Mycoflora and aflatoxin assessment of crude herbal drugs during storage in Haridwar, Uttarakhand, India

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ABSTRACT: The present paper explores the association of mycoflora and aflatoxins with stored crude herbal drugs that are most commonly used in ayurvedic preparations. Total of 10 drug samples collected from different locations of Haridwar were analysed for mold profile, aflatoxins and microbial load. All the samples were found contaminated with one or more fungal genera such as Alternaria, Aspergillus, Fusarium, Chaetomium, Cladosporium, Penicillium, Mucor, Rhizopus, Rhizoctonia and Verticillium. Among all the fungal isolates, Aspergillus flavus (23.60%) was isolated with highest frequency followed by A. niger (19.20%). Microbial load of all the samples was found beyond WHO limits. However, specific pathogenic bacteria Escherichia coli and Salmonella sp. were not reported during the present investigation. After aflatoxins analysis, presence of aflatoxin B1 (AFB1) was proved in two samples. The present study suggests that detection of fungi and aflatoxins poses a risk for consumer’s health and it is necessary to check the herbal drugs before allowing distribution for public use.

Key words: Aflatoxin, Aspergillus, crude herbal drugs, fungal contamination

The use of herbal medicines in human health care has been in vogue since antiquity in both developed and developing countries because of better cultural acceptability and compatibility with the human body and its lesser side effect as compare to synthetic Drug (Kamboj, 2000). According to the World Health Organization (WHO) about 70 -80% of world population particularly in developing countries still rely on traditional medicines for their primary health care (Akerele, 1993). Over 8000 plant species have been reported to prepare some 25,000 formulations, to treat various ailments (Dubey et al., 2004). Commercially, 30% of world wide sale of drug is based on plant derived medicines which worth about US $21.2 billion a year and by the year 2050 it would be about US $ 5 trillion (Aneesh et al., 2009).

WHO has listed about 20,000 plant species in the world which are used in manufacturing different medicines. In India, over 6000 plants are in use in traditional, folk and herbal medicine, representing about 75 % of the medicinal needs of the third world countries (Rajshekharan, 2002 and Sharma et al., 2008) and there are over 2500 plant species in India having documented medicinal value (Tewari et al., 2012). WHO has also recognized the importance of traditional medicine and has been active in creating strategies, guidelines and standards for herbal medicine, which provide a detailed description of the techniques and measures required for the appropriate cultivation and collection of medicinal plants. Despite such guidelines, there is still a lacuna between this available knowledge and implementation, because producers, handlers and processors of herbal drugs are not much aware of WHO’s guidelines and they continue their work as before without any quality control measures which results in inferior quality of herbal drugs with lots of contaminants like heavy metals, pesticides and microbes. The unscientific methods of harvesting, collection, storage of raw materials, post harvest processing, transport and storage of herbal drugs in unhygienic conditions, are the main causes considered to make both, raw materials as well as herbal drugs prone to microbial infections leading to deterioration in safety and quality and can also cause health hazard to consumer inspite to cure the disease.

The fungal contaminates has been reported to affect the chemical composition of the raw materials and thereby, decreases the medicinal potency of the herbal drugs (Roy, 2003), whereas mycotoxins produced by these fungal contaminants causes several ailments of liver, kidney, nervous system, muscular, skin, respiratory organs, digestive tract, genital organs etc (Muntanola, 1987; Durakovic et al., 1989). Mycotoxin contamination beyond the WHO permissible limits in some cases has been observed earlier in a study at National Botanical Reasearch Institue (NBRI), Luckow (Rai and Mehrotra, 2005). The present study is an effort to identify the fungal and mycotoxin contaminants of crude herbal drugs that are most commonly used in the manufacture of various commercial drugs and which can be toxic to human health, if any.

