J. Indian bot. Soc Vol. 89 (1 & 2) 2010 : 74-79

FUNGAL AND MYCOTOXIN CONTAMINATION OF SOME COMMON STORED HERBAL FRUIT SAMPLES.

AJAY K. GAUTAM AND REKHA BHADAURIA
School of Studies in Botany, Jiwaji University, Gwalior-474011

A total of 106 stored dried fruit samples of *Emblica officinalis* (Alma), *Terminalia bellerica* (Baheda) and *Terminalia chebula* (Haritiki), randomly collected from various retailers of Gwalior city, Madhya Pradesh (India), were analyzed for mould and mycotoxins contamination. Mycological examination revealed that 97.77% of the total samples examined, were found to be contaminated with different fungi. A total of 17 different fungal species were isolated from all the fruit samples. The predominant mycoflora obtained was distributed in five different genera comprising of *Aspergillus, Penicillium, Alternaria, Rhizopus* and *Syncephalastrum.* The *Aspergillus* (71.95%) was recovered as the most dominant genus followed by *Penicillium* (15.44%), *Rhizopus* (9.51%), *Alternaria* (1.67%) and *Syncephalastrum* (1.41%). During mycotoxicological investigation of fruit samples six mycotoxins namely aflatoxin B_1 & B_2 aflatoxin G_1 & G_2, citrinin and sterigmatocystin were detected. Aflatoxin B_2 was detected as an important contaminant (34.43%) in the tested fruit samples followed by sterigmatocystin (17.54 %) and aflatoxin B1 (12.88%). Alatoxin G1 and G2 are detected in 9.22% and 11.10% samples, respectively whereas; citrinin was from 3.07 % samples only. Aflatoxin B2 was detected maximum in 65.51% baheda fruits, 22% haritiki fruits and in 15.8% amla fruits only. The presence of wide range of fungi and their mycotoxins in the fruits of *E. officinalis, T. bellerica* and *T. chebula* showed the potential risk of the use of these herbal fruits and their products for the users.

Key words: Fungal contamination, Herbal fruits**,** Mycotoxins, Storage.

Herbal drugs have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments (Sharma *et al.* 2008). These are now in great demand in the developing world for primary health care because of the general belief that herbal drugs are without any side effects besides being cheap and locally available (Padh 2001, Trucksess and Scott 2008). Over 8000 plant species are reported to be used to prepare some 25,000 formulations to treat various ailments (Dubey *et al.* 2008). Different parts of the medicinal plants in crude as well powdered form are of equal importance in ayurveda and are being used as medicines to cure various health disorders. Amla (*Emblica officinalis* Gaertn.) is one of the richest sources of Vitamin C, used as a tonic, blood purifier, cardio-protective, diuretic, laxative. Fruits of baheda (*Terminalia bellerica* (Gaertn.) Roxb.) are used mainly as tonic, laxative in cough as well as in piles and dyspepsia. Haritiki (*Terminalia chebula* Retz.*)* fruit contains 24-32% tannins, can be used to cure acidity and to stop dysentery. Fruit powder is used as laxative and decoction is used in bleeding and ulceration of the gums. In ayurveda, the powdered mixture of these three fruits named as Trifla churn, is prescribed for the various stomach problems such as: constipation or indigestion, dyspepsia, anemia, impurity of blood, hyperlipidaemia, skin diseases, excessive heat, and irritation of eyes (Juss 1997). These plants are normally carrying a great number of bacteria and fungi, often of soil origin. In addition, pre and post harvest practices of these three medicinally important fruits like storage after harvesting, handling and production often cause additional contamination and microbial growth (Donia 2008). Post-harvest spoilage of these stored herbal fruits under unhygienic conditions by filamentous fungi is one of the most important threats associated with

Received on December 20, 2008 Accepted on July 14, 2009

processed and stored fruits (raw material) and herbal drugs. Discoloration, quality deterioration, reduction in commercial values as well as in therapeutic potential and mycotoxin production has been linked to mouldy contaminated herbal drugs (Roy 2003, Essono *et al*. 2007). The mycotoxins produced by toxigenic fungal contaminants are known to cause several ailments of liver, kidney, nervous system, muscular, skin, respiratory organs, digestive tract, genital organs, etc (Rai and Mehrotra 2005). Due to the importance of these herbal fruits in triphala churn preparation, the objective of the present study was to evaluate the predominant mycoflora and mycotoxins associated with these medicinally important stored herbal fruits (stored dry fruits of *E. officinalis, T. bellerica* and *T.chebula*) retailed in the market of Gwalior.

