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Detection of Aflatoxigenic *Aspergillus flavus* Isolates in Raw Materials of Some Indian Medicinal Plants

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A B S T R A C T

Raw materials of six medicinal plant species viz. *Terminalia arjuna* (Roxb. ex DC), *Acorus calamus* (L.), *Rauvolfia serpentina* (L.), *Holarrhena antidysenterica* (Roth.), *Withania somnifera* (L.) and *Boerhaavia diffusa* (L.) procured from herbal drug market were subjected to their mould profile. During mycoflora analysis, 906 fungal isolates were recorded from the raw materials. The least number of fungal isolates (96) were detected from *T. arjuna* while highest (217) from *B. diffusa*. The genus *Aspergillus* was found to be most dominant encountered in all the samples, followed by *Cladosporium herbarum*, *Penicillium italicum* and *Trichoderma viride*. The highest percent relative density was recorded in case of *Aspergillus flavus* (37.19) followed by *A. niger* (23.84) and *C. herbarum* (11.81) while the lowest was found in case of *A. nidulans* (0.55). Some of the *A. flavus* isolates were toxigenic secreting aflatoxin B_1. Highest content of aflatoxin B_1 (272.46 µg/kg) was recorded by *A. flavus* isolated from *B. diffusa* while least aflatoxin B_1 production (194.62 µg/kg) from *H. antidysenterica*. The survey reveals that the contamination of herbal drugs with storage fungi and mycotoxin is alarming and appropriate quality control measures should be taken urgently.

Introduction

Herbal drugs are used by nearly 75-80% population in the world for primary health care. Besides their high demand in Asian countries, European people also prefer herbal drugs in curing various sufferings due to their no or minor side effects as compared to modern medicines (Ekor, 2013; Gunjan *et al.*, 2016). Like food commodities, other agricultural products and herbal drugs are also susceptible to fungal spoilage and toxin production (Chen *et al.*, 2020). Different mycological problems with raw materials of herbal drugs viz. association of mycoflora with crude herbal drugs under storage, their role on biodeterioration and mycotoxin elaboration have drawn worldwide attention for quality maintenance and therapeutic potentials of plant drugs (Altyn and Twaruzek, 2020; Pallares *et al.*, 2022). India is one of the largest producers of traditional medicines comprising nearly 8,000 medicinal plants. Tropical

conditions i.e. hot and humid climate of the country is very conducive for growth, development and multiplication of mycoflora. The fungal contamination affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs (Singh *et al.,* 2022). There are reports on export of aflatoxin contaminated herbal raw materials from India and some of the samples have been reported to contain aflatoxin B_1 more than 20 μg/kg, the tolerance level fixed by WHO (Rajeshwari and Raveesha, 2016). Mycotoxins are thermostable in nature (Kabak, 2009) and cannot be completely degraded during drug formulation.

Fungal infestations and mycotoxin elaboration in various agricultural commodities has been studied by several workers (Balendres *et al.,* 2019; Kumar *et al.,* 2021). However, proper attention has not been paid towards fungal contamination of herbal raw materials. In the present piece of work we attempt to report the fungi associated with raw materials of medicinal plants viz. arjun tree (*Terminalia arjuna* Roxb.ex DC), sweet flag (*Acorus calamus* L.), serpentine (*Rauvolfia serpentine* L.), conessi (*Holarrhena antidysenterica* Roth.), winter cherry (*Withania somnifera* L.) and hog weed (*Boerhaavia diffusa* L.) to analyze the incidence of aflatoxin B_1 producing isolates of *Aspergillus flavus.*

Materials and Methods

Herbal drug collection and preparation

Raw materials of some selected herbal drug samples namely *T. arjuna, A. calamus, R. serpentina, H. antidysenterica, W. somnifera* and *B. diffusa* were collected from various shopkeepers of herbal drug market of Varanasi and adjoining areas of eastern Uttar Pradesh, India during August-October, 2021. These raw materials were chosen on the basis of their availability in the market and popularity of uses. The drug samples were collected in sterilized polythene bags to avoid further contamination. In the laboratory, materials were individually finely ground in a common household blender. The blender's cup was rinsed in 90% alcohol before and after grinding the individual sample. The powder was sieved through No. 50 mesh sieve, kept tightly packed in a new paper bags and stored at 5ºC for further analysis (Kumar *et al.,* 2013).

