The Presence of Aflatoxin B1 and Fungi in Traditional Drugs in Vietnam

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Abstract- To explore the presence of aflatoxin B1 (AFB1) and fungi in traditional drugs collected in Vietnam. Materials and Methods: 505 samples of 88 different traditional drugs were obtained from 10 hospitals in Nghe An, a central province of Vietnam. AFB1 contamination was determined by a high-performance liquid chromatography (HPLC) assay. Fungal contaminants were determined according to WHO regulations, and the obtained Aspergillus strains were characterized via morphological and molecular identification. Results: 24 samples (4.75% of the total samples) were contaminated with AFB1, and the average concentration was 0.062±0.030 µg/kg (ranging from 0.009 to 0.097 µg/kg). Fungal isolates were detected from 174 samples (34.45%). The genus Aspergillus was predominant (82.76% of the isolates), but Rhizopus, Alternaria, Corynespora, and yeast were also found in a few samples. Among 144 strains of Aspergillus recovered, A. niger (105 strains) was most frequently found, followed by A. tubingensis (31 strains), A. oryzae (4 strains), and A. flavus (4 strains). Conclusion: This study suggests a low risk of aflatoxin B1 exposure to consumers of traditional drugs in Vietnam.

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Keywords: Traditional drug; Mycotoxin; Aflatoxin; Fungi; Aspergillus; Vietnam

Introduction

Mycotoxins are low-molecular-weight natural compounds produced by filamentous fungi (molds) and are toxic to animals in low concentrations. Aflatoxins are a group of about 20 different mycotoxins. The four major naturally produced compounds are aflatoxin B1, B2, G1, and G2. Among them, aflatoxin B1 (AFB1) is the most toxic, carcinogenic, and mutagenic (1-3). Human exposure to aflatoxins is usually from the consumption of food or drugs that are contaminated by aflatoxin-producing molds. Ingestion of aflatoxins can result in acute or chronic toxicity, with the main target organ being the liver. Acute toxicity in humans (although less likely) can result in mortality, with the acute lethal dose for adults being approximately 10 to 20 mg of aflatoxin (4). Chronic exposure to aflatoxin can result in mutations that cause liver cancer (1). In developed countries, regulations on the levels of aflatoxin total and AFB1 in food work relatively well to protect human populations from significant aflatoxin ingestion. But in many developing countries, aflatoxin ingestion remains high, especially where similar regulations are not enforced or nonexistent (5).

Many species of the Aspergillus genus can produce aflatoxins. The most important aflatoxin-producers are A. flavus and A. parasiticus (2). Other less common species are A. bombycis, A. ochraceoroseus, A. nomius, A. pseudotamari, and A. niger (2,6). Aflatoxin - producing strains of Aspergillus generally produce 2-3 aflatoxins, one of which is always aflatoxin B1, so AFB1 is the most prevalent worldwide (2).

The aflatoxin-producing fungi can colonize a variety of natural products that are usually used as traditional (non-conventional) medicines (7,8). About 70-80% of the world's population rely on non-conventional medicine in their primary healthcare (9), so the risk of aflatoxin exposure after consumption of these drugs cannot be
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Vietnam is a developing country and traditional medicine still play an important role in the treatment of different diseases. About 75% of people use traditional drugs as their primary source of treatment (10). The ingredients for traditional medicine are derived from wholly natural sources such as plant, animal, and mineral products. About 1500 licensed and many more unlicensed remedies are used throughout the country (10). One reason for the popularity of traditional drugs is the belief that they produce few or no side effects (10). However, this kind of drugs may be contaminated with pathogens or toxins. The Vietnamese Ministry of Health has issued the National Technical Regulation on mycotoxin contamination in food, limiting the AFB1 to a maximum of 12 µg/kg (11). However, this regulation does not cover natural products used as medicine and there is a lack of awareness of the regulation among traditional medicine practitioners and consumers in Viet Nam (10). It is essential to provide the general public with adequate information to facilitate a better understanding of the potential risks associated with the use of natural products and to ensure that all medicines are safe and of adequate quality. This paper aimed at evaluating the occurrence of aflatoxin AFB1 and aflatoxin-producing molds in natural products stored in some hospitals of Nghe An, a central province of Vietnam.

