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Diversity of Fungi and Mycotoxins Associated with Stored Triphala Churn and its Ingredients

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Abstract: The present study explores the mycoflora associated with stored, Triphala churn and its raw material. Total 106 stored fruits and 68 powdered samples have been analysed for their fungal and mycotoxin association, if any. Results revealed that total 21 fungal species belonging to 11 different genera, in which eight species were only from genus *Aspergillus*, four from *Penicillium* and single species of each *Helminthosporium*, *Curvularia*, *Alternaria*, *Geotrichum*, *Fusarium*, *Rhizopus*, *Paecilomyces* and *Syncephalastrum* was recorded. About 97.36% of Triphala churn samples, 94.66% dried fruits and 89% powdered samples were found contaminated with various fungi. About 73.68% Triphala churn, 50% fruits and 20.58% powdered samples were found contaminated with six mycotoxins namely, aflatoxin B1 and B2, aflatoxin G1 and G2, citrinin and sterigmatocystin. All the six mycotoxins were detected from the samples of fruits and powder, whereas, five mycotoxins were detected from Triphala churn samples. After PCR analysis of most frequently occurring fungal species with two universal fungal primers ITS-1 and ITS-4, band pattern obtained on agarose gel clearly differentiating the different fungal species. Seven frequently observed species of *Aspergillus* namely, *A. niger*, *A. terreus*, *A. fumigatus*, *A. flavus*, *A. terreus*, *A. parasiticus*, *A. versicolor* were tested for their aflatoxigenic nature with *omt-1* and *ver-1* gene specific primers and amplification products of 895 and 596 bp were obtained only from *A. flavus* and *A. parasiticus* which indicate as aflatoxigenic nature. Presence of species of *Aspergillus* and *Penicillium* and mycotoxins in Triphala churn and its ingredients could represent a threat to consumer's health.

Key words: Triphala churn, constituents, associated mycoflora, mycotoxins, aflatoxigenic fungi

INTRODUCTION

Triphala churn is one of the most important traditional Indian ayurvedic formulations popular all over the world. It is a powdered herbal formulation originated thousands years ago with main goal to promote body health as medicine and as a rejuvenating mixture. It is an equiproportional (1:1:1) mixture of dried fruit powder of three important medicinal plants namely, *Emblica officinalis* (Garetn.) *Terminalia bellirica* (Gaertn.) Roxb. and *Terminalia chebula* (Retz.). This formulation is based on a tridoshic formula, it control Vata, Pitta and Kaph of body means can be used to bring balance to all body's constitutions. Triphala churn have a different therapeutic use, in which main is to regulate digestive system and helps to ensure that the digestive tract works at the optimal level. It also supports blood purification, bile secretion and maintains the health of gastrointestinal tract lining (Kulkami, 1995; Juss, 1997).

As Triphala churn is of plant origin i.e., prepared from dried fruit powder of *Emblica officinalis*, *Terminalia bellirica* and *Terminalia chebula*; the churn as well as its ingredients are prone to fungal

contamination at various steps of its preparation. The fruits also get contaminated by microorganisms during growth (while the fruits are on tree), after harvesting, processing (when fruits are dried) and during storage. In addition to pre and post-harvest practices, other factors like sterilization of instruments, environmental conditions like temperature, moisture and relative humidity of processing area and methods used for processing are also the main cause and sources of fungal association (Truckesses and Scott, 2008) with Triphala. This fungal contamination may leads to reduction in quality as well as therapeutic potential of herbal drugs. In addition, the mycotoxins produced by fungal contamination resulted into several ailments of liver, kidney, nervous system, muscular, skin, respiratory organs, digestive tract, genital organs, etc among users (Rai and Mehrotra, 2005; Dragan *et al.*, 2010). Because of several health effects it's necessary to investigate the associated mycoflora in and related mycotoxins with Triphala churn and its ingredients. Besides, the identification of toxigenic and nontoxigenic fungal species at early stages is also necessary to avoid possible health risk for users.

Therefore, present study was aimed to investigate the status of mycoflora and mycotoxins associated with stored Triphala churn and its ingredients. In addition an attempt was made to characterize the frequently occurring mycotoxic and non-toxic *Aspergillus* species at molecular level along with their morphological, microscopic identification.