MATERIALS AND METHODS

Isolation and identification of fungi

A total of 106 fruit samples of *E. officinalis* (n=37), *T. bellerica* (n=29) and *T. chebula* (n=40), stored by local retailers after harvesting, were collected during the year 2007-2008 from Gwalior market in clean sterilized polythene labeled bags to avoid further contamination. All the collected 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. The moisture content of all the collected samples was determined by oven drying at 80^0C for 2-3 h. Enumeration of mycoflora was determined by standard method on Potato-Dextrose Agar (PDA) and Czapek Dox Agar media. Identification of fungi was done on the basis of morphological and cultural characteristics (Gilman 1975). Frequently occurring fungal cultures were further confirmed at IARI, New Delhi.

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 4^0C (Houssou *et al*. 2009). Mycotoxin standards were obtained from the Sigma Chemical Co. (St Louis, MO, USA).

Mycotoxin analysis:

For mycotoxin analysis, fifty grams fine grounded powdered samples of each fruit were extracted with chloroform. Thin Layer Chromatography (TLC) technique was employed in 20x20 cm glass plates with gel silica to detect and identify mycotoxins (Singh 1988). For TLC, 10 µL of extracts were loaded in plates, together with specific standards, developed in benzene: methanol: acetic acid (24:2:1) and toluene: ethyl acetate: formic acid (6:3:1) solvents and then observed under long wavelength UV- light at 365 nm.

Statistical Analysis:

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

RESULTS

Fungal contamination:

Fungal populations isolated from fruit samples are shown in Tables 2-4. Maximum fruit samples were found to be associated with fungal contaminants. The mycological examination of fruit samples revealed that 100% fruit samples of *T. bellerica* followed by *T. chebula* (97.5%) and *E. officinalis* (86.48%) were found to be

Table 1: Description of the plant samples used and %age of contaminated samples

Sr. No.	Botanical Names	Part used	No. of contaminated samples / No. of samples examined	%age of samples contamination	Moisture content
1	*Emblica officinalis*	fruit	32/37	86.48%	8.21%
2	*Terminalia bellerica*	fruit	29/29	100%	9.29%
3	*Terminalia chebula*	fruit	39/40	97.5%	4.55%

highly contaminated with one or another fungal species. A variation in the percent moisture content was observed among three types of fruits. Highest 9.29% moisture content was observed in *T. bellerica* fruits whereas, it was 8.21% & 4.55% in *E. officinalis* and *T. chebula* fruit samples respectively (Table 1).

During investigation seventeen different fungal species belonging to six genera were isolated from all the fruit samples on PDA and CDA media. The predominant mycoflora obtained was distributed in six different genera comprising of *Aspergillus* with maximum nine species, *Penicillium* (3 species) and other like *Curvularia*, *Alternaria*, *Rhizopus* and *Syncephalastrum* with single species each. Some

Table 2: Percent Incidence of fungal genera isolated from fruit samples

Fungal genera	Amla fruits	Baheda Fruits	Haritiki fruits	Mean±SD
Aspergillus	83.78%	90%	100%	91.26±8.18308
Penicillium	13.51%	15%	20.68%	16.39±3.783548
Curvularia sp.	-	2.5%	-	2.5±0.00
Alternaria sp.	-	5%	3.44%	4.22±1.103087
Rhizopus sp.	13.51%	12.5%	24.13%	16.71±6.442844
Syncephalastrum sp.	2.70%	-	-	2.7±0.00

unknown fungal species are also isolated from fruit samples. The broadest spectra of fungal genera and species were recorded in Amla fruits (4 genera and 13 species), haritiki fruits (5 and 12) and (3 and 8) in baheda fruits (table 3&4). Based on incidences of occurrence of fungal isolates, isolated from fruit samples, *Aspergillus* species were most frequent (91.26%) followed by *Penicillium* species (16.39%) and *Rhizopus* species (16.71%). Three other genera like *Alternaria*, *Curvularia* and *Syncephalastrum* were observed in the range of 4.22%-2.5%, while, *Curvularia* and *Syncephalastrum* were isolated only from baheda and amla fruits respectively (table 2).