Materials and Methods

Sampling

Five hundred and five samples of natural products, composed of 88 different species, were evaluated to assess the presence of aflatoxin B1 and fungal strains. The products were chosen based on their availability and popularity of use and obtained from the stores of ten hospitals in Nghe An province, Vietnam. About 30 g of each kind of medicinal product were put into two sterile polythene bags and sealed properly. These bags were labeled with the code of hospital, type of medicine, and time of sampling. One bag of each medicine was transported to the Department of Laboratory, Nghe An General Friendship Hospital for fungal culture and the other to the National Institute of Medicinal Materials to analyze aflatoxin AFB1. The detailed information of each sample was given as Additional file 1: Table S1. The samples were kept at 4°C until use and processed as soon as possible to avoid second contamination.

| Vietnamese name         | Scientific name                                      | Number of samples |
|-------------------------|------------------------------------------------------|-------------------|
| Bac ha                  | Mentha arvensis                                      | 06                |
| Bach mao can            | Rhizoma imperatae cylindricae                        | 03                |
| Ba tu nhan              | Semen platycladi orientalis                         | 11                |
| Bach phuc linh          | Poria cocos                                         | 08                |
| Bach thuoc              | Radix paeoniae lactiflorae                          | 08                |
| Bach truat              | Rhizoma atractyloides macropetalae                   | 08                |
| Ban ha                  | Rhizoma pinelliae                                    | 08                |
| Bo cot toai             | Rhizoma drynariai                                    | 03                |
| Cau dang                | Ramulus cum unco uncarii                            | 08                |
| Cau ky tu               | Fructus lycii                                       | 08                |
| Chi tu                  | Gardeniae fructus                                   | 03                |
| Cho de rang cua         | Herba Phyllanthus amari                              | 03                |
| Co xuc                  | Radix achyranthis asperae                            | 03                |
| Cot khi cu              | Radix polygoni cuspidati                             | 03                |
| Dai tao                 | Fructus zizipha jujubae                              | 08                |
| Dan sam                 | Radix salviæ miltiorrhizae                           | 08                |
| Dang sam                | Radix codonopsis                                     | 08                |
| Day dau xuong           | Caulis tinosporeae tomentosae                        | 03                |
| Dia long                | Lumbricus.                                           | 08                |
| Do trong                | Cortex eucommiae                                     | 08                |
| Duong quy               | Radix angelicæ sinensis                              | 08                |
| Plant Name                  | Latin Name                              | Quantity |
|----------------------------|-----------------------------------------|----------|
| Ha diep                    | Folium nelumbinis                       | 03       |
| Ha kho thao                | Spica prunellae                         | 08       |
| Hanh nhan                  | Semen armeniacae amarum                 | 08       |
| Hau phac                   | Syzygii cuminii                         | 03       |
| Hoang cam                  | Radix scutellariae                      | 08       |
| Hoang lien                 | Rhizoma coptidis                        | 08       |
| Hong hoa                   | Flos carthami tinctorii                | 07       |
| Hoai son                   | Dioscoreae rhizoma                     | 03       |
| Hoang ba nam               | Cortex oroxyli indici                  | 03       |
| Hoe hoa                    | Flos Styphnolobii japonici imaturi     | 03       |
| Huyen sam                  | Radix scrophulariae                    | 07       |
| Huong phu                  | Rhizoma cyperi                         | 03       |
| Huyet giac                 | Lignum dracaenae cambodianae            | 03       |
| Hy thiem thao              | Herba siegesbeckiae                    | 03       |
| Ich mau                    | Herba leonuri japonici                 | 03       |
| Ich tri rhan               | Fructus alpiniae oxyphyllae            | 07       |
| Ke noi kim                 | Endothelium Corneum Gigeriae Galli     | 03       |
| Ke dau ngua                | Fructus xanthium strumarium            | 07       |
| Khuong hoat                | Rhizoma et radix notopterygii          | 07       |
| Khuong hoang               | Rhizoma curcumae longae                | 03       |
| Kim ngan hoa               | Lonicerae Flos                          | 03       |
| Kim tien thao              | Herba Desmodii styracifolii            | 03       |
| Kinh gioi                  | Herba Elsholtziae ciliatae             | 03       |
| La khoi tia                | Folium Adisae                           | 03       |
| Lac tien                   | Herba passiflorae                      | 04       |
| Moc thong                  | Caulis clematisidis                    | 03       |
| Ma hoang                   | Herba ephedrae                         | 07       |
| Mach mon                   | Radix ophiopogonis japonici            | 07       |
| Mau don bi                 | Cortex paoniaeae suffruticosae         | 08       |
| Moc huong                  | Radix saussureae lappae                | 08       |
| Ngoc truc                  | Rhizoma polygonati odorati             | 03       |
| Nhan tran                  | Herba adenosmatis caerulei             | 03       |
| Ngu vi tu                  | Fructus schisandrae                    | 08       |
| Ngu linh chi               | Feaces trogopterum -pteropodae         | 08       |
| Nguu tat                   | Radix achyranthis bidentatae           | 08       |
| O tac cot                  | Os sepiae                              | 03       |
| Phong phong                | Radix ledebouriellae seseloidis        | 08       |
| Phuc than                  | Poria                                  | 08       |
| Que chi                    | Ramulus cinnamomii                     | 03       |
| Que tam                    | Cortex cinnamomii                      | 03       |
| Sai dat                    | Herba wedeliae                         | 03       |
| Sai ho                     | Radix bupleuri                         | 08       |
| Sinh dia                   | Radix rehmanniae glutinosae            | 08       |
| Son thu                    | Fructus corni officinalis              | 08       |
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Quantitative determination of aflatoxin B1