MATERIALS AND METHODS

Sample collection: A total of 38 Triphala churn samples, 106 dried stored fruits (*E. officinalis* = 37, *T. bellirica* = 29 and *T. chebula* = 40) and 68 powdered (*E. officinalis* = 25, *T. bellirica* = 22 and *T. chebula* = 21) samples were randomly collected from different regions of Gwalior Madhya Pradesh, India during the year 2007-2009, transported to Mycology laboratory at School of Studies in Botany, Jiwaji University, Gwalior, (MP) India and stored for further analysis.

The moisture content of all the collected samples was determined by oven drying at 80°C until their weights remains constant (Essono *et al.*, 2007) and the difference in weight was calculated as:

$$MC = \frac{(W_i - W_f)}{W_i} \times 100$$

where, MC is Moisture content; W_i is Initial weight and W_f is Final weight.

All the collected (Fruit and powdered) samples were carefully examined morphologically for their physical appearance i.e. colour and texture.

Mycoflora isolation: One gram of each powdered sample was mixed aseptically in 9 mL of sterile distilled water and shaken vigorously. Appropriate tenfold serial dilution (10^{-5}) was 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. Triplicate of each sample were incubated at $25 \pm 2^\circ\text{C}$ for 7 days and examined visually as well as under a compound light microscope daily for preliminary identification of fungal genera. The identified genera were then, sub-cultured on suitable agar plates for species identification.

Macroscopic and microscopic identification of Mycoflora: Identification of fungal species was done on the basis of cultural and morphological characteristics. Macroscopic features like colony colour and margins as well as microscopic such as conidia and conidiophores

arrangements were examined for species differentiation (Gilman, 2001). Most frequently occurring fungal species were further validated by culture at Indian Agricultural Research Institute (IARI), New Delhi, India.

Molecular methods: Pure culture of most frequently occurring fungal isolates isolated from Triphala churn and its ingredients were maintained. For DNA isolation, the cultures were grown in potato dextrose broth (PDB; pH 5.5) for 7 days at $28 \pm 1^\circ\text{C}$. Mycelia were filtered through filter paper (Whatman No. 1) and DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980).

The fungus-specific universal primers ITS1 and ITS4 were used to amplify genes encoding the ITS region (Tarai *et al.*, 2006). In addition of universal primers, two mycotoxin specific primers *ver-1* and *omt-1* (Shapira *et al.*, 1996; Konietzny and Greiner, 2003) were also used to differentiate between mycotoxic and non toxic *Aspergillus* isolates. All the PCR reagents like Taq polymerase, 200 μM dNTP (dATP, dCTP, dGTP, dTTP), reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl_2) and primers used were of Merck Specialities Pvt. Ltd., India. Concentrations of DNA template, primer and deoxynucleotide triphosphates (dNTPs) and the optimum annealing temperature were standardized for each primer in preliminary trials. PCR was performed in a total reaction volume of 25 μL , which consisted of 7.5 μL of target DNA solution, 3 μL of each of the primers, 3.5 μL of Taq Buffer A, 1 μL of dNTP's and 7.3 μL of milliQ water. The amplified PCR products were electrophoresed on a 1% agarose gel in TBE buffer visualized by staining with ethidium bromide and photographed using a gel documentation system (UVI tec, UK).

Detection of mycotoxins: The standards of Aflatoxins B_1 , B_2 , G_1 and G_2 (Sigma, Chemical, St. Louis, USA) were prepared by dissolving the pure aflatoxins in acetonitrile: water (1:1, v/v) to give concentrations of 1 mg mL^{-1} for each. The solutions were stored at 4°C . Natural occurrence of mycotoxins in collected herbal drug samples were examined by Thin-Layer Chromatography (TLC) (Singh, 1988). Fifty grams of each powdered samples were extracted with chloroform. About 50 μL of chloroform extract was applied on 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) and observed under long wavelength UV-light at 365 nm. For mycotoxin identification, fluorescence and R_f value of the samples spot on TLC plates were matched with the fluorescent intensity and R_f value of standards.

Statistical analysis: The analysis of data was performed with Microsoft Excel 2007 (Window XP) for Mean and standard deviation. Descriptive analysis of percent relative density, frequency and incidence were performed on the collected data.

RESULTS

Morphological analysis and moisture content: All samples were found to have a water content ranging from 4.41±1.97 to 9.29±4.96%. However, significant differences could be noted between the mean water content of the different kinds of samples. The highest (9.29±4.96%) percent moisture content was recorded in *T. bellirica* fruits followed by Triphala churn (8.23±2.24%), *E. officinalis* fruits (8.21±2.18%) and powder (7.14±0.38%) samples while it was minimum (5.84±2.68 and 4.41±1.97%) in *T. bellirica* powder and *T. chebula* fruits, respectively. In the remaining samples of *E. officinalis* and *T. chebula* powder the values of percent moisture content were 7.14±0.38% and 6.41±3.01 (Table 1). No significant differences could be observed taking into account the geographic origin of samples.

