



## INCIDENCE OF TOXIGENIC FUNGI AND MYCOTOXINS IN MEDICINAL HERBS AND HERBAL DRUGS

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Incidence of toxigenic fungi and mycotoxin contamination studied in medicinal herbs namely *Aegle marmelos*, *Embllica officinalis*, *Terminalia bellerica*, *T. chebula*, and *Syzygium cumini* collected from Uttarakhand region under field and storage conditions. Natural contamination of mycotoxins in some commonly used herbal drugs Triphala, Chyawanprash, Isabbel, Amla ambari, Amla sada, Dolabi and Isabgol chaff was also evaluated. Mycotoxin producing fungi like *A. flavus*, *A. ochraceus*, *F. moniliforme* and *Penicillium citrinum* were recorded on all herb samples under study. Aflatoxins were the most common mycotoxins elaborated by different isolates of *A. flavus* isolated from samples under study. Mycotoxin contamination in fresh and stored samples of medicinal herbs under study showed aflatoxin B<sub>1</sub> as the most common natural contaminant. The concentration of aflatoxin B<sub>1</sub> in the fresh medicinal samples was quite high i.e. upto 0.45 µg/g in *A. marmelos* followed by *T. chebula* (0.22 µg/g), *E. officinalis* (0.21 µg/g) and *T. bellerica* (0.12 µg/g). Aflatoxin B<sub>1</sub> was the most common contaminant in most of the herbal drugs. The concentration of Aflatoxin B<sub>1</sub> in Triphala was quite high (0.62-1.83 µg/g) followed by Isabbel (0.59-1.13 µg/g), Amla ambari (0.52-1.69 µg/g) and Chyawanprash (0.49-1.24 µg/g).

**Key Words:** Mycotoxins, Toxigenic fungi, Medicinal herbs, Herbal drugs

Medicinal herbs have a long history of use in therapy throughout the world and make an important part of traditional medicine. A large section of population in India seek remedies to their health ailments through indigenous system of medicine namely Ayurveda, Unani, Siddha and Tibetan medicine. According to the World Health Organization (WHO), eighty percent of the population in developing countries relies on traditional medicine, mostly in the form of plant drugs for their health care needs.

The state of Uttarakhand in the western Himalayan region is a reservoir of herbs and has more than 700 species of medicinal plants used in traditional system of medicine. A substantial part of the demand of herbal based pharmaceutical industries is met through wild collections from the forests. Very often these herbal products are collected and stored haphazardly without caring for the quality of the produce. Medicinal samples may get contaminated by various fungi during harvesting, storage and distribution. Limited studies carried out on herbal samples indicate that these are capable of harbouring toxigenic fungi and threat of mycotoxin on them exists (Chourasia 1995, Khan and Singh 2000, Singh

2003). It has also been established that fungal infection and mycotoxin contamination cause deterioration in the chemical constituents of the plant material under storage which may result in reduced efficacy and unsafe herbal preparations (Dutta and Roy 1987).

Mycotoxins, secondary metabolites of mold fungi, have been reported in many agricultural/horticulture crops/edible & medicinal seeds of forest origin and Tree Borne Oil Seeds screened for toxigenic molds (Aziz 1987, Van Egmond 1981, Singh *et al.* 2001, Singh and Shukla 2008, Singh 2012, Singh 2014). These toxins may remain in the substrate even after the moulds responsible have died. Among these aflatoxins are the most harmful mycotoxins due to their carcinogenicity to men and animals and higher frequency of occurrence under natural conditions. Aflatoxins have been implicated in hepatocellular carcinoma, acute hepatitis, Reye's syndrome, cirrhosis and kwashiorkor (Wary 1981, Gaurama and Bullerman 1995).