MATERIALS AND METHODS

Sample collection

A total of 10 sun dried one year stored crude drug samples (100g), each of Emblica officinalis (amla), Terminalia chebula (harar), Terminalia belerica (bahera), Zingiber officinalis (sonth), Piper nigrum (kali mirch), Cinnamomum tamala (tejpatta), Piper longum (pippali),...
*Withania somnifera* (ashwagandha), *Asparagus racemosus* (shatavari) and *Chlorophytum borivilianum* (safed musli) were randomly collected from different drug dealers and pharmacies of Haridwar during December, 2012 to January 2013 in sterilized polyethylene bags and transported to laboratory. The moisture content of all the collected samples was determined by digital moisture meter and the samples were stored in cool place for further mycological analysis. Half of the quantity of each sample (50g) was ground into coarse powder with the help of a mechanical grinder and the powdered samples were also stored in air-tight containers at room temperature till further analysis. The pH value of 10% aqueous suspension of each sample was measured using a digital pH meter.

**Mycological analysis of crude samples**

The crude samples were screened for the association of mycoflora using agar plate method. Small pieces of each crude sample was surface sterilized with 2% Sodium hypochlorite solution (NaOCl) for one minutes, washed thoroughly with three changes of sterile distilled water, blotter dried and placed at equidistance in each plate containing potato dextrose agar medium. The plates were incubated at 28 ± 1°C for 7 days. Identification of fungal species was done on the basis of cultural and morphological characteristics (Ananthanarayan and Paniker, 1999; Gilman, 2001) and pure culture of some isolates used for aflatoxin study was maintained by following single spore isolation method. Percentage of incidence of individual fungus was determined using the formula suggested by Giridher and Ready (1997).

**Mycological analysis of powdered samples**

One gram of each powdered sample was mixed aseptically in 9 ml of sterile distilled water and shaken vigorously. Appropriate tenfold serial dilution was made and 1 ml of the dilution was transferred aseptically to sterilized Petri plate, containing potato dextrose agar media. Triplicate of each sample were incubated at 28 ± 1°C for 7 days and microbial load (total bacterial count, total yeast and mould count and specific pathogenic bacteria) was recorded in term of mean number of colony-forming units (cfu) as per prescribed WHO protocol (Ananymous, 1998).

**Detection of natural occurrence of aflatoxin**

Natural occurrence of aflatoxins in collected crude samples was evaluated by thin-layer chromatography (TLC). For aflatoxin analysis, five gram of each powdered sample was extracted with 50 ml chloroform (Singh 1988). Fifty µl chloroform extract of each sample was spotted on TLC aluminium plates coated with silica gel (0.25mm thickness) with the help of micropipette, together with specific standards (Sigma, Chemical, St. Louis, USA). 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 and the solutions were stored at 4°C. The chromatogram was developed at room temperature, in TLC chamber containing a solvent system composed of chloroform and acetone (9:1). Visualization was performed under long wavelength UV light at 365 nm. Qualitative detection of aflatoxins was done on the basis of their fluorescence and retention factor (RF) values (Scott et al., 1970).

**RESULTS**

**Moisutre content**

All samples were found to have a moisture content ranging from 1.32 to 12.03% (Table 1). The highest (12.03%) percent moisture content was recorded in ashwagandha roots samples followed by amla fruits (11.69%), tejpatta leaf (10.16 %) and bahera fruits (8.23%) samples, while it was minimum (1.32%) in sonth rhizomes followed by shatavari roots (1.46 %) and safed musli roots (1.53%).