During morphological examination a remarkable change in the colour and texture was observed in case of the Triphala churn and other powdered samples. Light brown and light greenish colour with fine powdered form was observed in fresh samples of Triphala as well as *E. officinalis*, *T. bellirica* and *T. chebula* powders, whereas solid clumps with darker shade were appeared in the old stored samples. Black spots were observed on the surface of old stored fruit samples as compared to freshly stored. The damages created by insects during storage were also observed in some fruit samples. Fungal infection in some fruits was also noticed.

Fungi associated with Triphala churn and its ingredients: The mycological examination of all the samples revealed that 97.36% of Triphala churn samples, 94.66% dried fruits and 89% powdered samples were found to be contaminated. All (100%) the samples of *T. bellirica* (fruits) and *E. officinalis* (powder); 97.5% samples of *T. chebula* (fruits); 86.48% of *E. officinalis*

(fruits) and 85.71% *T. chebula* (powder) were showing fungal contamination with various fungi. Minimum 81.11% fungal contamination was observed in *T. bellirica* powdered samples (Table 2). Percentage of contamination was significantly (p<0.001) higher in case of fruit and Triphala churn over powdered samples.

The mycological analysis of fruits and powdered samples revealed that total 21 fungal species belonging to 11 different genera and three major fungal groups i.e., Ascomycotina, Deteuromycotina and Zygomycotina were found associated with all the samples. The genus *Aspergillus* was found to be the most dominant one with eight species viz. *A. flavus*, *A. niger*, *A. fumigatus*, *A. terreus*, *A. parasiticus*, *A. versicolor*, *A. ochraceus* and *A. nidulens* whereas, four species of *Penicillium* viz. *P. citrinum*, *P. rubrum*, *P. chrysogenum* and *P. viridicatum* and single species of each *Helminthosporium*, *Curvularia*, *Alternaria*, *Geotrichum*, *Fusarium*, *Rhizopus*, *Paecilomyces* and *Syncephalastrum* was recorded in all the samples.

Seven genera were recorded from Triphala churn, while nine genera from *E. officinalis*, six from *T. bellirica* and six from *T. chebula* fruit and powder. About seventeen fungal species were isolated from Triphala churn samples, where as twenty from its ingredients i.e., eighteen from *E. officinalis*, thirteen from *T. bellirica* and *T. chebula*. The highest percentage relative density was shown by *Aspergillus* species. Among all the *Aspergillus* species, *A. niger* was most common and isolated with higher frequency and density followed by *A. fumigatus* from all the samples. Among the different fungal genera recorded, the highest percentage relative incidence was shown by *Aspergillus*, followed by *Penicillium* while others were recorded in moderate to low incidence (Fig. 1).

Aspergillus niger was recorded with higher relative density in *T. chebula* fruits (61.54%) followed by *T. bellirica* fruits and powder (57.06 and 58.17% resp.),

Table 1: Average moisture (%) content of different fruits and powdered samples

Sample	<i>Emblica officinalis</i>	<i>Terminalia bellirica</i>	<i>Terminalia chebula</i>	Triphala churn
Fruit	8.21±2.18	9.29±4.96	4.41±1.97	-----
Powder	7.14±0.38	5.84±2.68	6.41±3.01	8.23±2.247

Table 2: Description of the fruit, powdered and Triphala churn samples used with % age of contamination

Sample	Botanical names	No. of contaminated samples	No. of samples examined	% age of samples contamination	Mean±SD	t-test
Triphala churn		37	38	97.36	97.36±0.00	p<0.001 for # and \$
Fruits	<i>E. officinalis</i>	32	37	86.48		
	<i>T. bellirica</i>	29	29	100.00	94.66±0.071	
	<i>T. chebula</i>	39	40	97.50		
Powder	<i>E. officinalis</i>	25	25	100.00	89.00±0.098	
	<i>T. bellirica</i>	18	22	81.11		
	<i>T. chebula</i>	18	21	85.71		

= p-value between fruits and powder samples; \$ = p-value between Triphala churn and powder samples

