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MYCOLOGICAL ANALYSIS AND AFLATOXIN B_1 CONTAMINANT ESTIMATION OF HERBAL DRUG RAW MATERIALS

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Abstract

Background: The present study explores the fungal contamination of important herbal drug raw materials (HDRM), which are widely used in the preparation of many herbal drugs. Understanding of the microbial contamination status of HDRM is one of the important steps to ensure the safety and efficacy of herbal drugs.
Materials and Methods: Eighteen samples of six herbal drug raw materials (HDRM) viz., *Acorus calamus* Linn., *Cassia angustifolia* Vahl., *Centella asiatica* (Linn.) Urban, *Myristica fragrans* Houtt.,*Tinospora cardifolia* (Wild) Miers and *Withania somnifera* (Linn.) Dunal, were screened for fungal contamination, by employing serial dilution method. All the isolates of *Aspergillus flavus* were screened for their ability to produce aflatoxin B_1 (AB_1) and highly contaminated samples were subjected to AB_1 estimation by using Thin Layer Chromatography (TLC), spectrophotometric method and occurrence of Aflatoxin B1 was confirmed by Liquid Chromatography-Mass Spectrometry analysis (LCMS).
Results: A total of 302 isolates of 42 fungal species belonging to 17 genera were found in association with test the samples. More than 61% of *A. flavus* isolates tested positive for production of AB_1 and highest yield recorded was 5008.20 ppb from the isolates of *T. cordifolia.* Amongthesix highly contaminated samples three samples tested positive for AB_1. Highest AB_1 was recorded from *T. cordifolia* (104.19 µg/kg), followed by *A. calamus* (13.73 µg/kg) and *M. fragrans* (12.02 µg/kg).
Conclusion: Assessment of fungal and mycotoxin contamination should be a part of the quality check while selecting HDRM for manufacture of herbal products. Safe processing and storage practices are necessary.

Key words: Herbal drug raw material, Fungi, Contamination, *Tinospora cordifolia*, Aflatoxin B_1

Introduction

Plants have been the most important resource for the preparation of Nutraceuticals and medicines since time immemorial. Medicinal plants are widely used as raw material for dietetic supplements and pharmaceutical products, which are used to maintain good health and for the cure of diseases, specifically for self-medications (Moorthy et al. 2010). In recent times, the use of herbal products has increased in both developing and developed countries, in the belief that being natural they are safe and harmless (Kosalec et al. 2005). Of late, the ever increasing acceptability of herbal remedies and herbal food supplements, the quality, efficacy and safety have become important issue of concern for health authorities, herbal drug and Nutraceuticals industries (Anonymous, 2007).

Unfortunately, there is a considerable increase in the number of reports regarding the people experiencing negative effects, caused by the use of herbal preparations. One of the reasons for such problem is the poor quality of HDRM and insufficient attention being paid to the quality assurance and control of these products. Although WHO has framed guidelines for the quality control of herbal materials, which provide a detailed description of the techniques and measures required for the appropriate cultivation and collection of HDRM, there is still a lacuna between the available knowledge and implementation, because farmers and other relevant persons like producers, handlers and processors of HDRM and products are not professionally trained and are not much aware of the WHO's guidelines. They continue their work without any quality control measures resulting in inferior quality of herbal products with lots of contaminants including microbes (Anonymous, 2001).

HDRM are prone to infestation by microorganisms specially moulds, as they are organic in nature.Fungal contaminations are known to affect the medicinal potential of herbal drugs and nutritional value of the herbal preparations by alerting chemical composition of the HDRM(Singh et al. 2008). Some of these fungal contaminants are toxigenic in nature that involve potential hazard to human and animal health. *Aspergillusflavus* is the most important fungi, as it produces aflatoxins, a group of extremely toxic substances that exhibit acute or chronic hepatotoxicity and is a potent carcinogen and mutagen (Rizzo et al. 1998).

Mysuru is an important city of Karnataka, India from the point of view of herbal drugs and herbal Nutraceuticals manufacturing and sales. None of the earlier workers have made any attempt to screen the HDRM of Mysuru city for microbial contamination in general and fungal contamination in particular. Considering these, an attempt was made to screen the widely used HDRMfor fungal contaminationwith special reference to diversity, frequency and density of fungal contaminants. In addition, an effort has been made to identify the toxigenic isolates of *A. flavus* and estimate the AB_1 in herbalmaterials, so as to assess the risk associated with consumption of herbal formulations made out of such contaminated samples. The results of the study would help to formulate management strategies to prevent or minimize fungal contamination.