Present study was carried out to study occurrence of mycotoxin producing fungi, investigate the strains of fungi isolated for their ability to produce mycotoxins, and evaluate

natural occurrence of mycotoxins in some important medicinal herbs namely *Aegle marmelos*, *Emblica officinalis*, *Terminalia bellerica*, *T. chebula*, and *Syzygium cumini* as well as some commonly used herbal drugs i.e. Triphala, Chyawanprash, Isabbel, Amla ambari, Amla sada, Dolabi and Isabgol chaff based on the raw materials under study.

## MATERIAL AND METHODS

**Collection of Samples:** Fresh samples of *Aegle marmelos*, *Emblica officinalis*, *Terminalia bellerica*, *T. chebula*, and *Syzygium cumini* fruits/seeds were collected from different forest division of Uttarakhand during their harvesting season. Stored samples were procured from different storage centres of the state and sample of herbal drugs were procured from pharmaceutical industries.

**Mycoflora Counts:** In order to record the fungal flora associated with fresh and stored medicinal samples, blotter test as well as agar plate methods as recommended by International Seed Testing Association (ISTA 1999) was followed. Mycotoxin producing fungi were recorded, isolated, and maintained on culture media for determining their toxigenic potential.

**Screening for toxigenic Potential of isolated fungi:** The aflatoxin producing potentials of *Aspergillus flavus* Link ex Fries isolates were tested in SMKY liquid medium (Diener & Davis 1966). The constituents of the medium were, Yeast extract - 7g; Sucrose - 200g; Magnesium Sulphate ( $MgSO_4 \cdot 7H_2O$ ) - 0.5g; Potassium Nitrate ( $KNO_3$ ) - 3g; Distilled water- 1litre. All isolates of *Aspergillus ochraceus* and *Penicillium citrinum* were screened for their ochratoxin and citrinin level respectively by inoculating them in the liquid media composed of Sucrose - 40 g; Yeast extract-20 g; Distilled water-1 litre (Schwenk *et al.* 1958, Davis *et al.* 1972). Zearalenone producing ability of isolates of *Fusarium* was evaluated on moist - rice medium (Scott *et al.* 1970). All flasks were incubated at  $28 \pm 2^\circ C$  for 11 days and then filtered. The filtrate was extracted

with chloroform and the chloroform extract were used for qualitative and quantitative detection of mycotoxins.

**Natural Occurrence of Mycotoxins:** The herb samples were extracted chemically for the presence of aflatoxins by the method of Thomas *et al.* (1975). Few samples, in which fungi producing other mycotoxins were associated, were extracted by the method of Roberts and Patterson (1975). The chloroform extract were used for qualitative and quantitative detection of mycotoxins. The same procedure was followed for evaluating mycotoxin in the herbal drugs as well.

**Qualitative detection and chemical confirmation of Mycotoxins:** Qualitative and quantitative estimation of the mycotoxins were carried out using Thin Layer Chromatography (TLC). Silica Gel - G (with 13%  $CaSO_4$  as binder) was used as stationary phase for the TLC. 50  $\mu l$  of chloroform extract was spotted on TLC plates along with the standards obtained from Sigma, USA. The spotted chromoplate was developed in the solvent system comprising Toluene: isoamyl alcohol: methanol (90:32:2, v/v/v). After developing, the plates were air dried and were observed under long (360nm) and short (260 nm) wavelengths UV light for the detection of mycotoxins. Chemical confirmation of aflatoxin was done by Trifluoroacetic acid (TFA) as suggested by Stack and Pohland (1975). Presence of Ochratoxin on TLC plates was confirmed with ammonia fumes which changed blue green spot to deep blue colour (Davis *et al.* 1969). Confirmation of citrinin was done by spraying TLC plates with freshly prepared mixture of 0.5 ml p- anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of conc.  $H_2SO_4$  and then by heating the plate at  $130^\circ C$  for 10 minutes. This changed yellow streak of citrinin to yellowish green under long wave UV- light (Scott *et al.* 1970). Zearalenone was also confirmed by spraying TLC plates with acidic p- anisaldehyde solution (Scott *et al.* 1970) by which greenish blue fluorescence turned faint brown (in visible light) and faint yellow in long

wave UV-light.