Table 3 & 4 showing the percentage relative frequency and density of fungi infected fruit samples. Based on relative frequency as well density *Aspergillus niger* was the predominant fungal species recorded with highest frequency (77.58%) and density (51.82%). Another, most prevalent fungal species was *A. fumigatus*, having high relative frequency (42.43%) and relative density (23.32%). *A. flavus* was also a frequently recorded fungal species among all the fruit samples, with relative frequency17.25% followed by *Penicillium rubrum* (11.71%) and *Rhizopus sp.* (15.81%). Other fungal species were present at low frequency in the range of

Table 3: Percentage frequency of fungi in positive infected fruit samples

Fungal Species	Amla Fruits (n=37)	Baheda Fruits (n=29)	Haritiki fruits (n=40)	Overall mean
A.niger (#7414.09)	23 (62.16%)	27 (93.10%)	31 (77.5%)	77.58±15.47
A.flavus (#7413.09)	10 (27.02%)	5 (17.24%)	3 (7.5%)	17.25±9.76
A. anstelodani(#7411.09)	3 (8.10%)	1 (3.44%)	2 (5.0%)	5.51±2.37
A. fumigates (#7408.09)	17 (45.94%)	12 (41.37%)	16 (40.0%)	42.43±3.11
A. terreus (#7406.09)	1 (2.70%)	-	-	2.7±0.00
A. versicolor	5 (13.51%)	4 (13.79%)	1 (2.5%)	9.93±6.43
A. ochraceus	1 (2.70%)	-	-	2.7±0.00
A. nidulens	2 (5.40%)	-	1 (2.5%)	3.95±2.05
A. luchensis	1 (2.70%)	-	-	2.7±0.00
P. rubrum	2 (5.40%)	5 (17.24%)	5 (12.5%)	11.71±5.95
P. citrinum (#7410.09)	3 (8.10%)	1 (3.44%)	2 (2.5%)	5.51±2.35
P. chrysogenum	-	-	1 (2.5%)	2.5±0.00
Curvularia lunata (#7407.09)	-	-	1 (2.5%)	2.5±0.00
Alternaria alternate (#7405.09)	-	-	2 (5.0%)	5±0.00
Rhizopus sp.	4 (10.81%)	7 (24.13%)	5 (12.5%)	15.81±7.25
Syncephalastrum sp.	1 (2.70%)	-	-	2.7±0.00
Unknown	1 (2.70%)	4 (13.79%)	2 (5.0%)	7.16±5.85

9.93-2.5% (table 3). The highest percent relative density as shown by *A. niger* and *A. fumigatus*, moderate density was observed in *A. flavus* (7.9 %) and *Rhizopus* sp. (6.48%). The lowest relative density was recorded in case of *Aspergillus spp.*, *Penicillium spp.*, *Alternaria sp.*, *Curvularia sp.* and *Alternaria sp.* in the range of 4.37- 0.29% (Table 4).

Within the genus *Aspergillus*, *A. niger* was recovered from 93.10% of baheda fruits followed

Table 4: Percentage Relative density of fungi in positive infected fruit samples

Genera of moulds	Amla Fruits		Baheda Fruits		Haritiki fruits		Overall
	N	%	N	%	N	%	mean±SD
A.niger	97	36.88	214	57.06	211	61.54	51.82±13.13
A.flavus	*34*	12.92	35	9.33	5	1.45	7.9±5.86
A. anstelodani	7	2.66	5	1.33	9	2.62	2.20±0.75
A. fumigatus	83	31.55	49	13.06	87	25.36	23.32±9.41
A. terreus	2	0.76	-	-	-	-	0.76±0.00
A. versicolor	11	4.18	10	2.66	1	0.29	4.37±1.82
A. ochraceus	1	0.38	-	-	-	-	0.38±0.00
A. nidulens	4	1.52	-	-	2	0.58	1.05±0.66
A. luchensis	1	0.38	-	-	-	-	0.38±0.00
P. rubrum	2	0.76	18	4.8	16	4.66	3.40±2.29
P.citrinum	4	1.52	4	1.06	3	0.87	1.15±0.33
P. chrysogenum	-	-	-	-	3	0.87	0.87±0.00
Curvularia lunata	-	-	-	-	1	0.29	0.29±0.00
Alternaria alternata	-	-	2	0.53	2	0.58	0.55±0.35
Rhizopus sp.	12	4.56	34	9.06	20	5.83	6.48±2.32
Syncephalastrum sp.	4	1.52	-	-	-	-	1.52±0.00
Unknown	1	0.38	4	1.06	6	1.74	1.06±0.68

(N = number of positive infected fruit samples)

by haritiki fruits (77.50%) and amla fruits (62.16%). Fungal species like *A. terreus, A. versicolor* and *A. luchensis* were observed less frequently in amla fruits while other *Aspergillus* sp. like *A. fumigatus* and *A. flavus* were found to be more common in all the fruit samples (table 3).