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Materials and methods

Sample Collection

Eighteen samples of six HDRM viz., *Acorus calamus, Cassia angustifolia, Centella asiatica, Myristica fragrans, Tinospora cardifolia* and *Withania somnifera* were collected from 3 different market areas of the city of Mysuru. Plant materials were selected based on their wide usage in herbal medicine and Nutraceuticals. Samples were obtained in sterile airtight containers to avoid further contamination.

Mycological Analysis

Standard blotter method (ISTA, 2003) and serial dilution method (Koch, 1883) were employed for determining fungal association with the selected HDRM.

Standard Blotter Method (SBM)

Samples were placed on the blotters at the rate of 2 to 10 units per plate (based on the size of herbal raw material unit). Plates were incubated for seven days at 22±2^{o} C under alternating cycles of 12/12 hours NUV light and darkness, and observed for the associated fungi.

Serial Dilution Method (SDM)

One g of each sample was suspended in 9ml of sterile distill water and ten-fold serial dilutions (1:10) were prepared. One ml aliquot of each dilution was aseptically spread on Czapek Dox Agar (CDA) plates and were incubated at 28±2°C for 7 days and examined daily.

Identification of Fungi

After 7 days of incubation, fungi associated with HDRM were isolated on CDA to obtain a pure culture. Each fungus was observed under stereo-binocular microscope and identified primarily on the basis of their morphological and cultural characteristics. Further their identity was confirmed microscopically by staining with cotton blue in lactophenol using standard manuals.

Statistical Analysis

Percentage of fungal contamination of HDRM in SBM was calculated by using the formula:

$$\text{Percentage of fungal contamination} = \frac{\text{No. of units contaminated with fungi}}{\text{Total No. of units examined}} \times 100$$

Percent relative density of different fungi on the CDA resulted by surface washing of HDRM following SDM was calculated by using the formula:

$$\text{Relative density \%} = \frac{\text{No. of colony of individual fungus}}{\text{Total no. of colonies of all fungal species}} \times 100$$

Percent occurrence frequency of fungi with the herbal samples was determined using the following formula:

$$\text{Occurrence frequency \%} = \frac{\text{No. of individual fungal isolates on drug sample}}{\text{Total no. of fungal isolates on drug sample}} \times 100$$

Detection of Toxigenic Strain of *A. flavus* and Estimation of AB_1

All the isolates of *A. flavus* were screened for the production of the AB_1 using SMKY liquid medium (sucrose 200 g; magnesium sulphate, 0.5 g; potassium nitrate, 0.3 g; yeast extract, 7 g and 1 L of distilled water). TLC method (Singh et al., 2008) was employed for the estimation of AB_1 with slight modification. For quantitative estimation, spots of AB_1 on TLC were scraped out and mixed with 20 ml chloroform and filtered through Whatman (No.1) filter paper. The filtrate was concentrated through evaporation and dissolved in 4 ml of cold methanol, shaken well and the optical density was recorded at a wavelength of 360 nm and the amount of AB_1 was calculated.

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Estimation of AB_1 Content of Herbal Drug Raw Materials

Highly contaminated samples were selected and screened for AB_1 using TLC and spectrophotometric method (Thomos et al., 1975). Quantitative estimation was done using the following formula (Mohana et al., 2014).

$$AB_1 \text{ content } (\mu g/L) = [(D \times M) / (E \times L)] \times 1000$$

Where, D= absorbance; M= molecular weight of AB_1 (312); E= molar extinction coefficient of AB_1 (21,800) and L= path length (1 cm)