Moisture content and pH

For moisture content determination, the weighed amount of samples was dried at 100ºC for 24 h or until their weights remain constant and the difference in weight were calculated following Kumar *et al.,* (2013).

For pH measurement, a drug sample: distilled water suspension (1:10;w/v) for each sample was prepared and stirred for 24 h in 200 ml beaker. The pH of the suspension was measured using electronic pH meter device (Kumar *et al.,* 2013).

Mycological analysis

Ten gram of each sample was added separately to 90 ml sterile 0.85% saline solution in 250 ml Erlenmeyer flask and were thoroughly homogenized on electric shaker with constant speed for 15 min. Five fold serial dilutions were then prepared following Mishra *et al.,* (2015). One ml of suitable dilutions (10^{-4}) of each medicinal plant suspension was used separately to inoculate Petri dishes containing 10 ml freshly prepared Potato Dextrose Agar medium (Potato, 200g; Dextrose, 20g; Agar, 18g and distilled water 1000 ml). Ten replicates of each drug sample were used. Plates were incubated at 27±2 ºC for 7 days and examined daily but counts were recorded only after 3-4 days. After incubation, the plates were examined visually and with the help of compound microscope. Morphologically different mould colonies were individually sub-cultured on PDA medium. Identification of fungal species was done by cultural and morphological characteristics (Ravimannan *et al.,* 2016).

Percent relative density of different fungi on raw herbal drug samples was calculated following Singh *et al.,* (2008).

$$\text{Relative density of fungus (\%)} = \frac{\text{No. of isolates of a fungus}}{\text{Total no. of isolates of all fungi}} \times 100$$

Percent frequency of occurrence of mycoflora on individual raw herbal drug sample was determined following Singh *et al.,* (2008).

$$\text{Occurrence frequency (\%)} = \frac{\text{No. of fungal isolates on a drug sample}}{\text{Total no. of fungal isolates on all drug samples}} \times 100$$

Detection of toxigenic strains of *A. flavus* and estimation of aflatoxin B_1

Aflatoxin B_1 producing potential of different cultures of *A. flavus* isolates during mycoflora analysis was tested in SMKY medium (Sucrose, 200g; $MgSO_4.7H_2O$, 0.5g; KNO_3, 0.3g; Yeast extract, 7.0g; Distilled water, 1000ml). The method followed by Mishra *et al.,* (2015) was adopted for the estimation of aflatoxin B_1. 25 ml of medium was taken in 100 ml Erlenmeyer flask and inoculated separately with 5 mm diameter disc of seven days old culture of the *A. flavus* isolated from selected raw herbal drug samples. The flasks were incubated for 10 days at 27±2 ℃. After incubation content of each flask was filtered through Whatman filter paper no. 1. The filtered mycelium was dried at 100 ℃ for 24 h and their biomass was determined.

The filterate was extracted with 20 ml chloroform in a separating funnel. After separation chloroform extract was passed through anhydrous Sodium sulphate kept in Whatman filter paper no. 42. The extract was evaporated till dryness on water bath at 70 ℃. The amount of aflatoxin B_1 was determined by TLC technique. The residue left after evaporation was dissolved in 1 ml chloroform and 50 µl of chloroform extract spotted on TLC plate (20×20 cm^2 of silica gel) then developed in Toluene:Isoamyl alcohol:Methanol; (90:32:2;v/v/v) solvent system proposed by Kedia *et al.,* (2015). The intensity of aflatoxin B_1 was observed in Ultra Violate Fluorescence Analysis Cabinet at an excitation wavelength of 360 nm. The presence of aflatoxin B_1 was confirmed chemically by spraying trifluoroacetic acid. For quantitative estimation, spots of aflatoxin B_1 on TLC were scraped out and dissolved in 5 ml cold methanol, shake and centrifuge at 3000 rpm for 5 min. Optical density of supernatant was recorded at the wavelength of 360 nm and the amount of aflatoxin B_1 was calculated following Kedia *et al.,* (2015).

$$\text{Aflatoxin } B_1 \text{ content (µg/kg)} = \frac{D \times M}{E \times l} \times 1000$$

Where,

D - absorbance; M - molecular weight of aflatoxin B_1 (312); E - molar extinction coefficient of aflatoxin B_1 (21,800) and *l* - path length (1 cm cell was used)

Results and Discussion

The moisture content and hydrogen ion concentration (pH) of the raw materials are shown in Table 1. Moisture level in the examined materials ranged from 12.94% to 34.34%. It was least in *T. arjuna* and highest in *B. diffusa*. The hydrogen ion concentration (pH) of herbal drug samples showed acidic magnitude. The lowest mean pH level was detected in *H. antidysenterica* (4.89) and highest in *B. diffusa* (6.08) (Table 1).

