RESEARCH PAPER

Study on mycoflora and aflatoxins contamination in common spices in Dharmapuri district, Tamil Nadu

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Spices constitute an important group of agricultural commodities which are virtually indispensable in the culinary art. In India, some spices also possess strong anti-microbial and antibiotic activities. Samples of whole or ground black pepper from various sources yield numerous colonies of several species of *Aspergillus*. The mycolflora and mycotoxins of many agricultural products have been investigated by many researchers. The pH of the suspension was measured using a digital pH meter. Enumeration of fungal colonies in different sample sabourauds dextrose agar (SDA) (Hi-Media) was prepared and sterilized by autoclaving at 121^0 C for 15 minutes. Thin layer chromatography for detection of aflatoxin. The aflatoxin were isolated and characterized after the death of more that 1,00,000 turkey poults. Among 18 different types of and aflatoxins identified, major members are aflatoxin B1, B2, G1 and G2. Incidence of molds in different sample of spices showed fungal contamination with significance difference. The identified fungi assigned to 12 genera *Aspergillus niger*. These fungi are identified based on colony morphology and microscopic observation. TLC analysis of spices extracts revealed the presence of aflatoxin, once spices and food are contaminated by aflatoxins it is almost impossible to detoxifiy them by normal cooking methods.

Key words **:** *Aspergillus niger*, Aflatoxins, Spices, Sabouraud 's dextrose agar (SDA)

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INTRODUCTION

Spices constitute an important group of agricultural commodities which are virtually indispensable in the culinary art. In India, spices are important commercial crops from the point of view of both domestic consumption and export. Besides, huge quantities of spices are also being consumed within the country for flavouring foods and are also used in medicine, pharmaceutical, perfumery, cosmetics and several other industries.

There are over 80 spices grown in different parts of the world and around 50 spices are grown in India. The spices that India can offer in abundant quantities are pepper, ginger, turmeric, chilli, cardamom, celery, fenugreek, fennel, cumin, dill, coriander, cinnamon, ajowan (bishop's weed), cassia, clove, nutmeg and mace. Some spices also possess strong anti-microbial and antibiotic activities. Many of them possess medicinal properties and have a profound effect on human health, since they effect many functional processes. For instance, spices intensify salivary flow.

Fungi are the predominant contaminants of spices (Kneifel and Berger, 1994), but most such microbial populations are probably regarded as commensal residents on the plant that survived drying and storage. Most fungi are present on pepper of the post-harvest and storage type, which develop after harvest if relative humidity is not controlled during storage. Samples of whole or ground black pepper from various sources yield numerous colonies of several species of *Aspergillus* (Christensen *et al.*, 1967). The mycoflora of foods has traditionally been given considerably less attention than the bacterial flora (Kneifel and Berger, 1994; Sharma *et al.*, 1984).

Mycotoxins are secondary metabolites of mold fungi identified in many agricultural products screened for toxigenic molds (Van Egmond, 1981).

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Aspergillus fiavus, A. candidus, A. niger, A. luchuensis, A. ochraceus, A. nidulans, F. moniliforme. F. oxysporum, Alternaria alternata, Curvularia spp. and rhizopus stolonifer were reported as the most common fungi isolated from drug plants.

RESEARCH METHODOLOGY

The present investigation was carried out in the Department of Botany, Government Arts College, Dharmapuri, Tamil Nadu. The description of material used and the methods followed are detailed below:

Source of collected sample :

Five types of spices sample *viz*., chilli, black pepper and cumin were collected from local retailers agencies in Dharmapuri district in the month of December 2009 to February 2010.

Determination of moisture estimation (Alam *et al.*, 2001):

A moisture dish of appropriate size was weighed accurately. 10 g of the sample was added and reweighed. The container was placed in a hot air oven at 120^{0}C for approximately 2 hours. The dish was removed from the oven, covered, cooled in desiccator, and weighed.

$$\text{Percentage of moisture} = \frac{(P - A)}{P} \times 100$$

P= weight in g of sample;
A = weight in g of dried sample

Estimation of pH (Mandeel, 2005):

In preparation for the assay, each spice sample was mixed while in the bag and the required amount weighed for measurement pH, a 1:10 spice: distilled water suspension for each sample was prepared and stirred for 24 h in 200 ml beaker. Each sample was analyzed in triplicate. The pH of the suspension was measured using a digital pH meter.