Confirmation of Aflatoxin B_1 by LC-MS

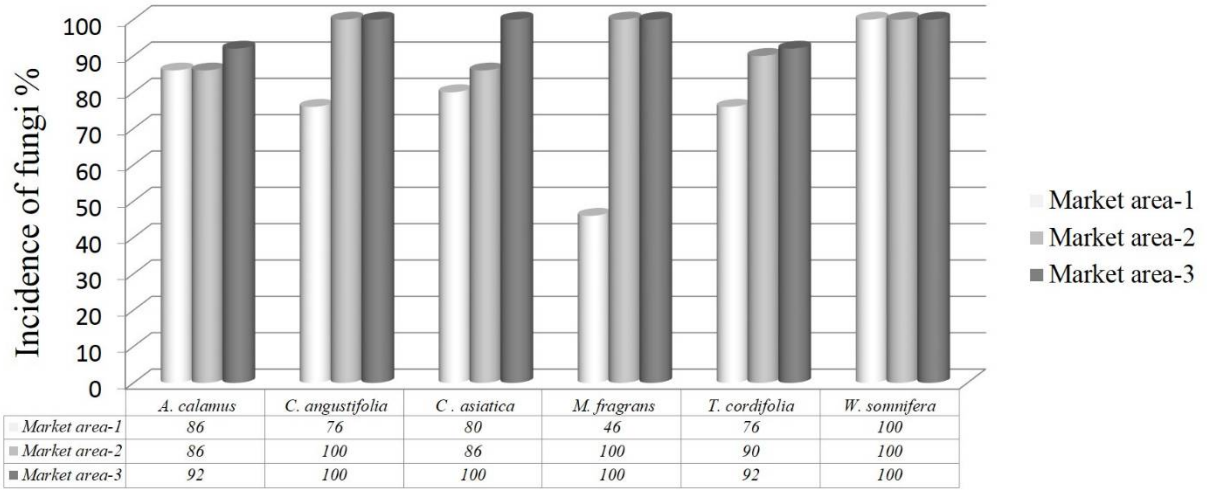
Positive samples were further confirmed by LC-MS analysis using aultra-performance liquid chromatography (UPLC) ACQUITY/SYNAPTG2S mass spectrometer. The samples were separated by the ACQUITY UPLCBEH C18 column (Waters, 1.7um 1.0 x 50mm particle size), using the mobile phase with aqueous 0.1% formic acid in deionized water and 0.1% formic acid in acetonitrile, at a flow rate of 0.3 ml/min. The volume of each sample injected was 2uL. The electrospray positive ionization (ESI+) source had the following settings: capillary voltage of 1.45 kV, cone voltage of 33-45 V, source temperature of 100 C, desolvation temperature of 150 C, cone gas flow rate of 00 l/h, desolvation gas flow rate of 500 l/h with nitrogen. Mass spectra ranging from m/z 100 to 500 were taken in positive-ion mode. Two characteristic fragmentations of the protonated molecular ion [M + H]+ were monitored for AB_1, the parent ion is m/z 313 and product ion was m/z 330.

Results and Discussion

Mycological analysis:

The use of herbal medicines and Nutraceuticals is continually expanding worldwide. In alternative system of medicine plant materials are commonly used as single drug or as ingredient of herbal formulations or as a part of food. The herbal materials used in these preparations have direct impact on the efficacy of the drug and nutraceuticals. The plant material generally used in herbal preparations provide nutrition to microorganisms and facilitates the growth and multiplication of microorganisms, specially moulds, which lead to contamination, deterioration and variation in chemical composition. This leads to inferior quality of herbal products with little or no therapeutic and nutritional efficacy. Understanding of microbial contamination status of HDRM is one of the important steps to ensure the safety and efficacy of herbal products. Reports on the risk-adverse effects associated with the use of herbal materials and products are available worldwide (Hitokoto et al., 1978; Lutz et al., 2006; Rizzo et al., 1998) including India (Mohana et al., 2014; Singh et al., 2008), but no report is available on microbial contamination of HDRM of this part of the state which is an important hub of the herbal preparations.Considering this the present investigation was carried out andfungal and aflatoxin B_1 contamination of HDRM collected from different market areas of Mysuru has been reported for the first time.

Results of the study revealed that all the 18 tested samples of 6 HDRM were found to be contaminated with various fungi. *W. somnifera* was found to be highly contaminated (100%) followed by *C. angustifolia*(92%), *C. asiatica* (88.6), *A. calamus* (88%), *T. cordifloia* (86%) and *M. fragrans* (82%) (Figure 1) Diversity and frequency of different fungi on HDRM is presented in figure 2. A total of 41 fungal species belonging to 16 genera were isolated and identified. The genus *Aspergillus* and *Penicillum* were more predominant. *A. niger* was found to be most frequently occurring fungi followed by *A. flavus.*



	A. calamus	C. angustifolia	C . asiatica	M. fragrans	T. cordifolia	W. somnifera
Market area-1	86	76	80	46	76	100
Market area-2	86	100	86	100	90	100
Market area-3	92	100	100	100	92	100

Figure 1: The percent incidence of fungi associated with herbal drug raw materials

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Fungal contaminants can be categorized into two groups, field (*Alternaria, Helminthosporium, Fusarium* and *Cladosporium*) and storage (*Aspergillus* and *Penicillium*) fungi (Domsch et al., 1981; Aziz et al., 1998). The results of the present study reveals the contamination of all the HDRM by both field and storage fungi indicating poor pre and post-harvest practices followed, especially during transportation and storage.