The HPLC assessment was performed on a Shimadzu (Kyoto, Japan) HPLC system equipped with an LC-20AD pump, SIL-20A HT autosampler, detector SPD-M20A, CTO-10AS VP column oven. All used reagents were of analytical reagent grade. AFB1 standard solution (250 ng/mL) was obtained from Rhone-diagnostics technologies (UK). Chromatographic separation was carried out on a Shim-pack GIST C18 column (250 x 4.6 mm, 5 µm). The reverse-phase HPLC assay was carried out using a C18 column with a flow rate of 1 mL/min, injection volume of 20 µL, and a column temperature of 25°C. The mobile phase was acetonitrile-phosphate, and the detection wavelength was set as 350 nm.

Evaluation of fungal contamination

The isolation and identification of fungi from herbal medicines were tested according to WHO regulations (9). Ten grams of each sample were mechanically homogenized in 90 mL 0.1% Tween-20 for 2 minutes. The mixture was filtrated through a disposable syringe with sterile cotton and then centrifuged at 2600×g for 10 min. The collected pellet was plated onto Petri dishes 9-10 cm in diameter containing Sabouraud dextrose agar (SDA) with chloramphenicol (0.1 g/L). Plates were incubated at room temperature and observed every day for a maximum of 7 days before negative results were noted. Colonies with different morphological characteristics were picked and subcultured onto fresh SDA slants to obtain pure cultures. The genus identification was based on the morphological characteristics of their colonies and spores. Those strains belonging to the Aspergillus genus were further identified into species-level followed the taxonomic schemes of Raper and Fennel (12).

To confirm the species identification, some representative strains of different Aspergillus species were subjected to molecular analysis in the laboratory of the Department of Parasitology, Vietnam Military Medical University. The fungal DNA was extracted using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp, Canada) under the manufacturer’s instructions. The primers ITS5 (13) and NL4 (5′- (14) were used to amplify a partial sequence of small subunit ribosomal RNA gene, the complete sequence of internal transcribed spacer (ITS) 1, 5.8S ribosomal RNA gene, ITS2, and partial sequence of large subunit ribosomal RNA. The yielded PCR products were sequenced, and the

| Cont. table S1 | 
|----------------|
| Tam sen | Embryo nelumbinis | 04 |
| Tan di | Flos magnoliae liliflorae | 08 |
| Tan giao | Radix gentianae macrophyllae | 08 |
| Tang ky sinh | Herba loranthi gracifolii | 08 |
| Thach vi | Herba pyroesi arae cheerei | 03 |
| Thach xuong bo | Rhizoma acori graminei | 03 |
| Thao quyet minh | Semen cassiae torae | 03 |
| Thien nien kien | Rhizoma homalomenae occulatae | 03 |
| Thuyen thoi | Pertostracum cicadidae | 03 |
| To moc | Lignum sappan | 03 |
| Trach ta | Rhizoma alismatis | 03 |
| Te tan | Herba asari | 07 |
| Thang ma | Rhizoma cimicifugae | 07 |
| Thien ma | Rhizoma gastrodiae elatae | 07 |
| Thien mon | Radix asparagi cochinchenensis | 07 |
| Tho phuc linh | Rhizoma smilacis glabrae | 07 |
| Thong thao | Medulla tetrapanacis | 08 |
| Thuoc dia | Radix rehmanniae glutinosae | 08 |
| Thuong truat | Rhizoma atractylodis lanceae | 08 |
| Ty giai | Rhizoma Dioscoreae | 08 |
| Vien chi | Radix polygalae | 08 |
| Xich thuoc | Radix paeoniae | 08 |
| Xuyen khung | Rhizoma ligustici wallichii | 08 |
obtained nucleotides were compared to database sequences in GenBank for the identification. Some of the sequences in the current study were deposited in GenBank under the code MF599709.1, MF599710.1, MF599715.1.