**Mycological analysis**

The present result with crude herbal drug samples suggests that none of the drug sample was found to be free from fungal contamination, although their incidence varied with the drug samples (Table 2). Bahera was found

<table>
<thead>
<tr>
<th>Samples</th>
<th>Part used</th>
<th>botanical name</th>
<th>Moisture content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amla</td>
<td>Fruits</td>
<td>Emblica officinalis</td>
<td>11.69</td>
</tr>
<tr>
<td>Harar</td>
<td>Fruits</td>
<td>Terminalia chebula</td>
<td>8.23</td>
</tr>
<tr>
<td>Bahera</td>
<td>Fruits</td>
<td>Terminalia belerica</td>
<td>9.52</td>
</tr>
<tr>
<td>Sonth</td>
<td>Rhizomes</td>
<td>Zingiber officinalis</td>
<td>1.32</td>
</tr>
<tr>
<td>Kali mirch</td>
<td>Fruits</td>
<td>Piper nigrum</td>
<td>2.88</td>
</tr>
<tr>
<td>Tej patta</td>
<td>Leaves</td>
<td>Cinnamomum tamala</td>
<td>10.16</td>
</tr>
<tr>
<td>Pippali</td>
<td>Fruits</td>
<td>Piper longum</td>
<td>6.22</td>
</tr>
<tr>
<td>Ashwagandha</td>
<td>Roots</td>
<td>Withania somnifera</td>
<td>12.03</td>
</tr>
<tr>
<td>Shatavari</td>
<td>Roots</td>
<td>Asparagus racemosus</td>
<td>1.46</td>
</tr>
<tr>
<td>Safed musli</td>
<td>Roots</td>
<td>Chlorophytum borivilianum</td>
<td>1.53</td>
</tr>
</tbody>
</table>
to be the most contaminated samples (100 %) followed by amla, sonth, ashwagandha (96% each) and tejpatta (92%). Kali mirch was found to have the lowest frequency of fungal contamination (64%). A total of eleven fungal genera belonging to twenty two species were identified from crude samples. Aspergillus was the most dominant genus recovered from 90% of the samples followed by Penicillium (50%), Alternaria and Fusarium (40% each), Rhizopus (30%), Chaetomium, Curvularia and Mucor (20% each), Cladosporium, Rhizoctonia and Verticillium (10% each). Aspergillus flavus (23.60%) was found to be the most frequently occurring fungal species isolated from all the samples except bahera and kali mirch. Aspergillus niger (19.20%) was the second dominant fungal species isolated from bahera, sonth, tejpatta, pippali, ashwagandha and safed musli followed by Penicillium oxysporum (7.60%) isolated from harad, sonth, pippali and shatavari and Fusarium oxysporum (5.60%) isolated from ashwagandha and safed musli. Rhizopus arrhizus (4.80%) was isolated from kali mirch. Other fungal species isolated from different samples were found to occur with less frequency (Table 2). Some sterile mycelia were also isolated during this investigation.

The powdered samples were analysed for microbial load (total bacterial count, total yeast and mould count and specific pathogenic bacteria) as per WHO guidelines. The results showed (Table 3) that total yeast and mould count in all the samples was beyond the range of WHO norms except in kali mirch in which no yeast and mould count was recorded. Total aerobic bacterial load was also beyond limit in all the samples. However, the specific pathogenic bacteria E.coli and Salmonella sp. were absent in all the samples.

The hydrogen ion concentration (pH) of samples falls in the magnitude of acidic condition. In all the samples the pH ranged from 3.17- 6.84 pH (Table 3). Among all the samples the highest pH level was observed in safed musli (6.84), followed by shatavari (6.65), tejpatta (6.66) and kali mirch (6.26). The lowest pH level was observed in amla (3.17).

Natural occurrence of aflatoxin

Most of the identified fungal species like Aspergillus, Penicillium and Alternaria are reported to have ability to produce mycotoxins like aflatoxins, ochratoxins and citrinine (Aziz et al., 1998). In present study, Aspergillus flavus and A. niger were predominantly isolated from the samples. Hence attention was also given for the natural occurrence of aflatoxin in herbal powdered samples.
during their storage. No significant aflatoxin contamination was recorded in the analysed powdered samples. Only two (20%) out of 10 samples analyzed were found contaminated with aflatoxins. On the basis of their fluorescence and retention factor (Rf) values, Aflatoxin B1 (AFB1) was identified in both the contaminated samples, which produce an intense blue fluorescence visible when exposed to long-wavelength (365 nm) ultraviolet light (Scott et al., 1970). Retention factor (Rf) values and fluorescence colour of samples and corresponding aflatoxin standards (Table 4).