Samples

Table 1: Fungal contaminants per gram of herbal drug raw materials (CFU/gm)

Herbal drug raw materials	**Herbal drug raw materials (CFU±SE)**					
	A. calamus	***C. angustifolia***	***C. asiatica***	***M. fragrans***	***T. cordifolia***	***W. somnifera***
Market area-1	$13x10^5$±0.81	$2.66x10^3$±0.47	$22.66x10^3$±2.05	$4.66x10^5$±0.94	$61.33x10^5$±2.49	$2.66x10^3$±0.47
Market area -2	$1.33x10^5$±0.47	$9x10^3$±0.81	$12x10^5$±1.63	$0.66x10^5$±0.47	$1.33x10^5$±0.47	$13.66x10^5$±2.05
Market area -3	$2x10^5$±0.81	$9.66x10^5$±1.69	$52x10^5$±1.63	$51.66x10^5$±1.24	$17.66x10^5$±1.24	$1.66x10^2$±0.47

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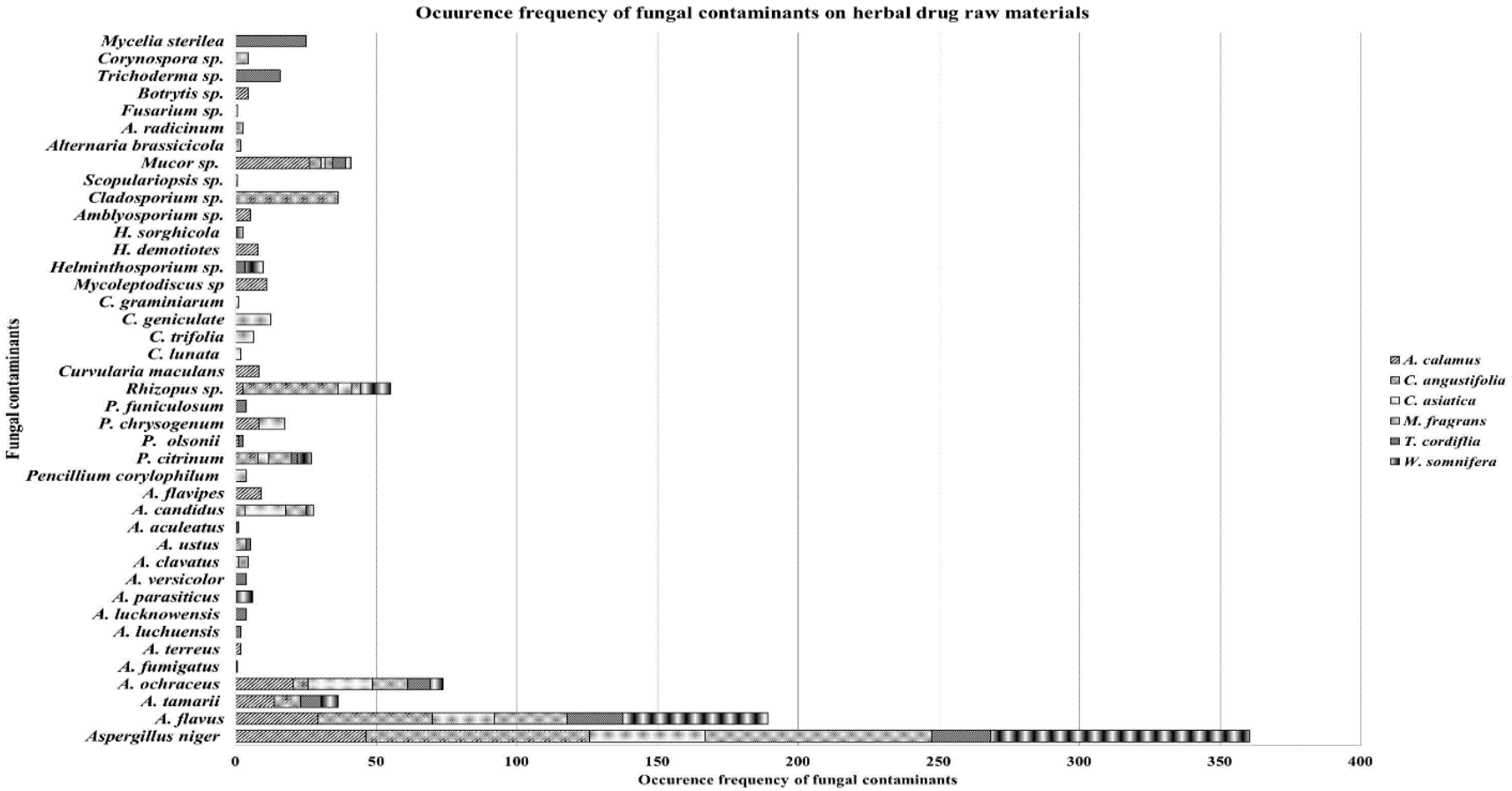