Mycotoxin Analysis:

Total one hundred and six fruit samples were analyzed for mycotoxin detection through thin layer chromatography (TLC). About 50% samples were found to be contaminated with various mycotoxins. A total of six mycotoxins namely aflatoxin B1 and B2, aflatoxin G1 and G2, citrinin and sterigmatocystin were detected during mycotoxin analysis of fruit samples. Aflatoxin B2 was detected as an important contaminant in 34.43% of the tested fruit samples followed by sterigmatocystin (17.54 %), aflatoxin B1 (12.88%). Aflatoxin G1 and G2 were detected in 9.22% and 11.10% fruit samples respectively whereas; only 3.07 % fruit samples were showing the presence of citrinin. Aflatoxin B2 was detected maximum in 65.51% baheda fruits, 22% in haritiki fruits and 15.8% in amla fruits only. Sterigmatocystin, the second major detected mycotoxin, was found only in 27.58% baheda and haritiki fruits (7.5%). All the six mycotoxins were detected from baheda fruits; whereas, amla fruits were showing the presence of all the mycotoxins except sterigmatocystin. In haritiki fruits only three mycotoxins namely aflatoxin B1 and B2 and sterigmatocystin were detected (table 5).

Table 5: Percentage of samples contaminated with different mycotoxins.

Mycotoxins	Amla fruits	Baheda fruits	Haritiki fruits	Mean±SD
AFB1	18.91	17.24	2.5	12.88±9.03
AFB2	15.8	65.51	22	34.43±27.08
AFG1	8.1	10.34	-	9.22±1.58
AFG2	16.21	6.0	-	11.10±7.21
Citrinin	2.7	3.44	-	3.07±0.52
Sterigmatocystin	-	27.58	7.5	17.54±14.19

DISCUSSION

During the survey and collection of samples it was found that these fruits are mostly transported from the field to market without taking any care regarding the quality of fruit / or damage. In addition, these fruits are stored within open and unclean tin containers in the market, thereby exposing them to microbial infection. Occurrence of *Aspergillus* and *Penicillium* as the most dominant fungal genera in the collected samples indicates the improper or unhygienic storage conditions which resulting into microbial contamination. These results are

in accordance with the previous reports (Abou *et al.* 1999, Gautam and Bhadauria 2008, 2009). The presence of wide range of fungi in these medicinally important herbal fruits showed that there is a potential risk for mycotoxins contamination, especially during prolonged storage in poor conditions of temperature and moisture (Bugno *et al.* 2006, Singh *et al.* 2008). Results of present investigation reveled that high moisture contents of the fruit samples may be responsible for the incidence of higher fungal counts (Essono *et al.* 2007). Most of the identified fungal species like *Aspergillus*, *Penicillium* and *Alternaria* are reported to have ability to produce mycotoxins like aflatoxins, ochratoxins and citrinin (Hitokoto *et al.* 1978, Bugno *et al.* 2006). Mycotoxins are causing toxicological and immunologic problems in animals and human beings through the contamination of cereals, food and other commodities (Shephard 2008). 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 (Noonimabc 2009). The presence of *A. flavus* in the stored fruits is also alarming since this fungus initially colonizes the substrate and predisposes the infected substrate to mycotoxin contamination (Diener *et al.* 1987). Aflatoxin contamination in crude samples of drug plants used in churn preparation (Efuntoye 2004) have also been reported.

The presence of different mycotoxins is also of the matter of concern because 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 (Shephard 2008). Presence of sterigmatocystin mycotoxin also makes a point of attention because this mycotoxin is reported to be a liver carcinogen and has immunosuppressive effects (Rotter *et al.* 1996). So, quality evaluation of these herbal fruits is necessary before being used in the preparation of Triphala churn.

The present pilot study on herbal fruits therefore indicates that the presence of *Aspergillus*, *Penicillium* and *Alternaria* toxigenic fungal species as well as mycotoxins like Aflatoxin B and G, citrinin and sterigmatocystin in these stored herbal fruits are alarming. Fungal contamination of these herbal fruits not only reduces the therapeutic potential of a drug but can also harm human health due to their mycotoxins. These also put a question mark on the use of these fruits as herbal medicine directly or as the raw material for the preparation of triphala churn. Therefore, it is necessary to improve the processing methods such as harvest, drying, transportation and storage. More studies are still required to find out more appropriate methods of decontamination of herbal raw materials.