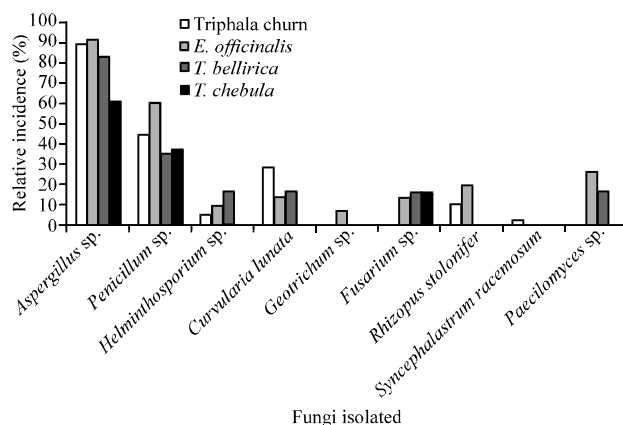


Fig. 1: The percent relative incidence of dominant fungal genera isolated from Triphala and its ingredients

Table 3: Percentage relative frequency of fungi in contaminated samples

Fungal species	Rel. freq. (%)							
	Triphala churn	<i>E. officinalis</i>		<i>T. bellirica</i>		<i>T. chebula</i>		
		F	P	F	P	F	P	
<i>A. niger</i>	68.42	62.16	76.66	93.10	66.66	77.50	36.11	
<i>A. flavus</i>	42.10	27.02	40.00	17.24	28.33	7.50	8.33	
<i>A. parasticvs</i>	18.42	8.10	10.00	3.44	16.66	5.00	-	
<i>A. fumigates</i>	44.73	45.94	55.00	41.37	30.00	40.00	50.00	
<i>A. terrelus</i>	5.26	2.70	-	-	-	-	-	
<i>A. versicolor</i>	13.15	13.51	43.33	13.79	2.24	2.50	4.16	
<i>A. ochraceus</i>	5.26	2.70	-	-	-	-	-	
<i>A. nidulens</i>	10.52	5.40	-	-	-	-	-	
<i>P. rubrum</i>	39.47	5.40	30.00	17.24	16.66	12.50	22.22	
<i>P. citrinum</i>	7.89	8.10	26.66	3.44	28.33	2.50	15.27	
<i>P. chrysogenum</i>	2.63	-	-	-	-	2.50	-	
<i>P. viridicatum</i>	5.26	-	11.65	-	-	-	-	
<i>Helminthosporium</i> sp.	5.26	-	5.00	-	-	-	-	
<i>C. lunata</i>	15.78	-	6.66	-	8.33	2.50	-	
<i>Geotrichum</i> sp.	-	-	3.33	-	-	-	-	
<i>A. alternata</i>	-	-	-	-	28.33	5.00	-	
<i>R. stolonifer</i>	10.52	10.81	5.00	24.13	-	13.33	-	
<i>S. racemosrm</i>	2.63	2.70	-	-	-	-	-	
<i>Fusarium</i> sp.	-	-	6.66	-	-	-	4.16	
<i>Paeleomyces</i> sp.	-	-	13.33	-	48.33	-	-	
<i>Chaetomium</i> sp.	5.26	-	-	-	-	-	-	
Unknown species	-	-	13.33	-	8.33	-	-	

Rel. Freq.: Relative frequency, F: Fruit, P: Powder

fruits of *E. officinalis* (36.88%) and Triphala churn (32.96%). The relative density of *A. flavus* was reported to be 26.49% from *T. chebula* powder and of *A. fumigatus* (31.55%) from *E. officinalis* fruits (Table 3). Similarly, the highest relative frequency was again shown by *A. niger* from *T. bellirica* and *T. chebula* fruits (93.10 and 77.50% resp.) followed by fruits and powder of *E. officinalis* (62.16 and 76.66% resp.) and Triphala churn (68.42%). The contribution of other fungal species was recorded to be varied from moderate (55%) to low (2.50%) relative frequency (Table 4).

Natural occurrence of mycotoxins: Total six mycotoxins namely aflatoxin B1 and B2, aflatoxin G1 and G2, citrinin and sterigmatocystin were detected during mycotoxins

analysis of collected samples. Presence of each mycotoxins was confirmed under long wavelength UV-light at 365 nm, spots giving bluish fluorescence refers to aflatoxin B, greenish for aflatoxin G, yellow for citrinin, while orange or reddish brown fluorescence indicated the presence of sterigmatocystin, as described by Scott *et al.* (1970).

A variable range (14.28-82.75%) of mycotoxins contaminated samples was recorded during the investigation. All the six mycotoxins were detected from the samples of fruits and powder, whereas, five mycotoxins were detected from Triphala churn samples. About 73.68% Triphala churn, 50% fruits and 20.58% powdered samples were found contaminated with various mycotoxins (Table 5).

