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RESEARCH ARTICLE

Investigations on the mycoflora and mycotoxin contamination of dried medicinal leaves of *Azadirachta indica* A. Juss. and *Justicia adhatoda* Linn. from Jammu and Kashmir state (India)

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Abstract

Dried leaves of two medicinal plants viz., *Azadirachta indica* and *Justicia adhatoda* were collected from various wholesale and retail shops of eight districts of Jammu and Kashmir. Mycoflora and mycotoxins associated with these leaves were investigated from 50 market samples. A total of 65 fungal species representing 30 genera were recovered using surface washing technique. Four measures of diversity are considered viz., species richness (*S*), Shannon-Wiener's diversity index (*H'*), Simpson dominance index (*Cd*), Pielou's evenness index (*J'*), Berger Parker's dominance index (*d'*). Presence of ample nutrients and similar kind of substrate, that is, plant leaves produces similar kind of diversity. Assessment of mycobial load of dried medicinal plants showed the presence of many such fungal species that are widely acknowledged as the most important mycotoxin producers. Analyses for mycotoxin contamination was done by multi-mycotoxin detection method. Aflatoxin B_1, aflatoxin B_2 and patulin were detected from dried leaves of *Azadirachta indica* whereas aflatoxin B_1, aflatoxin B_2 and zearalenol (ZOL) were detected from dried leaves of *Justicia adhatoda*. Among the mycotoxins detected, aflatoxins were found in both the leaf samples which is probably due to the presence of *Aspergillus flavus* toxin strains. However, no contamination of ochratoxin A, citrinin, zearalenone and deoxynivalenol was detected from the investigated samples.

Introduction

India is endowed with a rich wealth of medicinal plants and a strong base of many systems of medicines including Ayurveda, Unani, Sidha and other local health practices. The curative properties of medicinal plants are due to the presence of complex chemical substances including alkaloids, glycosides, corticosteriods and essential oils (Silva *et al*,. 2011). It has been observed that despite spectacular advancement in modern synthetic drugs, the viability and popularity of indigenous systems of medicine and use of medicinal plants in curing various minor and major health ailments is still intact in our country. However, despite the rich heritage of medicinal plants, India has not been able to capitalize on its medicinal wealth, chiefly because the quality of crude botanicals and finished medicinal plant products is adversely affected by a number of internal and external factors right from harvesting to processing (Dubey *et al*., 2004). In the biodeterioration of these dried commodities, fungi are known to play a significant role and probably rank second only to insects as a cause of deterioration and loss of field and stored plant products (Malaker *et al*., 2008).

Among the various fungi associated with dried commodities, xerophilic fungi are capable of growing at a water activity below 0.85 and are therefore, most important spoilage causing organisms of dried commodities (Singh, 1983). In addition, some of the fungal strains may even lead to the accumulation of a wide variety of toxic secondary metabolites commonly referred to as 'mycotoxins', such as

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aflatoxins, ochratoxins, citrinin, fumonisins, zearalenone, trichothecenes and sterigmatocystin (Al-juraifani, 2011). Fungal contamination of dried commodities is affected by the inter- relationship of several factors including climatic conditions, geographical location, chemical nature of the substrate, mode of commodity handling, type of dehydration process, type of storage containers used, spore load and microbial interaction (Roy, 2003). In India, there is a marked fluctuation of temperature and relative humidity in different seasons, which favours growth of storage fungi and thus increases their incidence (Singh and Saha, 1994). The highest incidence of storage fungi on dried herbals has been recorded during monsoon season when the temperature and relative humidity ranges between 29-32ºC and 79-91% respectively (Roy, 2003).