### Table 4. Colour of fluorescence and retention factor (Rf) values of samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Rf values of samples</th>
<th>Fluorescence colour</th>
<th>Rf values of aflatoxin standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amla</td>
<td>0.74</td>
<td>Blue</td>
<td>0.76 (B1)</td>
</tr>
<tr>
<td>Harar</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bahera</td>
<td>0.23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sonth</td>
<td>0.36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kali mirch</td>
<td>0.45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tej patta</td>
<td>0.45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pippali</td>
<td>0.70</td>
<td>Blue</td>
<td>0.76 (B1)</td>
</tr>
<tr>
<td>Ashwagandha</td>
<td>0.19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shatavari</td>
<td>0.23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Safed musli</td>
<td>0.30</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(-) Lack of blue/green fluorescence and Rf values of standards are not comparable to samples.

### DISCUSSION

The presence of high moisture content along with higher fungal counts in amla, bahera, tejpatta and ashwagandha samples justify favourable impact of high moisture and temperature on the fungal growth of stored herbal drugs (Hilt, 1998). Although, the moisture content of sonth is low but the fungal count is high, it may be due to improper harvesting and storage leading to fungal contamination. Species of *Aspergillus* and *Penicillium* dominating the mycoflora of stored herbal drugs with acidic pH level were already reported earlier (Aziz et al., 1998; Arab et al., 1999; Mandeel, 2005). The present investigation indicates that the contamination of crude herbal drugs with *Aspergillus, Penicillium, Fusarium and Alternaria* species is cosmopolitan in Haridwar region. This might be due to unavailability of proper harvesting and storage facility without temperature and moisture control, which expose them to microbial infection. Therefore, it is essential to scrutinize these herbal raw materials before processing for the presence of contaminants and only the raw materials of best quality should be allowed to use for the preparation of herbal drugs. Moreover, after processing these herbal drugs should also be tested for presence of toxigenic moulds and mycotoxins in order to reduce the risk for consumer’s health.

Presence of *A. flavus* is of main concern because fungus is a potent aflatoxin producer. The aflatoxins are reported to be carcinogenic, hepatogenic, nephrogenic and cause various nervous disorders (Shephard, 2008). In the present investigation, although a considerable mould contamination was detected but aflatoxins contamination was detected only in 20% powdered samples. However, the presence of wide range of fungal species in these medicinally important herbal crude drugs showed that there is a potential risk for mycotoxins contamination, especially during prolonged storage in poor conditions without temperature and moisture control. Several reports are available on aflatoxins contaminating raw materials of plant origin (Gautam and Bhadauria, 2008; Sareen et al., 2010) and herbal drugs (Khan et al., 2006; Okuniola et al., 2007; Enayatifard et al., 2010) which support the detection of aflatoxins in analysed samples during present investigation.

The potential risk of *A. niger* in stored herbal drugs should also be considered, because studies have shown that occasional isolates of *A. niger* can produce ochratoxin A and Fumonisins B2 (Abraca et al., 1994;
Noonimabc et al., 2009. Species of Aspergillus niger dominate the mycoflora of bahera was already reported as dominating mycobiont of stored herbal drugs (Arab et al., 1999). The presence of A. fumigatus was also found in the powdered herbal drugs analysed in present study. Therefore, there is an urgent need to prevent the entrance of such contaminated crude drug into direct use. To meet this demand, people should have to be aware of these contaminated herbal drugs and use the processed trade mark drug and should take advantage of modern storage system that improves the quality of crude herbal drug and decreases the probability of fungal contamination.

REFERENCES


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