Table 4: Percentage relative density of fungi in contaminated samples

Fungal specie	Rel. Den. (%)						
	Triphala churn	<i>E. officinalis</i>		<i>T. bellirica</i>		<i>T. chebula</i>	
		F	P	F	P	F	P
<i>A. niger</i>	32.96	36.88	28.15	57.06	58.17	61.54	8.60
<i>A. flavous</i>	10.92	12.92	8.73	9.33	2.40	1.45	26.49
<i>A. parasticvs</i>	10.88	2.66	9.22	1.33	12.98	2.62	-
<i>A. fumigates</i>	6.32	31.55	13.34	13.06	0.96	25.36	31.78
<i>A. terreus</i>	1.50	0.76	-	-	-	-	-
<i>A. versicolor</i>	2.56	4.18	9.22	2.66	0.48	0.29	0.66
<i>A. ochraceus</i>	0.92	0.38	-	-	-	-	-
<i>A. nidulens</i>	3.62	1.52	-	-	-	-	-
<i>P. rubrum</i>	14.91	0.76	9.46	4.80	11.53	4.66	14.56
<i>P. citrinum</i>	4.51	1.52	11.65	1.06	6.25	0.87	16.55
<i>P. chrysogenum</i>	0.08	-	-	-	-	0.87	-
<i>P. viridicatum</i>	0.84	-	0.97	-	-	-	-
<i>Helminthosporium</i> sp.	1.10	-	4.61	-	2.40	-	-
<i>C. lunata</i>	4.64	-	0.24	-	0.96	0.29	-
<i>Geotrichum</i> sp.	-	-	0.24	-	-	-	-
<i>A. alternata</i>	-	-	-	-	6.25	0.58	-
<i>R. stolonifer</i>	1.94	4.56	1.45	9.06	-	5.83	-
<i>S. racemosum</i>	0.30	1.52	-	-	-	-	-
<i>Fusarium</i> sp.	-	-	0.72	-	-	-	0.66
<i>Paecilomyces</i> sp.	-	-	5.82	-	1.92	-	-
<i>Chaetomium</i> sp.	1.90	-	-	-	-	-	-
Unknown species	-	-	0.48	-	0.96	-	-

Rel den: Relative density, F: Fruit, P: Powder

Table 5: Mycotoxins contamination in Triphala churn and its ingredients

Samples	Name	No. of samples analysis	No. of samples contaminated (%)	Mycotoxins detected	
				Mycotoxins	Positive samples (%afq)
Triphala churn		38	28 (73.68)	AfB1	13 (34.21)
				AfB2	12 (31.57)
				AfG1	4 (10.52)
				Sterigmatocystin	2 (5.26)
				Citrinin	4 (10.52)
				AfB1	7 (18.91)
				AFB2	6 (15.8)
				AFG1	3 (8.10)
				AFG2	6 (16.21)
				Citrinin	1 (2.70)
Fruits	<i>E. officinalis</i>	37	18 (48.64)	AFB1	5 (17.24)
				AFB2	19 (65.51)
				AfG1	3 (10.34)
				AFG2	2 (6.89)
				Sterigmatocystin	8 (27.58)
	<i>T. bellirica</i>	29	24 (82.75)	Citrinin	1 (3.44)
				AfB1	1 (2.56)
				AFB2	8 (20.51)
				Sterigmatocystin	3 (7.69)
<i>T. chebula</i>	39	11 (28.20)			
Total		106	53 (50)		
Powders	<i>E. officinalis</i>	25	7 (28)	AfB1	7 (28)
				AfB2	4 (16)
				AfG1	7 (28)
				AFG2	7 (28)
				Sterig.	4 (16)
	<i>T. bellirica</i>	22	4 (18.18)	AfB1	1(4.54)
				AFB2	6(27.27)
				Citrinin	11 (50)
				AfB1	2(9.52)
				AfG1	7 (33.33)
	<i>T. chebula</i>	21	3 (14.28)	AFG2	7 (33.33)
				Citrinin	7 (33.33)
Total		68	14 (20.58)		

Af: Aflatoxin, Sterig: Sterigmatocystin, fq: Frequency

Among the fruit samples, highest percentage of mycotoxin contaminated samples was observed in fruits of *T. bellirica* (82.75%) followed by *E. officinalis* (48.68%) and 28.20% samples of *T. chebula*. In case of powdered samples, *E. officinalis* showed highest (28%) mycotoxin contaminated samples followed by *T. bellirica* (18.18%) and *T. chebula* (14.28%).