**Results**

During 2017, a total of 505 samples, belonging to 88 species, of traditional medicines were examined for the presence of AFB1. Among them, 24 samples (4.75%) were AFB1-contaminated with the average concentration of 0.062±0.030 µg/kg (range of 0.009-0.097 µg/kg). The contaminated samples belonged to *Rhizoma imperatae cylindricae*, *Semen platycladi orientalis*, *Radix achyranthis asperae*, *Rhizoma dioscoreae persimilis*, *Endothelium corneum gigeriae galli*, *Herba desmodii styracifolii*, *Embryo nelumbinis*, *Rhizoma acori graminei*, *Radix salviae miltiorrhizae*, *Spica prunellae*, *Fructus schisandrae*, *Radix ledebouriellae seseloidis*, and *Rhizoma ligustici wallichii* species. Among them, *Endothelium corneum gigeriae galli* (3/3 samples), *Rhizoma imperatae cylindricae*, *Semen platycladi orientalis*, *Rhizoma dioscoreae persimilis*, *Rhizoma acori graminei* (2/3 samples) had the highest frequency of AFB1-contaminated samples (Table 1).

Among 505 investigated samples in this study, 174 specimens (34.45%) contained fungal contamination. The genus *Aspergillus* was predominant among the identified fungi (82.76% of the isolates) (Table 2).

Among 144 strains of *Aspergillus* recovered, *A. niger* (105 strains) was the most frequently encountered, followed by *A. tubingensis* (31 strains), *A. oryzae* (4 strains), and *A. flavus* (4 strains) (Table 3).

| Drug name                  | n | Positive |
|----------------------------|---|----------|
| *Rhizoma imperatae cylindricae* | 03 | 2        |
| *Semen platycladi orientalis* | 03 | 2        |
| *Radix achyranthis asperae*  | 03 | 1        |
| *Rhizoma dioscoreae persimilis* | 03 | 2        |
| *Endothelium corneum gigeriae galli* | 03 | 3        |
| *Herba desmodii styracifolii* | 03 | 1        |
| *Embryo nelumbinis*         | 04 | 2        |
| *Rhizoma acori graminei*    | 03 | 2        |
| *Radix salviae miltiorrhizae* | 08 | 2        |
| *Spica prunellae*           | 08 | 1        |
| *Fructus schisandrae*       | 08 | 2        |
| *Radix ledebouriellae seseloidis* | 08 | 2        |
| *Rhizoma ligustici wallichii* | 08 | 2        |
| **Total**                   | 65 | 24       |

**Table 1. Distribution of AFB1 among traditional drugs analyzed in this study**

Mean (µg/kg) 0.062
Standard deviation (µg/kg) 0.030

(*) the species without any positive samples were not expressed here

| Fungi          | n   | Percentage (%) |
|----------------|-----|----------------|
|                |     | Among infected samples (n=174) | Among total samples (n=505) |
| *Aspergillus*  | 144 | 82.76          | 28.51          |
| *Rhizopus*     | 33  | 18.97          | 6.53           |
| *Alternaria*   | 10  | 5.75           | 1.98           |
| *Corynespora*  | 9   | 5.17           | 1.78           |
| *Yeast*        | 24  | 13.79          | 4.75           |
| **Total**      | 220 | 100.00         | 34.46          |

**Table 2. Frequency of different fungi isolated from traditional drugs**

| Aspergillus species | n   | Percentage (%) |
|---------------------|-----|----------------|
|                     |     | Among *Aspergillus* strains (n=144) | Among the total of samples (n=505) |
| *Aspergillus niger* | 105 | 72.92          | 20.79          |
| *Aspergillus tubingensis* | 31  | 21.53          | 6.14           |
| *Aspergillus oryzae* | 4   | 2.78           | 0.79           |
| *Aspergillus flavus* | 4   | 2.78           | 0.79           |

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Discussion

The presence of aflatoxin in traditional drugs

Contamination with mycotoxins, especially AFB1, in natural products used for food or medicines has been extensively investigated worldwide, but data in Vietnam is scarce, despite the climatic conditions in Vietnam being very favorable for aflatoxin-producing fungi (15). The current study was carried out to investigate the occurrence of mycotoxins and fungi, focusing on AFB1 and AFB1-producing molds to assess the risk to consumers in Vietnam.