Storage fungi usually spoil the physical appearance and alter the chemical composition of the plant products on which they grow. As the dry products have always high concentration of nutrients, they form a very good substrate for the growth of a large number of moulds. In case of dehydrated drug plants, the fungal organisms thrive on them during storage period even by utilizing some active ingredients like phenols, alkaloids and proteins (Roy, 2003). Realizing the fact that mycoflora and mycotoxin contamination of dried medicinal plants has not received the attention that the magnitude of the problem warrants and since no such work has been attempted from Jammu and Kashmir State, which is a large reservoir of medicinal plants, an investigation was undertaken on two important medicinal plants viz., leaves of *Azadirachta indica* and *Justicia adhatoda*. Both these plants have leaves that are popularly used as ingredients in a large number of Ayurvedic formulations. Leaves of *Azadirachta indica* are effective against skin and mouth ailments. These are used as vermifuge, insecticides, tonic and antiseptic. These are also used in acne and blood purification. Nimbidin isolated from its leaves is used in medicinal cosmetics. Similarly, leaves of *Justicia adhatoda* are used in treatment of body inflammations and chest diseases. These leaves also have antioxidant properties.

Materials and Methods

(i) Isolation of mycoflora and diversity indices assessment

Dried market samples of selected medicinal plants were collected in pre-sterilized polythene bags from various wholesale and retail shops of eight districts of Jammu and Kashmir viz., Kathua, Jammu, Udhampur, Rajouri, Poonch, Doda, Srinagar and Leh. Sample bags were brought to the laboratory, sealed over flame to avoid external contamination and kept in the refrigerator at 5-7°C to prevent undesirable changes till further studies were conducted. Surface mycoflora associated with market samples of dried medicinal plants was determined by using surface washing technique (Singh and Kainsa, 1983). For isolating maximum number of fungal propagules from the surface of each sample, three different media – Dichloran Rose Bengal Chloramphenicol agar (DRBC), Dichloran 18% Glycerol agar (DG 18) and Malt Salt agar (MSA) were used. The petriplates were incubated for 7 days at 28 ± 2^{o}C till the proper growth of the fungal colonies was obtained. The recovered fungal species were identified by studying their cultural and morphological characters and using various keys and relevant literature.

Percent frequency of each fungal species was calculated by using the formula given below:

$$\text{Frequency (\%)} = \frac{\text{Number of samples from which an organism was isolated}}{\text{Total number of samples tested}} \times 100$$

The data was used in making comparison of different diversity indices. Shannon–Wiener's index (*H'*) (Shannon and Wiener, 1963) and Simpson's Dominance index (*Cd*) (Simpson, 1949) were calculated to determine the heterogeneity of sampled area (Table 1). Pielou's evenness index (*J'*) (Pielou, 1975) and Berger- Parker Dominance index (Berger and Parker, 1970) were also calculated to augment the interpretation of Shannon values.

(ii) Extraction of mycotoxins from dried leaf samples

Dried leaves of *Azadirachta indica* and *Justicia adhatoda* obtained from different markets were analysed for mycotoxin contamination by multi-mycotoxin detection method developed by Stoloff *et al*. (1971). In this method, 25g of finely ground sample was taken in 250ml Erlenmeyer flask containing 100ml mixture of acetonitrile and 4% potassium chloride (90:10v/v). This solution was put on a mechanical shaker for 30 minutes and then filtered through Whatman no. 41 filter paper. 50ml of this filtrate was taken in 250ml separating funnel, defatted and extracted twice with 50ml of iso-octane. The upper iso-octane layer was discarded. To the lower acetonitrile layer, 12.5ml of water was added, shaken and extracted thrice with 20ml of chloroform each time. The chloroform acetonitrile layer was filtered through Whatman no. 41 filter paper having a bed of anhydrous sodium sulphate. The extract was collected in a beaker and then evaporated to dryness

on a water bath. The residue was dissolved in 1ml of benzene: acetonitrile (98:2 v/v) solution and stored in a clean screw cap vial for thin layer chromatography and HPLC analysis.