PCR specificity with Universal fungal primers (ITS-1 and ITS-4):

Two universal primers namely ITS-1 and ITS-4 were utilized in the present investigation and amplification was obtained after PCR analysis, regardless the primers used. The PCR amplicon of each tested fungi namely, *A. niger*, *A. flavus*, *A. terreus*, *A. fumigatus*, *A. parasiticus*, *A. nidulens*, *P. rubrum*, *P. citrinum*, *P. viridicatum*, *Alternaria alternata*, *Syncephalastrum racemosum* and *Rhizopus stolonifer* showed different

banding pattern on agarose gel which clearly differentiating the different fungal species (Fig. 2).

Total 23 bands, 1-6 in number were recorded, with 0.3-1.185 kb size in ITS-1 and of 0.3-2.0 kb size in ITS-4. The variability in number and size of bands of different fungal isolates resulted from PCR reactions primed by ITS-1 and ITS-4 were clear in Fig. 2. With ITS-1, *Penicillium viridicatum* showed six bands from 300 bp-2000 bp whereas, four bands recorded in *Syncephalastrum racemosum* were of 500-1500 bp in size. In *A. niger* three bands of 300, 600 and 1100 bp were observed while three bands recorded in *A. versicolor* were in size of 500, 700 and 1100 bp. Two bands of 300 and 800 bp in *A. fumigatus* and two bands of 1000 and 1500 bp in *A. terreus* were recorded. The remaining fungal isolates like *A. flavus* showed single band of 800 bp size while it was 900 bp in *A. parasiticus*. Similarly with ITS-4, Six

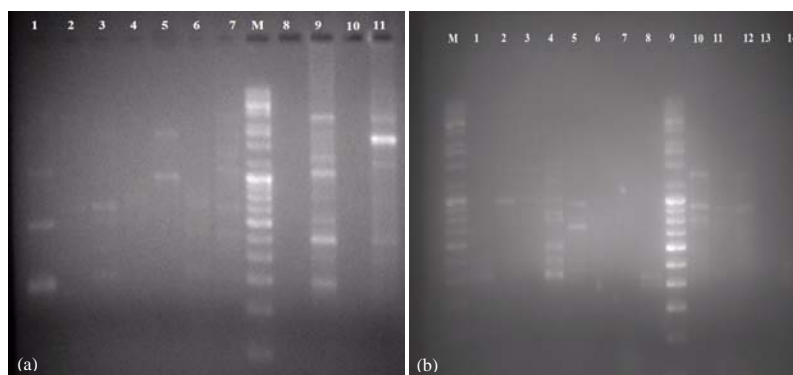


Fig. 2: Banding pattern of different fungal isolates resulted from PCR reactions primed by ITS-1 and ITS-4 universal fungal primer. (a) 1: *A. niger*, 2: *A. flavus*, 3: *A. fumigatus*, 4: *A. parasiticus*; 5: *A. terreus*, 6: control, 7: *A. versicolor*, M: Marker; 8: *P. citrinum*; 9: *P. viridicatum*; 10: *Rhizopus stolonifer*, 11: *Syncephalastrum racemosum*, (b) M: Marker; 1: *A. niger*, 2: *A. flavus*, 3: *A. terreus*, 4: *A. fumigatus*, 5: *A. parasiticus*, 6 and 7: Control, 8: *A. nidulens*, 9: Marker; 10: *P. rubrum*, 11: *P. citrinum*; 12: *P. viridicatum*; 13: *Alternaria alternata*, 14: *Rhizopus stolonifer*

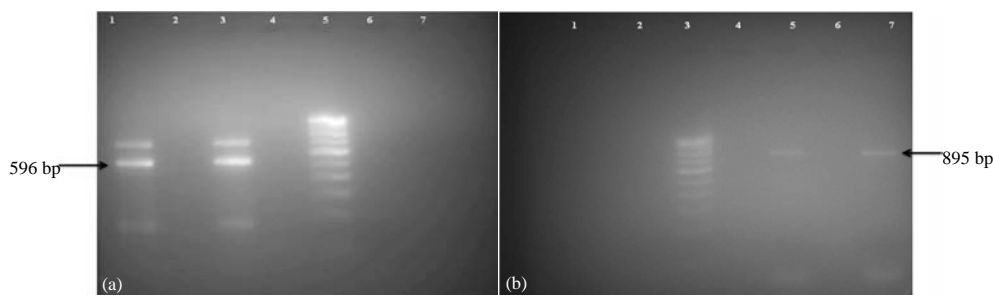


Fig. 3: Characterization of aflatoxigenic fungi by PCR: (a) *ver-1*, 1: *A. flavus*, 2: *A. niger*, 3: *A. parasiticus*, 4: *A. fumigatus*, 5: (M) marker, 6: *A. terreus*, 7: *A. versicolor*, (b) *omt-1*, 1: *A. niger*, 2: *A. fumigatus*, 3: (M) marker, 4: *A. versicolor*, 5: *A. flavus*, 6: *A. terreus*, 7: *A. parasiticus*