### Quantitative Estimations of Aflatoxin:

Aflatoxin being most potent mycotoxin, the quantitative estimation for the same was carried out. Quantity of aflatoxin was estimated spectrophotometrically (Nabney & Nesbitt 1965) with the help of UV Spectrophotometer.

### RESULTS

Mycotoxin producing fungi like *A. flavus*, *A. ochraceus*, *F. moniliforme* and *Penicillium citrinum* were recorded on all herb samples under study. Aflatoxins were the most common mycotoxins elaborated by different isolates of *A. flavus* isolated from samples of *Aegle marmelos*, *Emblica officinalis*, *Terminalia bellerica*, *T. chebula*, and *Syzygium cumini*. Out of 75 isolates from *A. marmelos*, 27 were toxigenic and produced aflatoxins in the liquid medium. Afl. B<sub>1</sub> was elaborated by 13 isolates whereas 6 isolates produced afl. B<sub>1</sub> and B<sub>2</sub>, only 3 elaborated B<sub>1</sub>, B<sub>2</sub> and G<sub>1</sub>. However, afl. B<sub>1</sub>B<sub>2</sub>G<sub>1</sub>G<sub>2</sub> were elaborated by 5 isolates. In *E. officinalis* 17 out of 52 isolates produced aflatoxin. Out of which 9 produced afl. B<sub>1</sub> and 8 afl. B<sub>1</sub>B<sub>2</sub>. In *T. bellerica* 7 produced afl. B<sub>1</sub> and 2 afl. B<sub>1</sub>B<sub>2</sub> out of 55 isolates of *A. flavus* screened for aflatoxin. On the other hand in *T. chebula* 12 isolates produced afl. B<sub>1</sub> out of 55 screened. In case of *S. cumini*, 3 isolates produced afl. B<sub>1</sub> out of 32 and 21 isolates screened (Table-1).

The amount of aflatoxins produced by the toxigenic isolates of *A. flavus* was in the range of 0.9 - 26 µg/ml in case of *A. marmelos*, 0.4 - 21.0 µg/ml in *E. officinalis*, 0.6 - 22 µg/ml in *T. bellerica*, 0.4 - 18 µg/ml in *T. chebula* and 0.3 - 6.2 µg/ml in *S. cumini*. The percentage of toxigenic isolates was comparatively lower in other mycotoxin producing fungi isolated from all the samples. 6 out of 22 isolates of *A. ochraceus* from *A. marmelos*, 7 out of 37 from *E. officinalis*, 5 out of 43 from *T. bellerica*, and 3 out of 37 from *T. chebula* elaborated och.A in liquid medium. Zearalenone was produced by 4 isolates of *F. moniliforme* from *A. marmelos* (out of 14). Citrinin was elaborated by 30.07%

isolates of *P. citrinum* obtained from *A. marmelos*, 14.81% in case of *E. officinalis*, 21.87% in *T. bellerica*, and 8.57% in *T. chebula* (Table-1).

Mycotoxin contamination in the samples of *Aegle marmelos*, *Emblica officinalis*, *Terminalia bellerica*, *T. chebula*, and *Syzygium cumini* showed aflatoxin B<sub>1</sub> as the most common natural contaminant. The concentration of aflatoxin B<sub>1</sub> in the fresh herbal samples also was high i.e. upto 0.45 µg/g in *A. marmelos* followed by *T. chebula* (0.22 µg/g), *E. officinalis* (0.21 µg/g) and *T. Bellerica* (0.12 µg/g). Aflatoxin B<sub>1</sub> was the most common mycotoxin encountered as natural contaminant in stored herb samples also. Aflatoxins were detected almost on all samples analyzed for mycotoxin contamination, however, zearalenone was detected in traces only on *A. marmelos*. The conc. of aflatoxin B<sub>1</sub> was in the range of 0.13 - 0.75 µg/g in *A. marmelos*, 0.13 - 0.75 µg/g in *T. chebula*, 0.08 - 0.30 µg/g in *E. officinalis*, 0.08 - 0.14 µg/mg in *T. bellerica* and 0.06 - 0.13 µg/g in *S. cumini* (Table-2).