The authors are thankful to the Head, School of Studies in Botany, Jiwaji University, Gwalior, for providing necessary laboratory facilities and Madhya Pradesh Council of Science and Technology, Bhopal (India) for financial assistance.

REFERENCES

Abou-Arab AA, Soliman KM, El-Tantawy ME, Ismail BR & Naguib K 1999 Quantity estimation of some contaminants in commonly used medicinal plants in Egyptian market. *Food Chemistry* **67** 357-363.

Bugno A, Adriana ABA, Tatiana CP, Terezinha AP & Myrna S 2006 Occurrence of toxigenic fungi *J Microbiol* **37** 47-51.

Diener UL, Cole RJ & Hill RA 1987 Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annu Rev Phytopathol* **25** 249–270.

Donia MA 2008 Microbial quality and aflatoxinogenesis of Egyptian spices and medicinal plants. *Global Vaternia* **2**1 75-181.

Dubey NK, Kumar A, Singh P & Shukla R 2008 Microbial contamination of raw materials: A major reason for the decline of India's share in the global herbal market. *Current Science* **95** 717-718.

Efuntoye MO 2004 Fungi associated with herbal drug plants during storage. *Mycopathologia* **136** 115-118.

Essono G, Ayodele M, Akoa A, Foko J, Olembo S & Gock J 2007 *Aspergillus* species on cassava chips in storage in rural areas of southern Cameroon: their relationship with storage duration, moisture content and processing methods. *African J Microbiol* 001-008.

Gautam AK & Bhadauria R 2008 Occurrences of toxigenic moulds and mycotoxins in ayurvedic medicine *trifla churn*. *J Mycol Pl Pathol* **38** 664-666.

Gautam AK & Bhadauria R 2009 Mycoflora and mycotoxins in some important crude and powdered herbal drugs. *Biological forum- An Int. Journal* **1** 1-7

Gilman JC 1975 A manual of soil fungi. Oxford & IBH Publishing Corporation, New Delhi, Bombay, Calcutta. Second edition.

Hitokoto H, Morozumi S, Wauke T, Saka S & Kurata H 1978 Fungal contamination and mycotoxin detection of powdered herbal drugs. *Appl and Environ Microbiol* **36** 252-256.

Houssou PA, Ahohuendo BC, Fandohan P, Kpodo K, Hounhouign DJ & Jakobsen M 2009 Natural infection of cowpea (*Vigna unguiculata* (L.) Walp.) by toxigenic fungi and mycotoxins contamination in Benin, West Africa. *J Stored Food Research* **45** 40-44.

Juss SS 1997 Triphala D The wonder drug. *Indian Med Gazette* **13** 194-96.

Noonimabc P, Mahakarnchanakulb W, Nielsend KF, Frisvadd JC & Samsona RA 2009 Fumonisin B2 production by *Aspergillus niger* in Thai coffee beans. *Food Additives and Contaminants* **26** 94-100.

Padh H 2001 Herbal medicine. *Current Science* **81** 15.

Rai V & Mehrotra S 2005 Toxic Contaminants in Herbal Drugs. *Environ News* **11(4)** 1-3.

Rotter BA, Prelusky DB & Pestka JJ 1996 Toxicology of Deoxynivalenol (vomitoxin). *J Toxicol Environ Health* **48** 1-34.

Roy AK 2003 Mycological problems of crude herbal drugs - Overview and Challenges. *Indian Phytopathology* **56** 1-13.

Sharma A, Shanker C, Tyagi LK, Singh M & Rao CV 2008 Herbal Medicine for Market Potential in India: An Overview. *Academic J Plant Scien* **12** 26-36.

Shephard GS 2008 Impact of mycotoxins on human health in developing countries. *Food Additives and Contaminants* **25** 146-151.

Singh P, Srivastava B, Kumar A & Dubey NK 2008 Fungal Contamination of Raw Materials of Some Herbal Drugs and Recommendation of *Cinnamomum camphora* Oil as Herbal Fungitoxicant. *Microbial Ecology.* DOI 10.1007/s00248-008-9375-x.

Singh PL 1988 Studies on deleterious effects of mycotoxin contaminated pulses and its control. P.G. Department of Botany, Bhagalpur. Pp 5-19.

Trucksess MW & Scott PM 2008 Mycotoxins in botanicals and dried fruits: A review. *Food Additives and Contaminants;* **25** 181-192.