During mycoflora analysis, 906 fungal isolates were recorded from raw materials (Table 2). The least number of isolates (96) were isolated from *T. arjuna* and highest (217) in *B. diffusa*. The genus *Aspergillus* (with six species) was found to be the most dominant encountered in almost all the samples, followed by *C. herbarum, P. italicum* and *T. viride*. Some mucorale were also isolated.

The relative density values (%) of each fungal species was calculated and ranged from 0.55% to 37.19% (Table 2). The highest percent relative

density was recorded in case of *A. flavus* (37.19), followed by *A. niger* (23.84) and *C. herbarum* (11.81). The lowest relative density was found in case of *A. nidulans* (0.55) followed by *A. candidus* and *Bipolaris* sp. (0.66).

The occurrence frequency (%) of recovered mycoflora on each herbal drug samples was calculated and found lowest (10.59%) in case of *T. arjuna* while highest (23.95%) in *B. diffusa*. Rest of the samples shared intermediate percent occurrence frequency (Table 2).

The natural production of aflatoxin B_1 by isolated *A. flavus* was detected from all the drug samples in variable amounts. The toxigenic isolates of *A. flavus* were minimum in case of *W. somnifera* (22.22 %) and maximum in *R. serpentina* (54.54 %). Highest aflatoxin B_1 elaboration (272.46 µg/kg) was produced by the *A. flavus* isolated from *B. diffusa* while the lowest aflatoxin B_1 (194.62 µg/kg) production recorded by *A. flavus* from *H. antidysenterica* (Table 2, Figure 1)

Varanasi (latitude 25.20° N and longitude 83.0° E) comes under sub tropical region, having generally higher relative humidity (49.0-86.6%) and temperature ranging from 25.8 °C to 33.65 °C as well as low evaporation i.e. 3.725 mm during August-October, providing highly conducive environment to fungal growth and proliferation. In the present study the moisture content of the samples of the herbal drugs was found to be slightly higher than that of the samples analysed by some earlier workers (Abba *et al.,* 2008; Singh *et al.,* 2008).

This may be because of the collection of the samples in rainy season. The findings of the present study are in accordance with Singh *et al.,* (2008) who reported maximum percentage of fungal association in samples having high moisture content and pH range 5.4 to 6.2. Higher incidence of aspergilli on the drug samples compared to other fungal forms in the present investigation may be due to their saprophytic nature and ability to colonize diverse substrate because secretion of various hydrolytic enzymes by these moulds as has been reported by Barberis *et al.,* (2014). Similarly the higher relative frequency of *A. flavus* and *A. niger* than the remaining fungal species supports the earlier observations made by Sham *et al.,* (2021).

In addition based on the findings of the present study a correlation between colonization of the drug samples with *A. flavus* and percent toxigenicity of the isolates may be established. The least percent toxigenicity (22.22) was observed in *W. somnifera* and highest (54.54) in *R. serpentina*. In some of the samples viz. *B. diffusa* and *R. serpentina* both colonization as well as percent toxigenicity of *A. flavus* was found to be high. Some samples viz. *H. antidysenterica* and *W. somnifera* showed high colonization of *A. flavus* than *T. arjuna* but the percent toxigenicity was vice-versa. Such a fluctuation in percent toxigenicity of *A. flavus* may be because of chemical profile of the individual raw materials (Vijayanandraj *et al.,* 2014; Safari *et al.,* 2020). The chemical ingredients of *W. somnifera* and *H. antidysenterica* may be suppressing the aflatoxin B_1 elaboration by the *A. flavus* isolates more than those of *T. arjuna.* The chemical nature of substrates and associated fungal population has been reported to affect aflatoxin elaboration in different cereals (Kumar *et al.,* 2022). Such facts may be pertinent in case of herbal raw materials which contain high amount of different secondary metabolites than the cereals.

The quantitative estimation of aflatoxin B_1 elaboration done in the present study reveals that the samples were highly contaminated by the toxigenic strains of *A. flavus.* The aflatoxin B_1 level recorded in the present study is much higher than the safest limit (20 µg/kg) as recommended by WHO (Rajeshwari and Raveesha, 2016).