Studies carried out for natural occurrence of aflatoxins on selected herbal drugs exhibited higher concentration of aflatoxin B<sub>1</sub>. The range of concentration of AFB<sub>1</sub> in Triphala was (0.62-1.83 µg/g), Isabbel (0.59-1.13 µg/g), Amla ambari (0.52- 1.69 µg/g), Chyawanprash (0.49-1.24 µg/g). It was in traces in Amla sada and Dolabi. The higher level of aflatoxin i.e. 0.98- 1.83µg/g in Triphala is a matter of great concern as the mycotoxin reduces the effectiveness of the medicine and poses serious threat to human health due to its hepatotoxic, nephrotoxic and cytotoxic effects.

### DISCUSSION

The results of the present investigation indicate that a large number of fungi were associated with the samples of *Aegle marmelos*, *Emblica officinalis*, *Terminalia bellerica*, *T. chebula*, and *Syzygium cumini*. Association of fungi as well as their incidence are governed by the nature of the substrates,

**Table 1:** Screening of fungi for mycotoxin producing potential

Substrate	Fungi	Isolates screened	Toxigenic isolates	% toxigenicity	Mycotoxin produced	Level of toxin (µg/ml)
<i>A. marmelos</i>	<i>Aspergillus flavus</i>	75	27	36.00	Aflatoxins	0.9 - 26
			13	17.33	Afl. B <sub>1</sub>	
			6	8.0	Afl. B <sub>1</sub> B <sub>2</sub>	
			3	4.00	Afl. B <sub>1</sub> B <sub>2</sub> G <sub>1</sub>	
			5	6.67	Afl B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	
	<i>A. ochraceus</i>	22	6	27.27	Ochratoxin	-
	<i>F. moniliforme</i>	14	4	28.57	Zearalenone	-
<i>P. citrinum</i>	26	8	30.07	citrinin	-	
<i>E. officinalis</i>	<i>A. flavus</i>	52	17	32.69	Aflatoxins	0.4 - 21
			9	17.30	Afl. B <sub>1</sub>	
			8	15.38	Afl. B <sub>1</sub> B <sub>2</sub>	
	<i>A. ochraceus</i>	37	7	18.91	Ochratoxin	-
<i>P. citrinum</i>	27	4	14.81	Citrinin	-	
<i>T. bellerica</i>	<i>A. flavus</i>	55	9	16.36	Aflatoxins	0.6 - 22
			7	12.72	Afl. B <sub>1</sub>	
			2	3.63	Afl. B <sub>1</sub> B <sub>2</sub>	
	<i>A. ochraceus</i>	43	5	11.62	Ochratoxin	-
<i>P. citrinum</i>	32	7	21.87	Citrinin	-	
<i>T. chebula</i>	<i>A. flavus</i>	55	12	21.81	Afl. B <sub>1</sub>	0.4 - 18
	<i>A. ochraceus</i>	37	3	8.10	Ochratoxin	-
	<i>P. citrinum</i>	35	3	8.57	Citrinin	-
<i>S. cumini</i>	<i>A. flavus</i>	32	3	9.37	Afl. B <sub>1</sub>	0.3 - 6.2

**Table 2:** Natural occurrence of mycotoxins in medicinal herbs

Medicinal herbs	Fresh samples		Stored samples	
	Mycotoxin detected	Level of mycotoxins (µg / g)	Mycotoxin detected	Level of mycotoxins (µg / g)
<i>A. marmelos</i>	Afl.B <sub>1</sub>	0.32- 0.45	Afl.B <sub>1</sub>	0.13 - 0.75
<i>E. officinalis</i>	Afl.B <sub>1</sub>	0.17 - 0.21	Afl.B <sub>1</sub>	0.08 - 0.30
<i>S. cumini</i>	Afl.B <sub>1</sub>	-	Afl.B <sub>1</sub>	0.06 - 0.13
<i>T. bellerica</i>	Afl.B <sub>1</sub>	0.08 - 0.12	Afl.B <sub>1</sub>	0.08 - 0.14
<i>T. chebula</i>	Afl.B <sub>1</sub>	0.15 - 0.22	Afl.B <sub>1</sub>	0.13 - 0.75