bands were observed in *A. fumigatus* (300-900), followed by *P. rubrum* with three bands of 700-1185 bp. Two bands were recorded in five fungal isolates namely, *A. niger* (300 and 400 bp), *A. terreus* (900 and 1185 bp), *A. parasiticus* (600 and 900 bp), *P. viridicatum* (800 and 1185 bp) and *Rhizopus stolonifer* (300 and 400 bp). Only single band was observed in *A. flavus* (900 bp), *A. nidulens* (300 bp), *P. citrinum* (800 bp) and *Alternaria alternata* (400 bp).

PCR specificity with aflatoxigenic primers *ver-1* and *omt-1*: *Aspergillus niger*, *A. terreus*, *A. fumigatus*, *A. flavus*, *A. terreus*, *A. parasiticus*, *A. versicolor* isolated, were tested for their aflatoxigenic nature with *ver-1* and *omt-1* gene specific primers. As expected, amplification products of 895 and 596 bp regardless of primer set used were obtained only from *A. flavus* and *A. parasiticus*. No such amplified products were not detected in remaining isolates of test organisms which means only two isolates were identified as aflatoxigenic and *A. niger*, *A. terreus*, *A. fumigatus*, *A. terreus* and *A. versicolor* as non-toxigenic strains during PCR analysis (Fig. 3).

DISCUSSION

During the survey for sample collection, samples were found stored in open metal/plastic containers/ wooden boxes/gunny bags or on the bare ground in local general stores, causes direct exposure to biotic environmental factors, air pollutants and other contaminants resulted into microbial contamination (Essonon *et al.*, 2007). Variations in morphological appearance i.e., change in colour texture and appearance was also found in Triphala churn and its ingredients, might be due to the improper storage in unfavourable environmental factors. Fungal infection in some fruits was also noticed which was possibly due to injuries during harvesting, transportation and drying. High moisture contents and incidence of higher fungal counts in *E. officinalis* (powdered) samples justify favourable impact of high moisture and temperature on the fungal growth stored herbal drugs (Roy *et al.*, 1988; Halt, 1998). 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).

Presence of 21 fungal species belonging to 11 genera revealed the broad mycobiota association with stored Triphala churn ingredients. Species of *Aspergillus* and *Penicillium* were reported as dominating mycoflora in

stored herbal drugs (Hitokoto *et al.*, 1978; Aziz *et al.*, 1998; Abou-Arab *et al.*, 1999; Elshafie *et al.*, 1999; Mandeel, 2005; Gautam *et al.*, 2009; Sareen *et al.*, 2010) which was also seen in present study. Similarly, the fungal contamination were found in other substrates like association of *Fusarium* with maize (Chehri *et al.*, 2010), sundried leaves of Jew's-Mallow and Okra fruits (Youssef, 2008).

The isolation of *A. niger* in high frequency from all drug samples, which are used by consumers in raw as well as product form, should be taken seriously as some strains can occasionally produce ochratoxin A and Fumonisin B2 (Abarca *et al.*, 1994; Noonimabc *et al.*, 2009; Gautam *et al.*, 2010; Edwin *et al.*, 2010; Gautam *et al.*, 2011). Isolation of *A. flavus* is also considerable due to aflatoxins production (Diener *et al.*, 1987). Production of sterigmatocystin also makes an attention point towards the presence of *A. versicolor* while citrinin and rubra toxin for *P. citrinum* and *P. rubrum*. Invasive and allergic aspergillosis due to inhalation of spores also justifies the risk of presence of *A. fumigatus* in collected samples.

