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MYCOBIAL CONTAMINATION AND MYCOTOXINOGENESIS OF *Tinospora cordifolia*: AN IMPORTANT MEDICINAL PLANT OF INDIA

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Abstract

An investigation of mycoflora and associated mycotoxins was carried out from dried market samples of stem portions of *Tinospora cordifolia,* an important medicinal plant of India. These samples were collected from various wholesale and retail shops of eight districts of Jammu and Kashmir state viz., Kathua, Jammu, Udhampur, Rajouri, Poonch, Doda, Srinagar and Leh. A total of 39 fungal species representing 18 genera were recovered by using surface washing technique. Assessment of mycobial load of *T. cordifolia* showed the presence of many such fungal species that are widely acknowledged as the most important mycotoxin producers. Analyses of samples for mycotoxin contamination was done by multi-mycotoxin detection method. The dried samples of *Tinospora cordifolia* were detected to be contaminated with aflatoxin B_1, aflatoxin B_2, ochratoxin A, patulin and citrinin. Among the various mycotoxins detected, aflatoxins were present in maximum number of samples, which is probably because *Aspergillus flavus* was recovered from all the investigated samples. However, fusarial species and their toxins were not detected from the investigated samples.

Keywords: Medicinal Plant, Mycoflora, Mycotoxins, Stem Portions, *Tinospora cordifolia*

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Introduction

Herbal medicines are used for primary health care by approximately 80 percent of the population in the developing countries (Akerele, 1993). These medicines have always remained the first choice of the people of developing countries because of low cost. Moreover, these herbal medicines have better cultural acceptability, better compatibility with human body and lesser side effects. People of the developed countries had more inclination towards chemical preparations, but during the last few years, there has been a considerable change in the medicinal system of the developed world also. Widespread toxicity and harmful after-effects associated with long-term use of synthetic drugs have forced the developed countries to think for some safe alternative healing methods (Gijtenbeek *et al.,* 1999). They have realized that 'natural is better' and thus there is an increasing demand for 'green medicine' which is now commonly sold in health stores.

The state of Jammu and Kashmir is the sixth largest state (area wise) of India. It is situated in the north and surrounded by North-Western Himalayas and has a rich heritage of over 300 medicinal plants (Kaul, 2010). These plants are frequently harvested, dried and used either in the system of Ayurveda or in local health care traditions practiced by hakeems of Kashmir, vaidyas of Jammu and amchis of Ladakh. However, due to lack of proper knowledge and use of unscientific methods of collection, drying and storage (Kaul, 1997), these dried medicinal plants may become prone to contamination with fungal spores. Another effect is that these medicinal plants also undergo deterioration even before they are used in making drugs. 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 one such important medicinal plant, *Tinospora cordifolia* Miers.

Tinospora cordifolia, which is known by the common name Guduchi, is an herbaceous vine (Fig. 1) of the family Menispermaceae (Hooker, 1897). It is used in the treatment of diabetes, high cholesterol, allergic rhinitis (hay fever), lymphoma and other cancers, hepatitis, fever, gonorrhea, syphilis, and to boost the immune system. Research has demonstrated that a combination of *T. cordifolia* extract and turmeric extract is effective in reducing the hepatotoxicity (Adhvaryu *et al.,* 2008).

Alcoholic extract of the stem shows activity against *Escherichia coli* (Nagaprashanthi *et al.*, 2012). A number of products of *T. cordifolia* are now available in the market, which are being used for curing different ailments (Table 1).

In spite of all these curative properties, it is not possible to capitalize the full medicinal wealth of *T. cordifolia* because of the associated fungal contamination of its market samples, which leads to altered efficacy of its drug products. Thus, an investigation was undertaken to isolate, purify and identify the mycoflora associated with the market samples of this plant and analyze the market samples for the presence of natural mycotoxin contaminants like aflatoxins (B_1 and B_2), ochratoxin A, patulin, citrinin, zearalenone, zearalenol and deoxynivalenol.

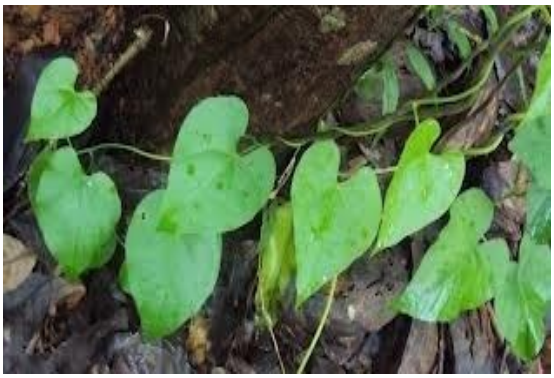

Fig. 1 Habit of *Tinospora cordifolia*

Fig. 2 Dried stem portions of *Tinospora cordifolia*

Materials and Methods

(i) Isolation of mycoflora and assessment of frequency (%)

Dried market samples of stem portions of *Tinospora cordifolia* 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 and surface mycoflora associated with the market samples of dried stem portions of *T. cordifolia* 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°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 by using various keys and relevant literature.

