# Detection of toxigenic fungi and mycotoxins in medicinally important powdered herbal drugs

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**Citation**

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**Abstract**

This investigation was designed to through light on the microbial status of some powdered herbal materials used in Triphala preparation. A total of 68 powdered samples Emblica officinalis (Amla), Terminalia belerica (Baheda) and Terminalia chebula (Harada) were collected from random sources in Gwalior market. Mycological analysis of powdered samples was carried out for the detection and enumeration of fungi using standard media. Samples were also investigated from mycotoxilogical point of view to identify mycotoxins associated. Fungal contamination was found in almost 91% of the samples. Aspergillus and Penicillium genera were detected more frequently than other genera i.e. Helminthosporium, Curvularia, Geotrichum, Fusarium, Rhizopus, Paecilomyces. In total, 771 fungal isolates belonging to 14 fungal species and 8 different genera were recorded. Overall, six species of genus Aspergillus and three of genus Penicillium were dominant. In spite of this natural infection of powder samples, only 14 (20.58%) out of 68 samples analyzed were found to be positive for mycotoxins including aflatoxins (B1, B2, G1 and G2), citrinin and sterigmatocystin. Therefore, study concluded that, as herbal drugs seems to be high risk products, thus require designing some more appropriate methods of their decontamination.

## INTRODUCTION

The use of Ayurveda is one of the oldest, richest and most diverse traditions, associated with the use of medicinal plants in India (Tandon et al., 2004). In this system of medicine, different parts of the medicinal plants are used in crude as well as powdered form. Over 8000 plant species have been reported to prepare some 25,000 formulations to treat various ailments (Dubey, 2004). According to Ayurveda, perfect digestion is the basis of all health. Trifla churn a wonderful ayurvedic drug act as perfect tonic for proper digestion (Pandey et al., 2008). Triphala literally means “three fruits, is a combination of Emblica officinalis (Garetn.) (Amla), Terminalia bellirica (Gaertn.) Roxb. (Baheda), and Terminalia chebula (Retz.) (Harada) are medicinally important in raw and powdered form. Amla, one of the three ingredients in Triphala, is the richest known natural source of vitamin C, which helps in the production of collagen, which is necessary to keep skin supple and thick. Baheda contain tannins, sitosterol, gallic acid used mainly as tonic, laxative in cough as well as in piles and dyspepsia. Haritiki contains 24-32% tannins used to cure acidity and dysentery. Fruit decoction is used in bleeding and ulceration of the gums (Wealth of India, 1952 & 1976). Trifla has many specific effects. It is particularly rejuvenating for the digestive tract, and also cure dyspepsia, anaemia, impurity of blood, hyperlipidaemia, skin diseases, excessive heat and irritation of eyes (Juss, 1997).

As the case with other herbal drugs, raw material and powdered ingredients of trifla i.e. E. officinalis, T. bellirica and T. chebula are also subject to operations of contamination by microorganisms during growth (while the fruits are on tree), after harvesting (when fruits are dried), processing and during storage. Post-harvest spoilage by filamentous fungi is one of the most common threats associated with processed and stored herbal products. Fungal contamination of stored herbal drugs not only linked to discoloration, quality deterioration, reduction in commercial values as well as in therapeutic potential but the mycotoxin produced by them in these herbal drugs can also cause several ailments of liver, kidney, nervous system, muscular, skin, respiratory organs, digestive tract, genital organs, etc. (Muntanola, 1987; Purchase, 1974; Durakovic et al., 1989; Rai and Mehrotra 2005; Trucksessand and Scott, 2008). Mycological studies of trifla churn have shown that fungal genera Aspergillus, Penicillium, Curvularia, Helminthosporium, Chaetomium, Rhizopus, Mucor and Aflatoxin (AFB& G), Citrinin and Sterigmatocystin are natural trifla churn contaminants (Gautam and Bhadauria,

2008).

Moreover, the occurrence of mycotoxin contamination in trifla churn seems to be very inevitable. The climatic conditions of Gwalior, where the average temperature and relative humidity are relatively high beside poor conditions and prolong duration of herbal drug storage, could also promote fungi i.e Aspergillus and Pernicillium. Therefore, present study was aimed to investigate the status of mould and mycotoxins contamination in some powdered herbal drugs (in relation to possible exposure of local users to the toxins of these contaminated herbal drugs.