**Table 3:** Natural occurrence of mycotoxins in herbal drugs

S.N.	Substrate	Mycotoxin detected	Level of mycotoxins (µg / g)
1	Triphala	Aflatoxin B <sub>1</sub>	0.62 -1.83
2	Chyawanprash	Aflatoxin B <sub>1</sub>	0.49 - 1.24
3	Isabbel	Aflatoxin B <sub>1</sub>	0.59 - 1.13
4	Amla ambari	Aflatoxin B <sub>1</sub>	0.52 - 1.69
5	Amla sada	Aflatoxin B <sub>1</sub>	in traces
6	Dolabi	Aflatoxin B <sub>1</sub>	in traces

methods of storage and prevailing environmental conditions.

The mycotoxigenic fungi viz. *Aspergillus flavus*, *A. ochraceus*, *Fusarium moniliforme* and *Penicillium citrinum* were commonly associated with the stored samples, however their incidence in fresh samples was

comparatively low. The range of toxin production by these fungi in liquid medium varied with the type of the substrate. It was also noted that aflatoxin B<sub>1</sub> was produced by almost all the toxigenic isolates of *A. flavus*, however, the frequency of aflatoxins other than B<sub>1</sub> was comparatively low. Toxigenic moulds have been reported as the most common fungi



isolated from medicinal herbs and their products (Ayres *et al.* 1980, Misra 1981, Aziz and Youssef 1991, Gupta *et al.* 2016). Fungal contamination in powdered samples of *Momordica charantia* and *Syzygium cumini* also reported by Gupta *et al.* (2016). The occurrence of aflatoxin in medicinal herbs and herbal products has been earlier reported by Rani and Singh (1990), Roy and Chourasia (1990), Khan and Singh (2000) and Singh (2003). The results of the natural occurrence of mycotoxin contamination of the stored fruit/seed samples under study indicate that most of the samples were contaminated with aflatoxins, although their incidence varied with type of the substrates. The variation in natural occurrence of mycotoxins may be due to differences in their moisture contents and constitutional make up. The natural contamination of mycotoxins in medicinal samples correspond to the incidence of toxigenic fungi associated with them and their respective potentiality to produce mycotoxins in the synthetic media.

The concentration of AFB<sub>1</sub> in the herbal drugs such as triphala and amla ambari was higher. Chourasia (1995) screened some herbal drugs of Indian pharmaceutical industries and reported that 64% of the finished commercial products showed the presence of Aflatoxin B<sub>1</sub> beyond the tolerance level fixed by W.H.O. Singh (2003) also reported higher level of aflatoxins in triphala (fruit triad) and its three ingredients. The higher level of aflatoxin i.e. 0.98-1.83 µg/g in Triphala in the present studies is a serious matter as it is widely used by rural & urban population of India to treat the common ailments like cold, cough, fever, constipation etc. However, more studies are required on herbal drugs to reach some definite conclusions.

## CONCLUSIONS

Presence of high concentration of aflatoxin in the medicinal herbs and herbal drugs is a matter of great concern as the mycotoxin reduces the effectiveness of the medicine and poses serious threat to human health due to its hepatotoxic, nephrotoxic and cytotoxic effects. There is a

need to restrict the use of mycotoxin contaminated samples in pharmacopoeial preparation, the raw herb samples must be graded and screened against *Aspergillus flavus*, a potent aflatoxin producing fungi. Better storage practices should be implemented to avoid fungal infection and mycotoxin contamination in the medicinal herbs.

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