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

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

(ii) Extraction of mycotoxins from dried samples

Dried stem portions of *T. cordifolia* (Fig. 2) obtained from different markets were analysed for mycotoxin contamination by multi-mycotoxin detection method developed by Stoloff *et al.* (1971). In this method, 25 g of finely ground sample was taken in 250 ml Erlenmeyer flask containing 100 ml 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. 50 ml of this filtrate was taken in 250 ml separating funnel, defatted and extracted twice with 50 ml of iso-octane. The upper iso-octane layer was discarded. To the lower acetonitrile layer, 12.5 ml of water was added, shaken and extracted thrice with 20 ml 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 1 ml 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.

Table1. Various valuable economic products of *Tinospora cordifolia*

Product name	Cure
Guduchi Tablets	General infections, immune disease, Hepatitis, Arthritis and anti- cancerous
Madhu Mehari	Dryness of mouth, Numbness debility, relieves Frequent urination, fatigue, Excessive thirst and maintains the blood sugar
Safe Herbs	Vaginal discharge and also helps in sexual debility
Mussaffen	Blood purifier and skin disease
Rebuild	Anti- stress and anti- oxidant
Septilin	Upper respiratory tract infection
Tonplex	Increases immunity and vitality
Joint & Muscle Excellence Tablets	Eliminate the toxins of joints
Natadadrol	Potent muscle-building androgen
Shila Pravang	Premature ejaculation, erectile dysfunction, to enhance the sexual stamina

Table 2. Qualitative and quantitative estimation of mycotoxins

Mycotoxin Estimated	Solvent System Used for TLC Analysis	Detection/ Wavelength	Colour	Quantitative Estimation	Detector (Wavelength)
Aflatoxins B_1&B_2	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	Fluorescence 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

Results and Discussion

During the period of investigation, 22 market samples of *Tinospora cordifolia* were collected in pre-sterilized polythene bags. These samples were screened for the mycobial load by using surface washing technique and three media (Dichloran Rose Bengal Chloramphenicol agar, Dichloran 18% 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, 39 fungal species representing 18 genera were recovered from the dried stem portions of *T. cordifolia* (Table 3). Assessment of surface mycoflora associated with *T. cordifolia* 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 4 shows that dried samples of *Tinospora cordifolia,* were contaminated with aflatoxin B_1 (0.11-1.27 $mgkg^{-1}$), aflatoxin B_2 (0.27-0.77 $mgkg^{-1}$), ochratoxin A (0.48-0.54 $mgkg^{-1}$), patulin (3.75 $mgkg^{-1}$) and citrinin (0.25-0.28). Aflatoxin B_1 was detected from the samples of 6 districts and aflatoxin B_2 was detected from the samples of 3 districts. Other mycotoxins viz., ochratoxins A and citrinin were detected form samples of only 2 districts, whereas patulin was detected from samples of only 1 district of Jammu and Kashmir. Presence of aflatoxins in maximum number of samples is probably because *Aspergillus flavus* was recovered from all these samples. In addition, no contamination of *Fusarium* species and their toxins viz., zearalenol, zearalenone and deoxynivalenol was detected from the investigated samples.

Table 3**.** Percentage frequency of mycoflora recovered from dried market samples of *Tinospora cordifolia* stem portions collected from various districts of Jammu and Kashmir state

Fungal species	Percentage frequency	Isolation of fungal species on different media		
		DRBC	DG-18	MSA
Alternaria alternata	12.5	-	+	+
Aspergillus candidus	12.5	+	+	+
A. deflectus	12.5	+	+	+
A. flavus	100.0	+	+	+
A. fumigatus	37.5	+	+	+
A. japonicus	62.5	+	+	+
A. niger	87.5	+	+	+
A. niveus	37.5	+	+	-
A. panamensis	12.5	-	+	-
A. sydowii	87.5	+	+	+
A. tamarii	62.5	+	+	+
A. terreus	12.5	+	+	-
A. terricola var. *americana*	12.5	-	+	+
Chaetomium brasiliense	12.5	-	+	-
C. globosum	12.5	+	-	-
C. indicum	12.5	-	+	-
C. olivaceum	12.5	+	+	+
Circinella simplex	12.5	+	-	-
Cladosporium cladosporioides	12.5	-	+	-
C. sphaerospermum	62.5	-	+	+
Curvularia brachyspora	12.5	+	-	+
C. lunata	37.5	+	+	-
Emericella nidulans	25.0	-	+	+
Eurotium amstelodami	87.5	+	+	+
E. chevalieri	62.5	+	+	+
E. rubrum	25.0	+	+	+
Geotrichum candidum	12.5	+	-	-
Mucor geophilus	12.5	+	+	+
Paecilomyces variotii	12.5	+	-	+
Penicillium chrysogenum	50.0	+	+	+
P. citrinum	62.5	+	+	+
P. oxalicum	12.5	-	+	+
P. paxilli	12.5	+	+	-
Phyllosticta sp.	12.5	+	-	-
R. stolonifer	75.0	+	+	+
Scopulariopsis brevicaulis	50.0	+	-	+
Sepedonium niveum	12.5	-	+	-
Syncephalastrum racemosum	37.5	+	+	+
Trichoderma harzianum	12.5	-	+	-
Total number of fungal species recovered	39			