As these plant materials are used for preparation of traditional medicines, the possibilities of aflatoxin B_1 contamination in these is a serious issue. This is certainly a matter of great concern because humans use these medicines to treat diseases.

Table.1 English name, scientific name, family, plant part used, moisture content and pH of selected herbal drug samples.

S. No.	English Name	Plant Taxon	Family	Plant part used	Moisture content (%)	pH of drug samples
1	Arjun tree	*Terminalia arjuna*	Combretaceae	Bark	12.94± 0.096	5.27± 0.113
2	Sweet flag	*Acorus calamus*	Araceae	Rhizome	27.33± 0.152	5.31± 0.085
3	Serpentine	*Rauvolfia serpentina*	Apocynaceae	Root	30.78± 0.500	5.49± 0.05
4	Conessi	*Holarrhena antidysenterica*	Apocynaceae	Bark	19.95± 0.106	4.89± 0.055
5	Winter cherry	*Withania somnifera*	Solanaceae	Root	14.67± 0.060	5.37± 0.01
6	Hog weed	*Boerhaavia diffusa*	Nyctaginaceae	Root	34.34± 0.193	6.08± 0.155

Values are mean (n=3) ± SD

Fig.1 Intensity of aflatoxin B_1 produced by toxigenic isolates of *A. flavus* recovered from stored herbal raw materials under UV.

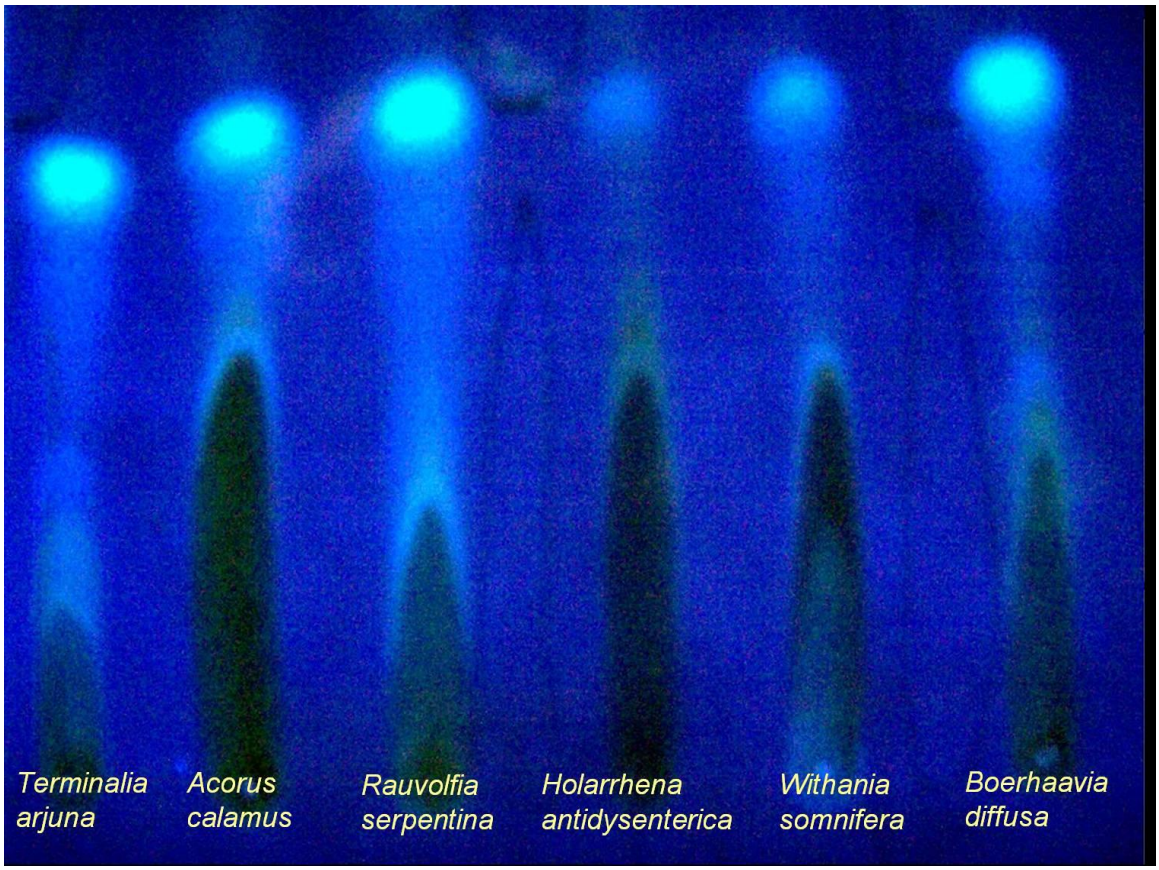

Table.2 Distribution of fungi isolated from herbal drugs their relative density, frequency of occurrence and Aflatoxin B_1 content.