	Aspergillus niger	A. flavus	A. tamarii	A. ochraceus	A. fumigatus	A. terreus	A. luchuensis	A. lucknowensis	A. parasiticus	A. versicolor	A. clavatus	A. ustus	A. aculeatus	A. candidus	A. flavipes	Penicillium corylophilum	P. citrinum	P. olsonii	P. chrysogenum	P. funiculosum	Rhizopus sp.	Curvularia maculans	C. lunata	C. trifolia	C. geniculate	C. graminiarum	Mycoleptodiscus sp	Helminthosporium sp.	H. demotiotes	H. sorghicola	Amblyosporium sp.	Cladosporium sp.	Scopulariopsis sp.	Mucor sp.	Alternaria brassicicola	A. radicinum	Fusarium sp.	Botrytis sp.	Trichoderma sp.	Corynospora sp.	Mycelia sterilea
A. calamus	46.6	29.3	14	20.7	0	2	0	0	0	0	0	0	0	0	9.33	0	0	1.33	8.33	0	2.66	8.66	0	0	0	0	11.3	0	8	0	5.33	0	0	26.6	0	0	0	4.66	0	0	0
C. angustifolia	79.3	40.7	9.33	5.33	0	0	0	0	0	0	0	4	0	3.33	0	0	8	0	0	0	34	0	0	0	0	0	0	0	0	0	0	36.7	0.66	4	2	2.66	0	0	0	0	0
C. asiatica	41	22	0	22.7	0	0	0	0	0	0	1.33	0	0	14.7	0	4	4	0	9.33	0	4.66	0	2	6.66	12.6	1.33	0	0	0	0	0	0	0	1.33	0	0	0.66	0	0	0	0
M. fragrans	80.7	26	0	12.7	0	0	0	0	0	0	3.33	0	0	7.33	0	0	8	0	0	0	3.33	0	0	0	0	0	0	0	0	0	0	0	0	2.66	0	0	0	0	0	4.66	0
T. cordiflia	21	20	7.33	8	0	0	2	4	0	4	0	1.33	0	0	0	0	2	1.33	0	4	0	0	0	0	0	0	0	3.33	0	0	0	0	0	4.66	0	0	0	0	16	0	25.3
W. somnifera	92	51.3	6	4.66	0.66	0	0	0	6.33	0	0	0	1.33	2.66	0	0	5	0	0	0	10.7	0	0	0	0	0	0	6.66	0	2.66	0	0	0	2	0	0	0	0	0	0	0

Figure 2: Diversity and occurrence frequency of fungi associated with the herbal drug raw material

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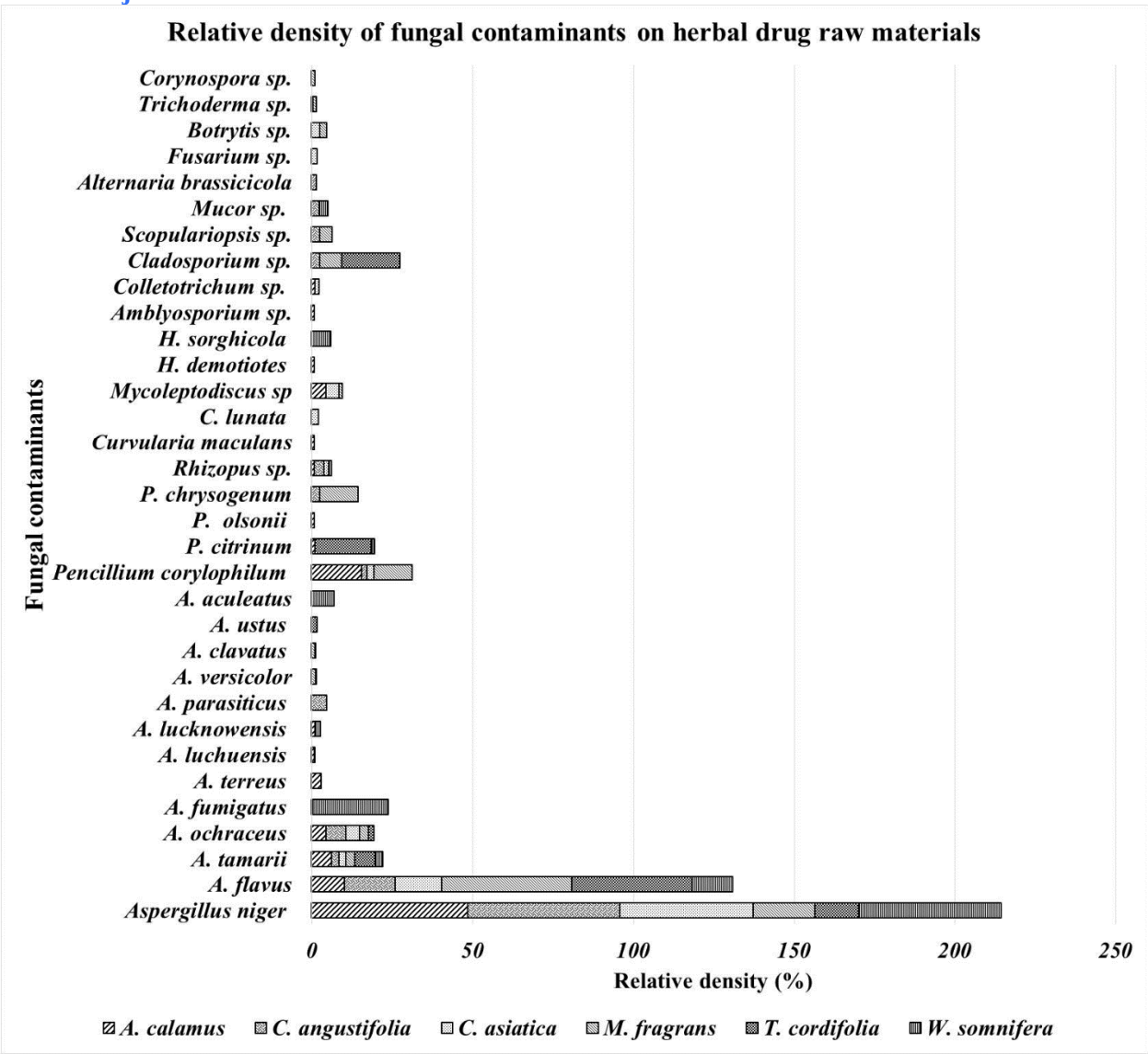