## MATERIALS AND METHODS

### SOURCE OF SAMPLES

A total of 68 powdered samples of E. officinalis (n=25), T. bellirica (n=22) and T. chebula (n=21) were randomly collected from different regions in Gwalior market during the year 2007-2008. The samples were transported to Mycology laboratory at School of Studies in Botany, Jiwaji University Gwalior, immediately and stored in air-tight containers at room temperature till further analysis.

### MYCOTOXINS STANDARDS

The standards of Alfatoxins $B_1$, $B_2$, $G_1$ & $G_2$ (Sigma, Chemical, St. Louis, USA) were used.

### MEASUREMENT OF MOISTURE CONTENT

Moisture content was measured prior to rinsing of raw materials in distilled water. For moisture content, weighed amount of individual samples were dried at 100 $^{O}$ C for 24 h and the difference in weight was calculated according to Essono et al. (2007).

$$MC = [(Wi - Wf)/Wi] \times 100$$

Where MC = Moisture content; Wi = Initial weight and Wf = Final weight

### MYCOFLORA ISOLATION

One gram of each powdered sample was mixed aseptically in 9 ml of sterile distilled water and shaken vigorously. Appropriate serial dilutions were made and 0.1 ml of the dilution was transferred aseptically to sterilized petri plates containing growth media. For mycobiota analysis, freshly prepared potato dextrose agar (PDA) and czapek dox agar (CZA) medium were used (Gautam and Bhadauria, 2008). Triplicate of each sample were incubated at 25±2$^{o}$C for 7 days and examined daily. The mean number of fungal colony-forming units (cfus) was recorded. After incubation, the plates were examined visually and under a microscope. Identification of fungal species was done by culture and morphological characteristics (Gilman, 1975).

### INCIDENCE OF FUNGAL SPECIES

The incidence of different fungal spp. was assessed calculating the percentage relative frequency relative density and incidence. The relative frequency is defined here as the percentage of samples within which a given species was found at least once. The relative density (%) is related to the number of isolates of genus or species was observed to occur in the samples analyzed. Their values were obtained according to Giridher and Ready (1997); Marasas et al., (1988).

### MYCOTOXIN STANDARD PREPARATION

The standard solutions were prepared by dissolving the pure aflatoxins (AfB1, AfB2, AfG1 and AfG2) in acetonitrile: water (1:1, v/v) to give concentrations of 1 mg/ml each for AfB1, AfB2, AfG1 and AfG2. The solutions were stored at 4C. Aflatoxin standards were obtained from the Sigma Chemical Co. (St Louis, MO, USA).

### DETECTION OF MYCOTOXINS

Natural occurrence mycotoxins in collected herbal drug samples were examined by thin-layer chromatography (TLC) (Singh, 1988). TLC technique was employed in 20x20 cm glass plates with silica gel (0.25mm thickness). The plates were dried in oven at 110C for about one hour and stored in dust and moisture proof containers. For mycotoxins analysis, fifty grams of each powdered samples were extracted with chloroform. About 50 µl of extracts were applied in silica gel plates with the help of micropipette, together with specific standards, developed with a mobile phase containing benzene: methanol: acetic acid (24:2:1), were observed under long wavelength UV-light at 365 nm. For mycotoxins identification, the fluoresce and Rf value of the samples spot on TLC plates were matched with the fluorescent intensity and Rf value of standards.

### STATISTICAL ANALYSIS

The analysis of data was performed with Microsoft Excel 2007 (Window XP) for Mean and standard deviation. The statistical analysis was performed using student's t-test. The p-value <0.05 was considered significant.

## RESULTS

## FUNGAL CONTAMINATION AND WATER CONTENT OF HERBAL POWDERED SAMPLES

All samples were found to have a water content ranging from 5.84±2.68 to 7.14±0.38. However, significant differences could be noted between the mean water content of the different kinds of samples. Highest mean water content was found in Amla powder samples followed by Haritiki and Baheda powder samples. The mycological examination of powdered samples revealed that 100% fruit samples of E. officinalis, T. chebula (85.71) and T. bellirica (81.11%) were found to be highly contaminated with one or another fungal species (table 1).