Our findings showed that 24 out of 505 tested samples (4.75%) were contaminated with AFB1. This prevalence was lower than that (19.1%) in some agricultural products (maize, rice, peanut, and sesame) in Vietnam (15) but still showed the potential for mycotoxin contamination in traditional drugs. Other surveys carried out in some neighboring countries showed a wide range of aflatoxin incidence in traditional drugs (from 8.7% to 35% of the tested samples) (16-19). The traditional drug samples were highly contaminated (64%-70.8% of herbal drugs) in some studies (20,21) and very low or even free of contamination in others (22). The formation of mycotoxins depends on many factors such as the fungus, substrate, environmental factors, and time, which may account for such variation (23). Our result did not reveal an association between specific types of traditional drugs and AFB1 contamination, given the low frequency of positive samples (Table 1).

The range of AFB1 in the current study was 0.009-0.097 µg/kg, lower than the regulatory limit of mycotoxin in Vietnam (11). The low concentration of aflatoxin in our finding is consistent with other surveys evaluating the concentration of AFs in traditional drugs (16-17,19-20). Nevertheless, the level of AFB1 (290.80 µg/kg) was high - enough to cause serious health problems, has been reported in Chinese products (18). These findings reveal the high variation of aflatoxin in traditional drugs, so local and updated data on that issue is needed.

The presence of fungi in traditional drugs

Molds are a large group of around 100,000 species that are widely distributed in nature. Given their ability to multiply on various raw materials or under unfavorable conditions, molds are one of the most widespread environmental contaminants. The growth of molds in traditional drugs may result in drug spoilage and the production of mycotoxins. In the current study, discoloration and musty smell due to fungal growth was noted in some samples at collection time. Results of the present study show that the traditional drugs were frequently contaminated by different fungal species, however, with lower prevalence (34.45%) compared with that in some other reports (7,21,24).

The observation of Aspergillus as the most frequent contaminant is consistent with previous reports (8,24-25). Among Aspergillus strains, A. niger was the most common, followed by A. tubingensis and others. The low prevalence of A. flavus, one of the most important producers of aflatoxin (26), is in line with other studies (7,22) and may explain the low concentration of AFB1 in traditional drugs in the present study. The dominance of A. niger aligns with some previous findings (8,22,24-25,27). A. niger can grow in a wide range of temperatures (6-47°C) and pH (1.4-9.8); thus, it is ubiquitous in the natural environment (28). Although A. niger is not a major aflatoxin-producer, some isolates of A. niger can produce aflatoxin B1, B2, and G2 (6). A. tubingensis has been isolated from traditional drugs, but their production of aflatoxin has not been reported (21). A. oryzae is a species of the section Flavi (containing important aflatoxin-producing A. flavus and A. parasiticus) and economically important in food fermentation and industry (26). A. oryzae species is usually considered atoxicigenic, but the production of aflatoxin B1, B2, or G2 by some strains of A. oryzae has been reported (29,30). From the present investigation and other studies, it is conceivable that A. niger is a mold that should be considered concerning mycotoxin production and fungal contaminant. The high contamination with fungi that are capable of producing aflatoxin in traditional drugs should raise attention because mycotoxins are stable chemical compounds and cannot be destroyed during most processing operations (7).

This study suggests that natural products used for medicinal purposes in Vietnam are usually contaminated with fungi that are capable of producing mycotoxins. There appears to be a low risk of aflatoxin to consumers who occasionally use traditional drugs because no samples used in this study had levels of AF contamination above current country regulation limits. However, people who use traditional drugs more frequently or with other sources of aflatoxin may be at a higher risk of exposure. The occurrence of aflatoxin B1 in tested samples should raise public awareness of the potential health hazards associated with traditional medicines. More studies are needed to investigate the contamination of traditional drugs by mycotoxin and mycotoxin-producing fungi in more areas of Vietnam.
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