Enumeration of fungal colonies in different sample (Mandeel, 2005):

Sabouraud's dextrose agar (SDA) (Hi media) was prepared and sterilized by autoclaving at 121^{0}C for 15 minutes and allowed to cool to 50^{0}C and poured into sterile Petriplates. For culturing, 10 g of ground feed sample was added to 90 ml of 0.1 per cent peptone water, allowed to stand 15 minutes, and shaken well for for 15 minutes. From this diluted *Chaetomium* sp., *Penicillium citrinum* and *Rhizopus stolonifer* were reported as the most common fungi isolated from drug plants (Ayres *et al*., 1980). sample, 0.1 ml was pipetted onto the surface of Petriplates containing the Sabouraud's dextrose agar. The Petriplates were incubated at 25^{0}C for 5 to 7 days. All colonies were counted and multiplied by the dilution factor (100) to calculate colony forming unit (CFU) for per g of spices. Duplicate samples from each feed culture were used to check repeatability of the sampling and incubation procedure.

Thin layer chromatography for detection of aflatoxin (A.O.A.C., 1984):

Procedure for separation of extract:

Forty ml of distilled water was added to 10 g of the ground spices sample and beated for 2 minutes in the blender. 60 ml of acetone was added and again beated it for two minutes. The contents were filtered. To 30 ml of the filterate and approximately 0.6g of cupric carbonate was added in beaker (A).

In another beaker (B), 34 ml of 0.2 M NaOH and 6m1 of $FeCl_3$ (0.41M) were added and the contents were swirled. The content of beaker (B) was added to beaker (A) and was mixed it slowly by swirling movements. The contents were filtered through Whatman No. 1 filter paper.

Forty ml of the filtrate was taken in a 250 ml separating funnel and 40 ml of (0.03%) H_2SO_4 and 10 ml of chloroform were added and mixed slowly. The chloroform layer was collected in a 100 ml beaker, added again 10 ml of chloroform and mixed thoroughly, and allowed it to settle and the chloroform was collected in the same 100 ml beaker.

Forty ml of 0.02 M KOH and 1 per cent KCl mixture were taken in a second separating funnel. To this, added the collected 20 ml chloroform extract and mixed it slowly and collected through anhydrous sodium sulphate bed drop by drop to remove any traces of moisture. The chloroform extract was kept in an oven at 50^{0}C till it has been dried. The dry aflatoxin film is rediluted with 0.2 ml chloroform and spotted on the TLC plate 40 µl.

Development of TLC plates:

For examination of extracts; aluminium- packed slica gel 60 F 254 (Merck, type 1.05554.0007) was used as supplied for 20 x 20 cm^2 plates according to AOAC (1984). Plates were spotted along 1.5 cm from the bottom with 40 µL aliquots of extract and different volume (2, 4, 6 and 8 µL) of aflatoxin Bl and B2 standard (4 µg ml) on another TLC plates. The plate was developed in acetone chloroform : water (88:12:1) solvent in equilibrated, TLC chromotanks at room temperature until the solvent front had reached a line marked 2 cm from the top of the plate. After development, plates were removed and air dried in a fume- cabinet and then examined in a UV long wavelength (364 nm) light in a UV cabinet.

Detection of aflatoxin:

Developed chromatograms were examined under UV light, since aflatoxins BI and B2 visualized blue fluorescence without treatment.

Identification and quantification of aflatoxin:

The chemical confirmation of aflatoxin was performed

by spraying the chromatoplate with 25 per cent sulphuric acid (Sinha *et al*., 2002) and dried at 110^{0}C in a hot air oven and viewed under long UV light. Amount of each toxin was calculated visualized on TLC plate by comparing known concentration of standard solution.

Each toxin was also confirmed on the basis of RF values and standard toxins that were given below:

Sr. No.	Toxin name	Rf values	Colour under UV light	Confirmation test	Confirmation colour
1	Aflatoxin B1	0.73	Blue	25% H_2SO_4 acid spray	Yellow colour
2	Aflatoxin B2	0.71	Blue	25% H_2SO_4 acid spray	Yellow colour

Calculation:

$$\text{Aflatoxin (ppb)} = \frac{S \times C \times D \times 1000}{T \times E}$$

RESEARCH FINDINGS AND ANALYSIS

The results of the present investigation have been presented in Table 1, 2, 3, 4 and 5.

Incidence of molds in different samples:

All spices showed fungal contamination with significance difference. The identified fungi assigned to 12 genera (*Acremoniurn, Aspergillus, Cladosporium, Colletotrichum, Geotrichum, Gliocladium, Rhizopus, Rhizomucor, Mucor, Pennicillium, syncephalastrum* and yeast; The incidence of mold in chilli is presented in (Table 1).