### Figure 1

Table 1: Description of the powdered samples used their moirture content and %age of contamination

| Powder Sample | Part used | No. of contaminated samples / No. of samples examined | %age of samples contamination | Mean Water content |
|---|---|---|---|---|
| *E. officinalis* | fruit | 25/25 | 100 | 7.14±0.38 |
| *T. bellirica* | fruit | 9/11 | 81.11 | 5.84±2.68 |
| *T.chebula* | fruit | 18/21 | 85.71 | 6.41±3.01 |

## FUNGAL MYCOFLORA

A total of 771 isolates were identified and recorded during the present study among all samples.

Fungal load of samples, determined by fungal colony count on agar plates revealed strong differences between samples. Table 2 is showing mean fungal load of the samples. E. officinalis powder sample with highest load of fungalisolates (412), while it was 208 and 151 in samples of T. bellirica and T. chebula, respectively. Total 14 fungal species belong to 8 different genera from 95 powdered samples were obtained on potato dextrose agar (PDA) and czapek dox agar (CZA) medium. The predominant mycoflora obtained was distributed in six different genera comprising of Aspergillus with maximun five species, covering 35.71% of the total species isolated followed by Penicillium (21.42%) and other like Helminthosporium, Curvularia, Geotrichum, Fusarium, Rhizopus and Paecilomyces with single species contributed 7.14% each. Some unknown fungal genera yet to be identified were also isolated in during this investigation. The broadest spectra of fungal genera and species were recorded in samples of E. officinalis (8 genera and 14 species) followed by T. bellirica (6 and 11) and least (3 and 7) in T. chebula (table 2-4). The genus Aspergillus was found to be the most dominant genus encountered, with five species viz. A. flavus, A. niger, A. parasiticus. A. fumigates and A. versicolor while only three species viz. P. Rubrum, P. Citrinum and P. viridicatum were found in all the samples.

### Figure 2

Table 2: Distribution of mycoflora in powdered samples

| | Fungi isolated | *E. officinalis* | *T. bellirica* | *T. chebula* | Total isolates | Relative Density (%) |
|---|---|---|---|---|---|---|
| Mean fungal load ($\times 10^3$CFU/g) | *A.n.* | 116 | 121 | 13 | 250 | 32.42 |
| | *A. fl.* | 36 | 5 | 40 | 81 | 10.50 |
| | *A.p* | 38 | 27 | - | 65 | 8.43 |
| | *A. f* | 55 | 2 | 48 | 105 | 13.61 |
| | *A. v* | 38 | 1 | 1 | 40 | 5.18 |
| | *P.r.* | 39 | 24 | 22 | 85 | 11.02 |
| | *P.c.* | 48 | 13 | 25 | 86 | 11.15 |
| | *P.v.* | 4 | - | - | 4 | 0.51 |
| | *He.* | 1 | 5 | - | 6 | 0.77 |
| | *Cu.* | 1 | 2 | - | 3 | 0.38 |
| | *Ge.* | 1 | - | - | 1 | 0.12 |
| | *Fu.* | 3 | 2 | - | 5 | 0.64 |
| | *Rh.* | 6 | - | - | 6 | 0.77 |
| | *Pec.* | 24 | 4 | - | 28 | 3.63 |
| | *UN* | 2 | 2 | 2 | 6 | 0.77 |
| | Total Isolates | 412 | 208 | 151 | 771 | |
| | Total Species | 14 | 11 | 8 | - | |

Fungal species are *A. n. – Aspergillus niger; A. fl. – Aspergillus flavus; A. p. – Aspergillus parasiticus; A. f.- Aspergillus fumigatus ; A. v.- Aspergillus versicolor; P. r. – Penicillium rubrum; P. c. – Penicillium citrinum; P.v.- Penicillium viridicatum; He.- Helminthoaporium; Cu.- Curvularia; Ge.- Geotrichum; Fu.- Fusarium; Rh.- Rhizopus; Pec. –Paecilomyces and UN- Unknown.*