	Aspergillus niger	A. flavus	A. tamarii	A. ochraceus	A. fumigatus	A. terreus	A. luchuensis	A. lucknowensis	A. parasiticus	A. versicolor	A. clavatus	A. ustus	A. aculeatus	Pencilliium corylophilum	P. citrinum	P. olsonii	P. chrysogenum	Rhizopus sp.	Curvularia maculans	C. lunata	Mycoleptodiscus sp	H. demotiothes	H. sorghicola	Amblyosporium sp.	Colletotrichum sp.	Cladosporium sp.	Scopulariopsis sp.	Mucor sp.	Alternaria brassicicola	Fusarium sp.	Botrytis sp.	Trichoderma sp.	Corynospora sp.
A. calamus	48.5	10.2	6.12	4.54	0.37	3.04	0.83	1.04	0	0	0	0	0	15.6	1.13	0.78	0	0.78	0.75	0	4.4	0.78	0	0.75	1.13	0	0	0	0	0	0	0	0
C. angustifolia	47.4	15.8	2.38	6.08	0	0	0	0	4.76	0	0	0	0	1.55	0	0	2.46	3.15	0	0	0	0	0	0	0	2.48	2.46	2.38	1.55	0	0	0	0
C. asiatica	41.6	14.5	2.15	4.29	0	0	0.14	0	0	0	0	0	0	2.15	0	0	0	1.36	0	2.15	4.16	0	0	0	1.25	0	0	0	0	1.66	2.5	0.41	0
M. fragrans	19.2	40.3	2.71	2.71	0	0	0	0	0	1.28	1.07	0	0	12.1	0	0	12.1	0	0	0	1.07	0	0	0	0	6.91	3.86	0	0	0	2.15	1.07	1.07
T. cordifolia	13.7	37.6	6.51	1.76	0	0	0	0	0	0.27	0.31	1.7	0	0	17.4	0	0	0	0	0	0	0	0	0	0	18.2	0	0	0	0	0	0	0
W. somnifera	44.2	12.5	2.33	0	23.4	0	0	1.75	0	0	0	0	7.01	0	0.98	0	0	0.98	0	0	0	0	5.88	0	0	0	0	2.73	0	0	0	0	0

Figure 3: Diversity and relative density of fungi associated with herbal drug raw materials

Occurrence of *Aspergillus* and *Pencillium*, as most predominant species on HDRM, correlate with the previously reported results (Abou-Arab et al., 1999; Aziz et al., 1998; Elashafie et al., 1999; Gautam and Bhadauria 2009; Hitakoto et al., 1978; Mandeel 2005; Sareen et al.,2010). *A. niger* has the ability to grow in a wide range of temperature (6-47 ^{0}C) and pH (1.4-9.8) (Gautam et al., 2010). This ability makes ubiquitous occurrence of species. Isolation of *A. niger* in high frequency and density from all HDRM, which are commonly used by manufacturers should be taken seriously as some strains can produce mycotoxins like ochratoxin A and Fuminosin(Abarca et al., 1994; Gautam and Bhadauria 2011; Noonim et al., 2009; Palencia et al., 2010). *A. niger*may also be attributed to its ability to produce multitude of hydrolytic enzymes that can degrade structural and chemical constituents of the HDRM (Gupta et al., 2013).