Names of herbal drugs	Fungal Isolates[a]															Mucorales[b]	Total isolates	Total species	Frequency of occurrence (%)	Toxigeni-city of *A.flavus* (%)	Aflatoxin B_1[c] content (µg/kg)
	A.f.	***A.n.***	***A.fu***	***A.t.***	***A.ni***	***A.c.***	***P.c.***	***P.i.***	***T.v.***	***F.o.***	***A.a.***	***C.l.***	***C.h.***	***B.s.***	**UI**						
Terminalia arjuna	39	25	5	-	-	-	-	8	-	-	-	1	17	-	1	(3) Genera	96	7	10.59	14/5 (35.71)	**210.64 ± 30.93**
Acorus calamus	69	38	6	-	-	2	7	13	12	2	3	3	23	-	2	(3) Genera	180	12	19.86	12/5 (41.66)	**247.27 ± 32.98**
Rauvolfia serpentina	64	30	10	11	5	-	-	14	10	3	1	2	19	-	-	(2) Genera	169	11	18.65	11/6 (54.54)	**263.30 ± 54.66**
Holarrhena antidysenterica	47	15	4	-	-	1	-	9	5	-	2	2	13	2	-	(2) Genera	100	10	11.03	13/3 (23.07)	**194.62 ± 52.46**
Withania somnifera	51	22	6	-	-	3	-	11	6	7	5	8	19	4	2	(4) Genera	144	12	15.89	9/2 (22.22)	**206.06 ± 48.57**
Boerhaavia diffusa	67	86	8	4	-	-	-	12	11	6	3	4	16	-	-	(5) Genera	217	10	23.95	10/5 (50.00)	**272.46 ± 52.71**
Total isolates	**337**	**216**	**39**	**15**	**5**	**6**	**7**	**67**	**44**	**18**	**14**	**20**	**107**	**6**	**5**		**906**				
Relative density (%)	**37.2**	**23.8**	**4.3**	**1.65**	**0.55**	**0.66**	**0.77**	**7.39**	**4.85**	**1.98**	**1.54**	**2.2**	**11.8**	**0.66**	**0.55**						

a Fungal isolates: *A.f.-Aspergillus flavus; A.n.- Aspergillus niger; A.fu.-Aspergillus fumigatus; A.t.- Aspergillus terreus; A.ni.- Aspergillus nidulans; A.c.- Aspergillus candidus; P.c.- Penicillium carneum;*
P.i.- Penicillium italicum; *T.v.- Trichoderma viride*; *F.o.- Fusarium oxysporum; A.a.- Alternaria alternata; C.l.- Curvularia lunata; C.h.- Cladosporium herbarum; B.s.- Bipolaris* species; U.I.- Unidentified

b Mucorales including *Rhizopus nodosus, Rhizopus* sp., *Mucor* sp., *Mortierella* sp. and *Absidia corymbifera* were recovered but not included among the total isolates.

c Mean of Aflatoxin B1 content produced by toxigenic isolates of *A. flavus* from each herbal drug samples.(-) Not Detected

The findings of the present study reveal that the contamination of herbal drugs with storage fungi and mycotoxin is alarming and appropriate quality control measures should be taken urgently. The work on quality control measures to combat fungal contamination can only be undertaken after the identity of the associated microorganisms. Since most of the fungi encountered are post harvest contaminants rather than the normal mycoflora of the native plants, better methods of storage will reduce their incidence or eliminate them.

Storage of crude herbal drug samples under hygienic conditions with low moisture content (about 8%) and low temperature may protect them from moulding of these samples and consequently reduce the risk of aflatoxin contamination. Modification of storage environment at high scientific level and transportation of the raw materials under safe condition will be effective in reducing risk of mycotoxin contamination in herbal drugs.

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