(iii) Qualitative and quantitative estimation of mycotoxins

For qualitative estimation, known amount of sample extracts and standards of investigated mycotoxins obtained from Sigma Aldrich Co. were spotted on activated TLC (Thin Layer Chromatographic) plates and developed with different solvent systems (Table 2). Quantitative estimation of detected mycotoxins was done through high performance liquid chromatography (Table 2). The analytical equipment of HPLC (CLASS-LC10 SHIMADZU) consisted of a liquid chromatographic pump LC-10AT, an auto injection system SIL – 10A with a 50 µl sample loop, a variable wavelength absorbance UV – VIS detector SPD – 10 set at 365 nm. The analytical component was CLC – ODS. The mobile phase consisted of water: acetonitrile: methanol (59: 29: 12v/v). Analysis was performed at room temperature (25-30 ^{o}C) and data was recorded in HP DeskJet 670C.

Table 1. Diversity indices.

Diversity indices	**Formula used**
Shannon–Wiener's index (*H'*)	$-\sum_{i=1}^{s} p_i \ln p_i$
Simpson's dominance index (*Cd*)	$\sum_{i=1}^{s} (p_i)^2$
Pielou's evenness index (*J'*)	$\frac{H'}{H'_{\max}}$
Berger- Parker dominance index (*d'*)	$\frac{n}{N}$

Where, p_i is the relative importance value of species i; $H'_{\max}$ is the maximum value of H'; n = number of individuals in a species and N = total number of individuals of all species.

Results

During the period of investigation, a total of 50 market samples of dried leaves of *Azadirachta indica* and *Justicia adhatoda* were collected in pre-sterilized polythene bags from eight districts of J&K State (viz., Kathua, Jammu, Udhampur, Rajouri, Poonch, Doda, Srinagar and Leh). These samples were screened for the mycobial load by using surface washing technique and three media (Dichloran Rose Bengal Chloramphenicol agar, Dichloran 18 percent Glycerol agar and Malt Salt agar) of different chemical composition. While recovering surface mycoflora, Dichloran Rose Bengal Chloramphenicol agar medium could trap maximum number of fungal species, Dichloran 18% Glycerol agar medium helped to recover a wide range of non-fastidious xerophilic fungi including most of the *Penicillium* and *Aspergillus* species, whereas, Malt Salt agar was more useful in recovering the members of *Aspergillus glaucus* group. This indicates that nutritional requirements of various fungi differ and there is no single medium, which can help in the recovery of all the fungi.

During the present investigation, a total of 65 fungal species representing 30 genera were recovered from the market samples. Of these, 49 fungal species were recovered from dried leaves of *Azadirachta indica*, whereas 46 fungal species were recovered from dried leaves of *Justicia adhatoda*. Recovery of a large number of fungal species indicates that dried leaves of these medicinal plants provide ample nutrients to the surface invading fungi. However, their presence on the two medicinal plants was not uniform. *Aspergillus* and *Penicillium* dominated the fungal flora with representation of 12 and 9 species respectively. Dominance of these two fungal genera may be due to their widespread occurrence and ability to grow on low moisture commodities. Some of the common xerophilic aspergilli and penicillia recovered from the samples included *Aspergillus flavus, A. candidus, A. fumigatus, A. niger, A. sydowii, A. terreus, A. versicolor. A. ochraceus, A. tamarii, Penicillium brevicompactum, P. citrinum* and *P. chrysogenum.*

Besides aspergilli and penicillia, some other xerophiles recovered from dried medicinal plants included *Eurotium amstelodami, E. chevalieri, E. rubrum, E. tonophilum, Emericella nidulans, Paecilomyces variotii* and *Wallemia sebi* (Table 3).

