoxin concentration detected from YJWP ranged from 29.5 to 819 ng/g (Ren et al., 1997; Li et al., 2000). According to the aforementioned findings,

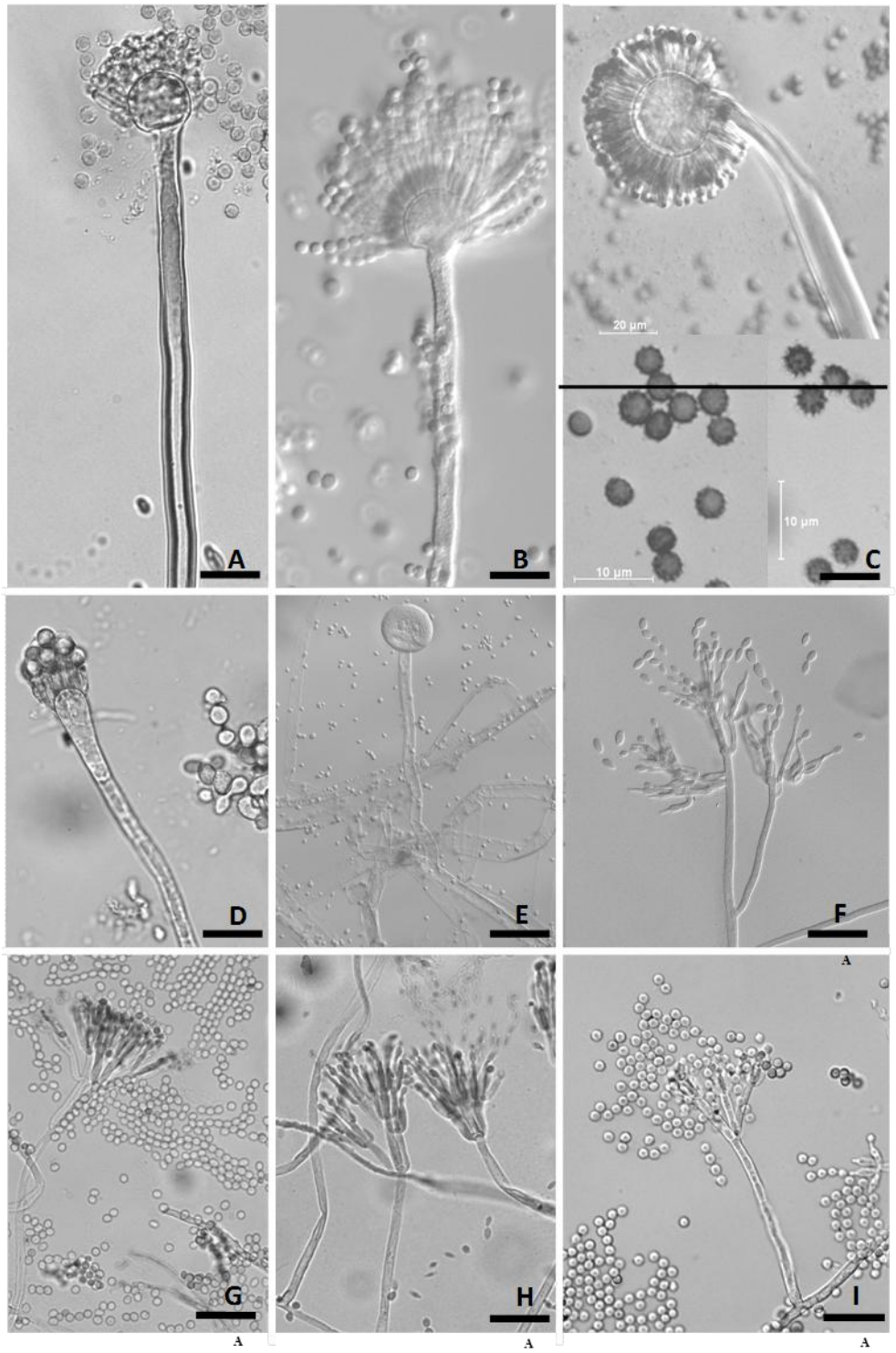

Figure 2. Morphology of contaminated fungal species. (A) *A. foetidus* ; (B) *A. flavus*; (C) *A. niger*; (D) *E. rubrum*; (E) *M. racemosus* ; (F) *P. varioti* ; (G) *P. chrysogenum*; (H) *P. variable* ; (I) *P. corylophilum*. Bars (A-C, F-I), 20 μm; (D) 10 μm; (E) 100 μm.

fungal contamination of TCPM containing fermented material demands urgent attention, because many species of *Aspergillus* and *Penicillium* were able to produce toxins such as aflatoxin and ochratoxin A (Chen et al., 2010, 2011), and small quantity of toxigenic fungi may produce enough toxin that cause health hazards. To

insure the medicine safety, standard operation should be applied in the fermentation, including pure inoculation, standardized fermentation period, humidity, temperature, and so on.

Besides toxigenic fungi, other species may also cause health hazards under special condition. For example, *Aspergillus* and *Paecilomyces* were reported to cause systemic infections in immunosuppressed patients. The *Aspergillus* species most often implicated in causing invasive disease were *Aspergillus fumigatus* and *A. flavus* (Manuel et al., 1998; Marjolein et al., 1999). *P. variotii* was one of the most common species of *P. aecilomyces* reported to cause infections. Various infections of hosts by *P. variotii* have been reported, including cellulitis, pyelonephritis, endophthalmitis, pneumonia, cerebrospinal shunt infection, prosthetic valve endocarditis and peritonitis (Wang et al., 2005). Immunosuppressed patients have high risks of systemic or localized infections including those of the gastrointestinal tract (Helderman and Goral, 2002). *A. flavus* and *P. variotii* were isolated from 67% of fungal contaminated TCPM. So, prescription of TCPM to immunosuppressed patients should be treated with cautions.

Conclusions

With comprehensive application of Chinese medicines, special attention has been paid on its safety. Fungal contamination is one of the factors threatening medicine quality and safety. This study investigated mycoflora associated with 27 commonly used TCPM. The results revealed that most TCPM were qualified in fungal counts, few were contaminated with *Aspergillus*, *Penicillum* etc. species. Raw herbal materials especially fermented materials were supposed to be the main source of fungal contamination. In order to ensure safety of TCPM, strict sterilization should be applied on raw herbal materials to reduce fungal contamination to a minimum.

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