## OCCURRENCE AND INCIDENCE OF FUNGI IN POWDERED HERBAL DRUGS

Relative density value (%) was estimated to determine the abundance of fungal isolates of given species among all samples (Table 2). The highest percentage relative density was shown by Aspergillus species. Among all the five Aspergillus species, A. niger was most common and isolated with higher density (32.42%). Other fungal species like A. fumigates, P. Citrinum, P. Rubrum, A. flavus, A. parasiticus and A. versicolor were recorded with moderate density i.e. 13.61%, 11.15%, 11.02%, 10.50%, 8.43% and 5.18% respectively. The remaining fungal species were isolated in low density. The occurrence value varied from as low as 0.12% for Geotrichum to as high as 3.63% for Paecilomyces.

Like in case of relative density, the highest relative frequency occurrence was recovered in Aspergillus niger (59.81%) followed by A. fumigates (45%), Paecilomyces (30%). The relative frequency of A. niger is significantly higher ($p < 0.001$) than other fungal species. Similarly, the occurrence of A. flavus is also significant ($p< 0.001$) except for P. rubrum ($p= 0.262$) and P citrinum ($p= 0.360$). Fungal species like A. flavus, A. versicolor, P. citrinum and P. rubrum were also recorded with high frequency of 25.55%, 23.75%, 23.42% and 22.96%, respectively. However, the occurrence frequency of P. rubrum over P. citrinum was not

significant (p= 0.722). The relative frequency of remaining fungi were found comparatively low in the range of 3.33-14.44%.

### Figure 3

Table 2: Relative frequency (%) of fungi in positive infected fruit samples

| | %age relative frequency | | | |
|---|---|---|---|---|
| **Fungal isolates** | ***E. officinalis*** | ***T. bellirica*** | ***T. chebula*** | **Mean±SD** |
| *A.niger* | 76.66±4.71 | 66.665±47.14281 | 36.11±19.64 | 59.81±21.12504 |
| *A.flavus* | 40±28.28 | 28.33±16.50387 | 8.33±8.33 | 25.55±16.01654 |
| *A.parasiticus* | 10±0 | 16.665±16.665 | Nd | 13.33±4.709331 |
| *A. fumigatus* | 55±63.63 | 30±30 | 50±50 | 45±13.22876 |
| *A. versicolor* | 43.33±32.99 | Nd | 4.16±4.16 | 23.75±27.69949 |
| *P. rubrum* | 30±14.14 | 16.665±16.665 | 22.22±22.22 | 22.96±6.700716 |
| *P.citrinum* | 26.66±18.85 | 28.33±16.50387 | 15.27±9.82 | 23.42±7.107327 |
| *P. viridicatum* | 11.65±2.35 | Nd | Nd | 11.65±0.00 |
| *Helminthosporium* | 5.0±0 | 8.33±8.33 | Nd | 6.665±2.354666 |
| *Curvularia* | 6.66±0 | 8.33±8.33 | Nd | 7.495±1.180868 |
| *Geotrichum* | 3.33±0 | Nd | Nd | 3.33±0.00 |
| *Fusarium* | 6.66±0 | 28.33±16.50387 | 8.33±8.33 | 14.44±12.06207 |
| *Rhizopus* | 5.0±0 | Nd | Nd | 5.0±0.00 |
| *Pecleomysis* | 13.33±0 | 48.33±44.78814 | Nd | 30.83±24.74874 |
| *UN* | 13.33±0 | 8.33±8.33 | 20.33±29.45 | 13.99±6.027714 |

Nd= Not detected; UN= Unknknown.

Based on incidences of occurrence, Aspergillus species were most frequent (78.70%) followed by Penicillium species (44.27%), Pecleomyces (28.32%) and Rhizopus species (20%), which were observed in almost all the samples. The incidences of Aspergillus and Penicillim were significantly higher ($p<0.001$) than Helminthosporium over curvularia ($p=0.018$) and Fusarium ($p=0.001$). The incidence of other genera like Fusarium, Curvularia, Helminthosporium and Geotrichum were observed to be less than 20% (table 3).