Diversity indices computed for the fungal species recovered from market samples of dried leaves of *Azadirachta indica* and *Justicia adhatoda* are given in table 4. These diversity indices show little differences in values of Shannon-Wiener's diversity index (*H'*), Simpson dominance index (*Cd*) , Pielou's evenness index (*J'*) and Berger Parker dominance index. For both *Azadirachta indica* and *Justicia adhatoda,* high values of Shannon-Wiener's diversity index (*H'* = 1.569 and 1.537 respectively) and Pielou's evenness index (*J'* = 0.928 and 0.925 respectively) were obtained. High values of (*H'*) indicates that leaves of these plants provide ample nutrients to the surface invading fungi thus leading to their high diversity. In addition, high values of evenness showed that there is less variation between fungal species due to similar kind of substrate, that is, plant leaves. Contrary to this, low values for Simpson dominance index (*Cd* = 0.033 and 0.035) and Berger Parker dominance index ((*d'* = 0.061 and 0.065 respectively) were obtained, which implies that no fungal species was dominant in either of the samples and different fungal species occurred together in both the samples.

Table 2. Qualitative and quantitative estimation of mycotoxins.

Mycotoxin Estimated	**Solvent System Used TLC Analysis**	**Detection/ Wavelength**	**Colour**	**Quantitative Estimation**	**Detector (Wavelength)**
Aflatoxins B1 & B2	Toluene:Isoamyl alcohol: Methanol (90:32:2 v/v)	Long UV	Blue	HPLC	UV/VIS 365nm
Ochratoxin A	Toluene:Ethyl acetate:90% Formic acid(50:40:10v/v)	Long UV	Bluish Green	HPLC	Ex. 333nm, Emm.470nm
Patulin	Toluene:Ethyl acetate: Chloroform(80:70:50v/v) with 1 ml of 90% formic acid	Visible light	Yellow	HPLC	UV/VIS 276nm
Citrinin	Toluene:Ethyl acetate: Chloroform(80:70:50v/v) with 1 ml of 90% formic acid	Long UV	Sky blue	HPLC	Fluorescence Ex. 325nm, Emm.385nm
Zearalenone	Toluene:Ethyl acetate: Formic acid(6:3:1v/v)	Long UV	Blue	HPLC	Fluorescence Ex. 274nm, Emm.440nm
Zearalenol	Toluene:Ethyl acetate: Formic acid(6:3:1v/v)	Long UV	Blue	HPLC	UV/VIS 236nm
Deoxynivalenol	Toluene:Ethyl acetate: Formic acid(6:3:1v/v)	Long UV	Sky blue	HPLC	UV/VIS 229nm

Table 3: Percentage frequency of mycoflora recovered from market samples of dried medicinal leaves of *Azadirachta indica* and *Justicia adhatoda.*

Fungal species	*Azadirachta indica*	*Justicia adhatoda*
Absidia corymbifera	12.5	-
Mucor mucedo	12.5	-
Rhizopus stolonifer	25.0	37.5
Syncephalastrum racemosum	62.5	25.0
Chaetomium cochliodes	37.5	-
C. globosum	37.5	37.5
C. indicum	-	12.5
C. olivaceum	25.0	25.0
Emericella nidulans	75.0	75.0
E. rugulosa	12.5	-
E. striata	-	12.5
E. variecolor	12.5	37.5
Eurotium amstelodami	87.5	75.0
E. chevalieri	62.5	50.0
E. rubrum	25.0	12.5
Microascus cinereus	-	12.5
Monascus ruber	12.5	-
Talaromyces helicus var. *major*-II	-	12.5
Alternaria alternata	37.5	25.0
A. dianthicola	-	12.5
Arthrinium phaeospermum	-	12.5
Aspergillus candidus	12.5	-
A. flavus	87.5	75.0
A. flavus var. columnaris	12.5	-
A. fumigatus	75.0	75.0
A. japonicus	12.5	12.5
A. niger	100.0	100.0
A. niveus	50.0	50.0
A. ochraceus	37.5	12.5
A. pencillioides	-	12.5
A. puniceus	-	12.5
A. sydowii	100.0	87.5
A. terreus	62.5	12.5
A. ustus	-	12.5
A. versicolor	-	12.5
Aureobasidium pullulans	12.5	-
Botryophialophora sp.	-	12.5
Cladosporium cladosporioides	50.0	12.5
C. sphaerospermum	37.5	75.0
Curvularia brachyspora	25.0	-
C. lunata	12.5	12.5
C. pallescens	37.5	-
Drechslera australiensis	12.5	37.5
D. hawaiiensis	12.5	-
Fusarium. Solani	12.5	37.5
Geotrichum candidum	-	12.5
Lasiodiplodia theobromae	12.5	-
Monodyctis paradoxa	-	12.5
Nigrospora oryzae	37.5	-
Nodulisporium didymosporum	-	37.5
Paecilomyces herbarum	12.5	12.5
P. variotii	12.5	-
Penicillium chrysogenum	12.5	50.0
P. citrinum	37.5	87.5
P. corylophilum	-	12.5
P. fellutanum	12.5	-
P. funiculosum	12.5	-
P. islandicum	12.5	12.5
P. oxalicum	12.5	37.5
P. pinophilum	12.5	-
P. purpurogenum	75.0	25.0
Pestalotiopsis palmarum	12.5	-
Scopulariopsis brevicaulis	50.0	87.5
Ulocladium consortiale	-	12.5
Wallemia sebi	12.5	-
No. of fungal species recovered from the leaves of each medicinal plant	49	46