+, Presence -, Absence

This study clearly indicates that xerophilic aspergilli and penicillia are commonly associated with the dried stem of *Tinospora cordifolia.* Among the various *Aspergillus* species, *Aspergillus flavus* was detected from all the samples. In addition, some other xerophilic species belonging to *Eurotium, Phyllosticta, Geotrichum, Emericella, Scopulariopsis, Trichoderma, Paecilomyces* and *Sepedonium* were also recovered from the investigated stem of the medicinal plant. 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 (Toma and Abdullah, 2013; Stevic *et al*., 2012). These workers also reported diverse range of fungal species belonging mainly to *Aspergillus, Penicillium, Rhizopus, Chaetomium, Fusarium, Eurotium* and *Cladosporium.*

Table 4. Analysis of mycotoxin contamination in market samples of dried stem portions of *Tinospora cordifolia*

Place of Collection	Samples found positive for mycotoxin contamination (mgkg^{-1})							
	Aflatoxins		Ochratoxin A	Patulin	Citrinin	Zearalenone	Zearalenol	Deoxynivalenol
	Afla B_1	Afla B_2						
Kathua	-	-	-	-	-	-	-	-
Kathua	-	-	-	-	-	-	-	-
Kathua	-	-	-	-	-	-	-	-
Jammu	-	-	-	-	-	-	-	-
Jammu	0.48+0.09	-	-	-	-	-	-	-
Jammu	-	-	-	-	-	-	-	-
Jammu	-	-	-	-	-	-	-	-
Jammu	-	-	-	-	0.25+0.06	-	-	-
dhampur	-	-	-	3.75+0.39	-	-	-	-
dhampur	0.22+0.03	-	-	-	-	-	-	-
Rajouri	-	-	0.54+0.03	-	-	-	-	-
Rajouri	0.30+0.09	0.27+0.05	-	-	-	-	-	-
Poonch	-	-	-	-	0.28+0.20	-	-	-
Poonch	0.73+0.13	0.50+0.08	-	-	-	-	-	-
Poonch	-	-	-	-	-	-	-	-
Doda	0.11+0.06	-	0.48+0.07	-	-	-	-	-
Doda	-	-	-	-	-	-	-	-
Srinagar	-	-	-	-	-	-	-	-
Srinagar	-	-	-	-	-	-	-	-
Srinagar	-	-	-	-	-	-	-	-
Leh	1.27+0.37	0.77+0.19	-	-	-	-	-	-
Leh	-	-	-	-	-	-	-	-
Number of positive samples	6	3	2	1	2	0	0	0
Percent of positive samples	27.3	13.6	9.1	4.5	9.1	0.0	0.0	0.0

-, Not detected

The presence of mycoflora, which are known to produce toxic metabolites (mycotoxins) were detected from the dried samples of *T. cordifolia* and many of them have hazardous effects (IARC, 1993). A survey of literature indicated occurrence of mycotoxin contaminants from dried medicinal plants of various Indian states viz., Bihar (Roy and Chourasia, 2001), Uttaranchal (Singh, 2003), Jammu and Kashmir (Sharma *et al.,* 2013).

In the present investigation, dried stem samples of *T. cordifolia* 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 varies with the type of dehydrated medicinal plant, storage practices, geographical factors, seasonal changes and varying aflatoxigenic potential of associated *A. flavus* strains (Bilgrami, 1984) 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). Similarly, there are reports of dried medicinal plants with aflatoxin contamination from other countries also (Elshafie *et al.,* 2003; Wongwiwat *et al.,* 2004).

Very few samples of dried stem portions of *T. cordifolia* were detected to be OTA contaminated (0.48 to 0.54 mgkg^{-1}) and they belonged to Rajouri and Doda districts. Reports of OTA contamination from other medicinal plants and their crude products have been provided by other ethanomycologists also (Aziz *et al.*, 1998; Efuntoye, 1999; Santos *et al.*, 2013; Singh, 2003). IARC (1993) has classified OTA in group 2B because of its toxicity on humans. Committee on Toxicity of Chemicals in Food, Consumer Products and Environment (COT) considered OTA as a genotoxic carcinogen and proposed that its level in food be reduced to the lowest (COT, 1997). The joint expert committee of Food Additives of the WHO and FAO set a provisional maximum intake of 100 µgkg^{-1} body weight. However, few countries have legislative limits ranging from 5 to 50 µgkg^{-1} (Webley *et al.,* 1997).

Another mycotoxin detected from the dried sample of *T. cordifolia* consisted of patulin. There is no earlier report of patulin contamination from dried medicinal plants. However, very high patulin contamination has been reported from rotted rosaceous fruits and their products (Beretta *et al.,* 2000).

In the present investigation, detection of aflatoxins B1 and B2, ochratoxin, patulin and citrinin from market samples of dried stem of *T. cordifolia* clearly indicates that its powdered formulations are not completely safe for direct human consumption. In view of the detected mycotoxin contamination, an urgent need for proper drying and storage of crude herbals is urgently required.

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