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Association of a total of 33 fungal species belonging to 17 genera was recorded by employing SDM. Table-1 represents the CFU of fungal contaminants on HDRM ranging from $1.66x10^2$±0.47/g in *T. cordifolia* to $61.33x10^5$±2.49/g in *W. somnifera*. Highest number of fungal species was found in *A. calamus* (17) followed by *M. fragrans* (14), *C. asiatica*(13)and *C. angustifolia* (11) *W. somnifera* (10) and *T. cordifolia* (9). The relative density of each fungus on different HDRM is presented in figure 3.

According to WHO, maximum permissible limit of mouldpropagules on herbal material is 10^5 CFU/g (WHO, 2001). Results of the present study reveals that all the samples of *A. calamus*, *M. fragrans* and *T. cordifoli*, 2 samples of *C. asiatica* and 1 sample of *C. angustifolia*are contaminated with fungi above the permissible limits. Hence they are not fit for human consumption. Presence of 42 fungal species belonging to 17 genera revealed the broad mycobiota association with HDRM. Although high fungal load may be acceptable due to the natural origin of those materials, they indicate the potential for spoilage and mycotoxigenesis (Lutz et al., 2006). *A. versicolor*, *P. citrinum* and *A. fumigatus* were also detected on test materials (Figure 2 and 3). *A. versicolor* and *P. citrinum* are reported to produce toxins sterigmatocystin and citrinin respectively. Inhalation of spores of *A. fumigatus* is known to cause invasive and allergic aspergillosis (Efuntoye 1999) which suggests the possible risk associated with use of these materials. The occurrence of mycotoxin producing fungal species(*Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Rhizopus*, *Mucor* and *Helminthosporium*) (Robbins et al., 2000; Stevic et al., 2012) indicates the possibilities of mycotoxin contamination of HDRM.

Estimation of Aflatoxin B_1 from *A. Flavus* Isolates and Herbal Drug Raw Materials

Reports on mycotoxins points out their effect on organisms in many ways, including interference with functioning of the cellular system, metabolism of carbohydrate and lipid, and direct binding with nucleic acids (Robbins et al. 2000). Among the mycotoxins, AB_1 has been considered as class Icarcinogen by International Agency of Research on Cancer (IARC) and also well known for its immunosuppressive and mutagenic potential (Anonymous, 1993).

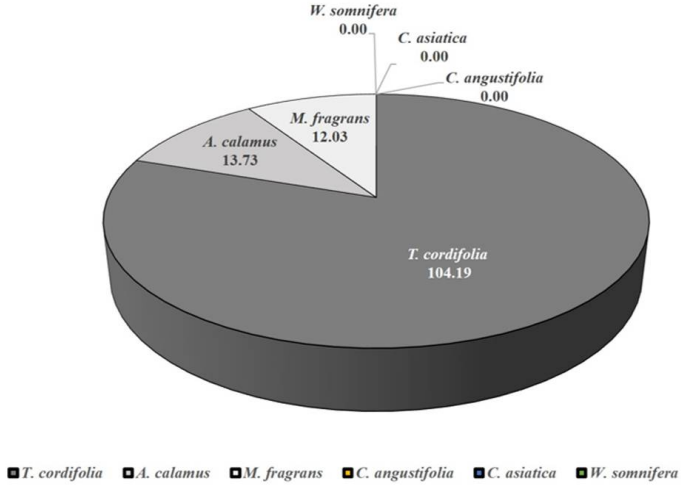

Figure 4: Aflatoxin B_1 content of herbal drug raw materials

In the present investigation 39 isolates of *A. flavus* were screened for their ability to produce AB_1. Among the 39 isolates tested, 24 (61.53%) isolates were found positive for AB_1 production. Highest number of isolates were from *T. cordifolia* i.e. 5 (83.33%) out of 6 isolates recorded positive, followed by *C. asiatica* (71.42%), *W. somnifera* (60%), *C. angustifolia* (60%) and *A calamus* (55.5%). Lowest number of isolates tested positive for AB_1 production from *M. fragrans*(42.85%). Highest yield of AB_1 (5008.20 µg/kg) was obtained from isolates of *T. cordifolia* followed by isolates of *M. fragrans* (1343.19 µg/kg) and least yield was recorded from the isolates of *W. somnifera*(144.16 µg/kg). Table-2 represents the percentage of AB_1 producing strains of *A. flavus* isolated from the HDRM. Further investigation was carried out to determine the AB_1 content of HDRM. Among the six HDRM tested three were found to be contaminated with AB_1 (Figure 4). Occurrence of AB_1in *T. cordifolia*,*A. calamus* and *M. fragrans*was confirmed by LC-MS analysis, by comparing their retention times and MS spectra with the standard (Figure 5).