-, means Not detected

Table 4: Diversity indices computed for the recovered fungal species.

Diversity indices	***Azadirachta indica***	***Justicia adhatoda***
Species richness (*S*)	49	46
Shannon-Wiener's diversity index (*H'*)	1.569	1.537
Simpson dominance index (*Cd*)	0.033	0.035
Pielou's evenness index (*J'*)	0.928	0.925
Berger-Parker's dominance index (d)	0.061	0.065

Table 5: Mycotoxins detected from dried leaf samples of *Azadirachta indica* and *Justicia adhatoda.*

Medicinal Plants	Samples found positive for mycotoxin contamination (mg/kg)							
	AFB_1	AFB_2	PAT	OTA	CIT	ZEN	ZOL	DON
Azadirachta indica	2 (0.22 - 0.28)	2 (0.09 - 0.21)	3 (2.25 – 4.60)	-	-	-	-	-
Justicia adhatoda	3 (0.20 – 0.34)	1 (0.13)	-	-	-	-	1 (0.26)	-

-, Not detected

Assessment of surface mycoflora associated with the spoilage of dried leaves of *Azadirachta indica* and *Justicia adhatoda* showed the presence of many such fungal species that are widely acknowledged as the most important mycotoxin producers. In view of this, an investigation was undertaken to verify contamination of 8 major mycotoxins from the market samples. These included aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), ochratoxin A (OTA), patulin (PAT), citrinin (CIT), zearalenone (ZEN), zearalenol (ZOL) and deoxynivalenol (DON).

Perusal of data presented in table 5 shows that dried leaves of *Azadirachta indica*, were contaminated with aflatoxin B_1 (0.22 - 0.28 mg/kg), aflatoxin B_2 (0.09 - 0.21 mg/kg) and patulin (2.25 – 4.60 mg/kg), whereas dried leaves of *Justicia adhatoda* were contaminated with aflatoxin B_1 (0.20 – 0.34 mg/kg), aflatoxin B_2 (0.13 mg/kg) and zearalenol (0.26 mg/kg). Among the various mycotoxins detected, aflatoxins were present in the leaves of both plants, which is probably due to the fact that *Aspergillus flavus* was recovered from these samples. In addition, no contamination of ochratoxin A, citrinin, zearalenone and deoxynivalenol was detected from the investigated samples.