### Figure 4

Table 3: Percentage incidence of fungal genera in herbal powdered samples

| | %age relative incidence | | | |
|---|---|---|---|---|
| Fungal genera | ***E. officinalis*** | ***T. bellirica*** | ***T. chebula*** | **Overal Mean±SD** |
| *Aspergillus* | 91.665 | 83.33 | 61.11 | 78.70±15.79 |
| *Penicillium* | 60.33 | 35 | 37.495 | 44.27±13.95 |
| *Helminthisporium* | 10 | 16.66 | Nd | 13.33±4.703 |
| *Curvularia* | 13.33 | 16.66 | Nd | 14.99±2.35 |
| *Geotrichum* | 6.66 | Nd | Nd | 6.66±0 |
| *Fusarium* | 13.33 | 16.66 | 16.66 | 15.55±1.92 |
| *Rhizopus* | 20 | Nd | Nd | 20±0 |
| *Pecleomyces* | 26.66 | 16.66 | Nd | 21.66±7.071 |
| *UN* | 26.66 | 16.66 | 41.66 | 28.32±12.58 |

## MYCOTOXIN CONTAMINATION OF SAMPLES

It is known that, under favourable conditions, some fungi can synthesize orally toxic metabolites – mycotoxins. Hence in our present study, we paid our attention to herbal powdered samples contamination with mycotoxins also along with mould contamination. A total of ninety six powdered samples were analysed for mycotoxin detection through thin layer chromatography (TLC). No significant mycotoxin contamination was recorded in the analysed powdered samples. Only 8 (14.03%) out of 96 samples analyzed were found positive for mycotoxins.

### Figure 5

Table 4: Number of samples contaminated with different mycotoxins

| Samples | AfB1 | AfB2 | AfG1 | AfG2 | Citrinin | Sterig. | Total |
|---|---|---|---|---|---|---|---|
| *E. officinalis* | 2 | Nd | 2 | 2 | Nd | 1 | 7(25) |
| *T. bellirica* | Nd | 2 | Nd | Nd | 2 | Nd | 4(22) |
| *T. chebula* | Nd | Nd | 1 | 1 | 1 | Nd | 3(21) |
| Total | 2 | 2 | 3 | 3 | 3 | 1 | 14(68) |
| Fq (%) | 14.28 | 14.28 | 21.42 | 21.42 | 21.42 | 7.14 | |

Af= aflatoxin; Sterig. = sterigmatocystin; Fq= frequency; Nd= not detected.

Total six mycotoxins namely aflatoxin B1 & B2, aflatoxin G1 & G2, citrinin and sterigmatocystin were detected during mycotoxins analysis of powdered samples. Table 4 showing the number of samples contaminated and percentage frequency of mycotoxins occurrence in different powdered samples. Among the six mycotoxins detected, more frequently mycotoxins were aflatoxins G1, G2 & Citrinin (21.42% each) followed by AfB1 & B2 (14.28 each) and then sterigmatocystin with 7.14% frequency of occurrence. Maximum numbers of contaminated samples (7/25) as well as mycotoxins (4/6) were found in Emblica powder samples. In T. bellirica the number of contaminated samples and mycotoxins was recorded to be four, while it was three in T. chebula powder samples. These results represent a considerable health risk for consumers of these herbal powders as drug, especially of trifla churn which is usually prepared by the mixture of these three ingredients.

## DISCUSSION

The presence of higher fungal counts and high moisture contents in powder E. officinalis samples justify favourable impact of high moisture and temperature on the fungal growth stored herbal drugs (Roy, 1989; Halt, 1998). Diverse abiotic factors operating in the processing and storage conditions as well as chemicals constituents of the herbal drugs might have resulted in variation in mycopopulation in different substrates (Chourasia et al., 2008). Although, the moisture content in powder samples of T. chebula is high but the fungal load is low as compared to T. bellirica perhaps due to its greater antifungal activity (Dutta et al., 1998; Ray and Majumdar, 1976; Chattopadhyay and Bhattacharyya, 2007). During the survey for sample collection, it was found that necessary precautions were not taken during processing, packing and storage of these herbal drugs. At some places

these herbal products were prepared under open environment under unhygienic conditions. Some of the collected samples were packed in transparent polythene bags rather than in air tight containers. All these practices may contaminate these products by exposing them to microbial infection.