Table 2: Aflatoxin B_1 production by toxigenic strains of *A. flavus*tested from each sample

	Herbal Drug Raw Materials	Toxigenic/Total Isolates	Percentage of AB_1 Producing *A. flavus* strains	Aflatoxin B_1 Production (ppb)[a]
1	*Acoruscalamus*	5/9	55.5	743.82±326.58
2	*Cassia angustifolia*	3/5	60.00	558.96±182.97
3	*Centellaasiatica*	5/7	71.42	487.54±266.92
4	*Myristicafragrans*	3/7	42.85	1343.19±498.81
5	*Tinosporacordifolia*	5/6	83.33	5008.20±1867.67
6	*Withaniasomnifera*	3/5	60.00	144.16±70.86

[a] Each data point represents the mean ± SEM

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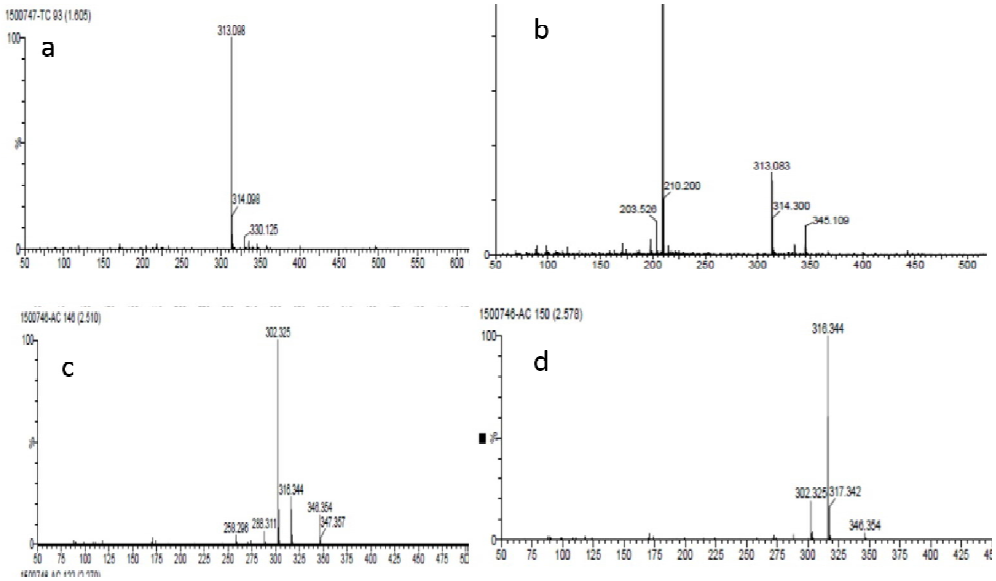

Figure 5: Mass spectra of aflatoxin B_1 in standard (a); *T. cordifolia*(b); *A. calamus* (c); *M. fragrans*(d)

According to European Pharmacopoeia (EDQM, 2011) the maximum permissible limit for AB_1 in herbal materials is 2 µg/kg and Food Safety and Standards Authority of India (FSSAI, 2011) has set 30 µg/kg as maximum permissible limit for Aflatoxinon food articles. In our study, *T. cordifolia*, possess AB_1 (104.19 µg/kg) more than the maximum permissible limit prescribed by European Pharmacopoeia and FSSAI. *A. calamus* (13.73 µg/kg) and *M. fragrans* (12.02 µg/kg) showed AB_1 content more than the prescribed limit of European Pharmacopoeia. Occurrence of AB_1 on HDRM positively correlates with the detection of more number of AB_1 producing *A. flavus* strain on the same materials (Table 2).

It is a matter of great concern, because the materials contaminated with these toxins are not fit for consumption. Since aflatoxins are reportedto be stable up to 269^0C, even routine boiling will not be able to detoxify the materials (Gupta et al., 2013). The prolonged storage of HDRM with mycotoxigenic fungi and related mycotoxins under poor storage conditions may pose a potential risk on consumer's health. The importance of HDRM as remedy for various ailments and as nutraceuticals for maintenance of good health is rapidly increasing with the consumers relying on it. Considering these it is necessary to undertake safety measures to prevent fungal contamination and mycotoxins production in HDRM. Thus there is a need for constant quality assessment of HDRM in the market in order to ensure safety and suitability for human consumption.

Conclusion

Presence of high density and frequency of fungi in the tested HDRM indicate the risk associated with the use of these materials. Concentration of AB_1 in *T. cordifolia, A. calamus* and *M. fragrans* exceeds the maximum permissible limit prescribed by WHO should be taken seriously because of its proven mutagenic, carcinogenic, teratogenic, neurotoxic, nephrotoxic and immunosuppressive activities. Contamination of the HDRM is due to the unscientific method of transportation, handling, processing and storage. The study reveals the need to educate the HDRM handlers to reduce the moisture content of HDRM to less than 13% and store them in hermetically sealed containers. This will avoid fungal contamination during transportation and storage.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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