Discussion

This study clearly indicates that xerophilic aspergilli and penicillia including *Aspergillus flavus, A. candidus, A. fumigatus, A. niger, A. sydowii, A. terreus, A. versicolor. A. ochraceus, A. tamarii, Penicillium brevicompactum, P. citrinum* and *P. chrysogenum* are commonly associated with the dried leaves of *Azadirachta indica* and *Justicia adhatoda. In addition,* some other xerophiles including *Eurotium amstelodami, E. chevalieri, E. rubrum, E. tonophilum, Emericella nidulans, Paecilomyces variotii* and *Wallemia sebi* were *also recovered* from investigated medicinal plants. Realizing the importance of the quality of dehydrated medicinal plants, a large number of workers have recently

engaged themselves in the study of surface mycoflora of various herbal drug plants during storage and marketing (Aziz et al., 1998; Roy, 2003; Donia, 2008; Stevic et al., 2012; Abdulla , 2013). These workers reported diverse range of fungal species belonging mainly to *Aspergillus, Penicillium, Rhizopus, Chaetomium, Fusarium, Eurotium* and *Cladosporium.*

Although medicinal plants are gaining popularity among all the sections of society on account of natural healing capacity and less side effects, yet the presence of mycoflora detected in dried samples cannot be ignored as they are known to produce toxic metabolites (mycotoxins), which have numerous hazardous effects (IARC, 1993). A survey of literature indicated occurrence of mycotoxin contaminants from dried medicinal plants of Bihar (Roy and Chourasia, 2001) and Uttaranchal (Singh, 2003) but no such investigation has been attempted from Jammu and Kashmir State. Usually these dried botanicals are consumed in small doses but the contaminated ones can pose a health problem for such innocent consumers who consume them regularly (IARC, 2002). It is commonly observed that most of these botanicals and their formulations are recommended by the Hakeems, Amchis and Vaidyas for a long period of duration and this may be sufficient to produce variable ill effects in the body system of the consumers who are mainly rural dwellers.

In the present investigation, dried leaves of both the medicinal plants were detected to be contaminated with very high concentration of aflatoxins B_1 and B_2 in comparison to the permissible tolerance limits. However, the magnitude of aflatoxin contamination varied with the type of dehydrated medicinal plant, storage practices, geographical factors, seasonal changes and varying aflatoxigenic potential of *A. flavus* strains. Similar observations were recorded by Bilgrami *et al.* (1984) while investigating some dehydrated commodities of Bihar. The LD 50 level of aflatoxins for animal and human consumption ranges from 0.3 mg/kg body weight for very sensitive species to 9-10 mg/kg for others (Krogh, 1987).Few other Indian researchers have also reported aflatoxin contamination in amounts exceeding permissible limits from varied types of dehydrated medicinal plants and their formulations (Roy and Chourasia, 2001; Singh, 2003). There are reports of dried medicinal plants with aflatoxin contamination from other countries also (Elshafie *et al*., 2003; Wongwiwat *et al*., 2004).

Patulin was detected as an important contaminant of *Justicia adhatoda.* There is no earlier report of patulin contamination from dried medicinal plants. Currently, there is no evidence to prove that patulin has the potential to produce adverse human health effects, yet the finding in animals that this mycotoxin is a possible carcinogen and teratogen, emphasizes the need for concern. So far, very high patulin contamination has been reported only from rotted rosaceous fruits and their products (Beretta *et al*., 2000; WHO, 2000; Singh, 2002).

Similarly natural occurrence of zearalenol (ZOL), a fusarial toxin derived from zearalenone was detected from *Justicia adhatoda.* Earlier, zearalenol has been detected from medicinally important dried fruits (Sharma, 2005), rhizome and root tubers (Koul and Sumbali, 2008) along with other fusarial toxins (ZEN and DON). Zearalenol is known to show three to four times more severe oestrogenic properties than zearalenone (Bottalico *et al*., 1985).

Conclusion

In the present investigation, detection of aflatoxins B_1 and B_2, patulin and zearalenol from the dried medicinal leaves of *Azadirachta indica* and *Justicia adhatoda* clearly indicates that their formulations are not completely safe for human consumption. In view of the mycotoxin contamination detected from the market samples, an ardent need for proper storage of crude herbals to minimize the mould and mycotoxin contamination is urgently required.

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