Detection of aflatoxin B1 and B2; G1 and G2 form Triphala churn and its ingredients justifies the contamination of all the analysed samples with *A. flavus* and *A. parasiticus*. Several reports are available on aflatoxins contaminating raw drugs of plant origin (Aziz *et al.*, 1998; Martins and Martins, 2001; Ali *et al.*, 2005; Singh *et al.*, 2008; Gautam and Bhadauria, 2008; Sareen *et al.*, 2010) and herbal drugs (Singh, 2003; Govender *et al.*, 2006; Khan *et al.*, 2006; Okunlola *et al.*, 2007; Enayatifard *et al.*, 2010) which support the detection of aflatoxins in analysed samples during present investigation. Presence of mycotoxins form other food and feed materials like corn (Youssef, 2009; Tinyiro *et al.*, 2011), food stuffs (Jimoh and Kolapo, 2008) and rice (Murthy *et al.*, 2009) were also reported. Presence of aflatoxins is of a point of attention because they are hepatotoxic, Immunosuppressive, carcinogenic and mutagenic for consumers. Aflatoxin B1 is the most potent mycotoxin evaluated as a Group 1 carcinogen, considered as 'Carcinogenic to humans' (Shephard, 2008). Sterigmatocystin and citrinin produced by *A. versicolor*, *P. citrinum* respectively, was also detected in collected samples is of great concern because former is a liver carcinogen and has immunosuppressive effects (Vesonder and Horn, 1985) while later is nephrotoxic nature as well as teratogenic (Benneth and Klich, 2003; Yu *et al.*, 2006; Singh *et al.*, 2007).

Fungal contamination of herbal drugs by toxigenic strain of *Aspergillus* with high frequency during storage, posing subsequent danger for consumers health and

highlight the importance of rapid detection of aflatoxin producers such as *A. flavus* and *A. parasiticus* using molecular strategy. Out of seven *Aspergillus* isolates subjected to PCR analysis with aflatoxigenic primers *ver-1* and *omt-1* and only two fungal species, *A. flavus* and *A. parasiticus* showed the amplification indicating their potential aflatoxin producing ability. The use of *ver-1* and *omt-1* as probe can discriminate between aflatoxin producing toxigenic and non-toxigenic fungi. Similar observations using *ver-1*, *omt-1* and *aflR* (Shapira *et al.*, 1996) and *nor1*, *ver1* and *omt1* (Geisen, 1996) RAPD genetic markers have been reported to differentiate, aflatoxigenic and non aflatoxigenic strains of *A. flavus* and *A. parasiticus*. Maheshwar *et al.* (2009) has also reported the association of toxigenic endophytic *Fusarium verticillioides* in maize by PCR analysis.

It is believed that conventional morphological and microscopic characterization methods are inaccurate because of intra- and interspecific morphological divergences, time consuming and not sufficient to characterize the different fungal species (Klich and Pitt, 1988; Samson *et al.*, 2004). Molecular characterization on the other hand, is a rapid, quick procedure requires minimal handling. Two universal fungal primers (ITS1 and ITS4) used in the present study, variation in the number and sizes of these ITS regions with different band patterns were observed. Several such studies on the use of PCR technology for the detection and diagnosis of fungi by using the Internal Transcribed Spacer (ITS) have already been published (Henson and French, 1993; Marek *et al.*, 2003; Haughland *et al.*, 2004; Druzhinina *et al.*, 2005). With the help of RAPD- PCR, Khan *et al.* (2007) studied diversity in *Aspergillus niger* isolates collected from pigeon pea field in Aligarh region. After RAPD analysis a set of different band patterns of amplified DNA with PCR were observed for different genome. Similar studies were also carried out recently by Godet and Munaut (2010) in differentiating *A. flavus*, *Aspergillus parasiticus*, *Aspergillus tamaris* and *Aspergillus nomius* by PCR- RAPD markers. Likewise, Leema *et al.* (2010) confirmed the *A. flavus* by molecular methods i.e., amplification of the internal transcribed spacer 2 (ITS 2) regions.

CONCLUSION

The prolonged storage of medicinally important herbal raw material and drugs with mycotoxic fungi and related mycotoxins in unfavourable climatic conditions, may pose a potential risk on consumers health. Looking into the importance of Triphala churn and its ingredients in curing various human ailments and an alarming increase

among consumers relying on it, it a necessity to undertake safety measures against fungal contamination and mycotoxins that might be present in raw materials during harvesting, storage and processing. Therefore, it is essential to scrutinize these herbal raw materials before processing for the presence of contaminants and only the raw materials of high grade (showing absence of mould/ mycotoxin and other contaminants) should be allowed to use for the preparation of herbal drugs. Moreover, after processing these herbal drugs should also be tested for presence of mycotoxins, prior packing and launching for public use.

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