Species of Aspergillus and Penicillium dominate the mycoflora of collected powdered samples of of E. officinalis, T. bellirica and T. chebula, were already reported as dominating mycobiota of stored herbal drugs (Hitokoto et al., 1978; Ayres et al., 1980; Aziz et al., 1998; Arab et al., 1999; Elshafie et al., 1999; Mandeel, 2005). Isolation of toxigenic Penicillim species (P. Rubrum and P. citrinum) is also of great concern due to mycotoxic effects of their secondary metabolites. P. citrinum is a mesophilic species known to produce citrinin, a renal toxin causes diarrhoea, increased food consumption and reduces weight due to kidney degradation in chickens. Rubra toxin produced by P. rubrum causes abdominal pain, jaundice, convulsions (Banu, 2005). A. fumigates, major cause of both invasive and allergic aspergillosis affects individuals who are immune compromised (Doory et al., 1984; Morey et al., 1993). The presence of A. fumigates was also found in the powdered herbal drugs analysed in present study. Hence, the presence of wide range of fungi in these medicinally important herbal drugs showed that there is a potential risk for mycotoxins contamination, especially during prolonged storage in poor conditions without temperature and moisture control (Efuntoye 2004; Bugno et al., 2006; Singh et al., 2008).

Most of the identified fungal species like Aspergillus, Penicillium and Alternaria are reported to be known producer of mycotoxins like aflatoxins, ochratoxins and citrinine (Bugno et al., 2006, Aziz et al., 1998, Hitokoto et al., 1978). The isolation of such toxic mycoflora form powdered samples, which are used as herbal drugs by consumers, should be taken seriously. The growth of mycoflora on the stored herbal drugs can lead to the contamination with mycotoxins, which can pose potential health risks (Dereje et al., 2009). The potential risk of A. niger in stored herbal drugs due to mycotoxin production should also be considered, because studies have shown that occasional isolates of A. niger can produce ochratoxin A and Fumonisin B2 (Abraca et al., 1994, Noonimabc et al.,2009). The presence of A. flavus in the powdered samples is considerable since this fungus initially colonizes the substrate and predisposes the infected substrate due to aflatoxins B1 and B2 production (Diener et al., 1987). Aflatoxins are highly toxic, mutagenic, carcinogenic and teratogenic metabolites produced mainly by A. flavus and have been implicated as causative agents in human hepatic and extrahepatic carcinomas. Aflatoxin B1 has been evaluated as a Group 1 carcinogen, that is, 'Carcinogenic to humans' (Shephard, 2008). Detection of AfB1, AfG1 & G2 in E. officinalis samples revealed their susceptibility to these mycotoxins. High frequency of occurrence of A. versicolor also makes a point of attention because important mycotoxin (sterigmatocystin) is produced by them which is a liver carcinogen and has immunosuppressive effects (Vesonder and Horn, 1985). Aflatoxin contamination of common medicinal plants (Roy et al., 1988), mould profile and mycotoxin contamination in crude samples of drug plants (Choursia, 1990), mycotoxin contamination of some herbal drugs curing kidney diseases (Kumar and Roy, 1994) have also been reported. The presence of wide range of fungi in these medicinally important herbal drugs showed that there is a potential risk for mycotoxins contamination, especially during prolonged storage in poor conditions without temperature and moisture control (Bugno et al., 2006; Singh et al., 2008).

## CONCLUSION

In the present investigation, although a considerable mould contamination was detected but mycotoxins contamination was detected only in 14.03% powdered samples suggesting good storage conditions of herbal powder samples. This is because the mould presence itself does not imply that the stored material is also contaminated with mycotoxins. Certain mycotoxin-producing fungi can be present without mycotoxins being present itself (Brodnik et al., 1977; Stojanovic et al., 1977). But, the prolong storage of these herbal drugs with isolated toxigenic fungal population may contaminate with mycotoxins. Therefore, these powdered herbal drugs should be carefully stored and the growth of naturally found toxic fungi should be inhibited. Besides that, these herbal drugs must be tested for mycotoxins presence prior to their use.

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