Table 1 : Incidence of mold and their abundance on different sample of chilli

Sr. No.	Name of the fungus	Sample-I		Sample-II		Sample-III		Sample-IV		Sample-V	
		No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %
1.	*Alternaria* spp.	-	-	3	7.14	-	-	4	9.75	1	2.5
2.	*Aspergillus flavus*	31	77.5	19	45.23	26	44.82	21	51.21	23	57.5
3.	*Aspergillus niger*	7	17.5	17	40.47	31	53.44	6	14.63	7	17.5
4.	*Cladosporium* spp.	-	-	1	2.38	-	-	3	7.31	-	-
5.	*Geotrichum* spp	2	5	-	-	1	1.72	2	4.87	3	7.5
6.	*Rhizopus* spp.	-	-	-	-	-	-	5	12.19	-	-
7.	Yeast	-	-	-	-	-	-	-	-	2	5
8.	Unidentified	-	-	2	4.76	-	-	-	-	4	10
	Total	40	-	42	-	58	-	41	-	40	-

Table 2 : Incidence of mold and their abundance on different sample of black pepper

Sr. No.	Name of the fungus	Sample-I		Sample-II		Sample-III		Sample-IV		Sample-V	
		No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %
1.	*Acremonium* spp	2	4.34	-	-	-	-	3	5.17	-	-
2.	*Aspergilus fumigatus*	12	26.08	2	8.69	3	20	13	22.41	2	12.50
3.	*Aspergillus flavus*	5	10.86	3	13.04	2	13.33	7	12.06	3	18.75
4.	*Aspergiluis flavus*	3	6.52	8	34.78	1	6.66	4	6.89	1	6.25
5.	*Aspergillus glaucus*	-	-	1	4.34	-	-	2	3.44	3	18.75
6.	*Aspergillus* spp.	-	-	-	-	2	13.33	3	5.17	1	6.25
7.	*Cladosporium* spp.	7	15.21	2	8.69	1	6.66	6	10.34	2	12.50
8.	*Colletotrichum* spp.	-	-	2	8.69	-	-	1	1.72	-	-
9.	*Epicoccium* spp.	1	2.17	-	-	-	-	-	18.96	1	6.25
10.	*Penicillium* spp.	3	6.52	1	4.34	2	13.33	11	8.62	1	6.25
11.	*Phoma* spp.	8	17.39	4	17.39	3	20	5	5.17	2	12.50
12.	*Rhizomucor* spp.	2	4.34	-	-	-	-	3	-	-	-
13.	Unidentified	3	6.52	-	-	1	6.66	-	-	-	-
	Total	46	-	23	-	15	-	58	-	16	-

Table 3 : Incidence of mold and their abundance on different sample of cumin

Sr. No.	Name of the fungus	Sample-I		Sample-II		Sample-III		Sample-IV		Sample-V	
		No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %	No. of fungi (x 10CFU)	Abundance %
1.	*Aspergillus* spp.	8	37.78	-	-	-	-	3	15.78	1	3.57
2.	*Aspergillus fumigatus*	2	8.69	7	38.88	4	19.04	-	-	-	-
3.	*Aspergillus niger*	3	13.04	2	11.11	3	14.28	1	5.26	2	7.14
4.	*Aureobasidium* spp	-	-	-	-	1	4.76	4	21.04	2	7.14
5.	*Chrysosporium* spp.	-	-	-	-	-	-	2	10.52	1	3.57
6.	*Cladosporium* spp.	5	21.73	3	16.66	6	28.57	2	10.52	7	25
7.	*Curvularia* spp.	-	-	-	-	-	-	1	5.26	2	7.14
8.	*Mucor* spp.	1	4.37	-	-	2	9.52	-	-	-	-
9.	*Penicillium* sp.	-	-	1	5.55	-	-	2	10.52	3	10.71
10.	*Rhizopus* spp	1	4.37	1	5.55	-	-	-	-	5	17.85
11.	*Trichoderma* spp.	1	4.37	4	22.22	5	23.8	2	10.52	1	3.57
12.	Unidentified	2	8.69	-	-	-	-	2	10.52	1	3.57
	Total	23	-	18	-	21	-	19	-	28	-

In black pepper, sample-IV had high 580 fungal colony forming unit, followed by sample - I (460 CFU/g), sample - 11(230 CFU/g), sample - V (160 CFU/g) and sample - III (150 CFU/g) (Table 2). The highest fungal colonies were found in sample- V, followed by sample-I, III, IV and II of different sample of cumin (Table 3 and Fig. 1).

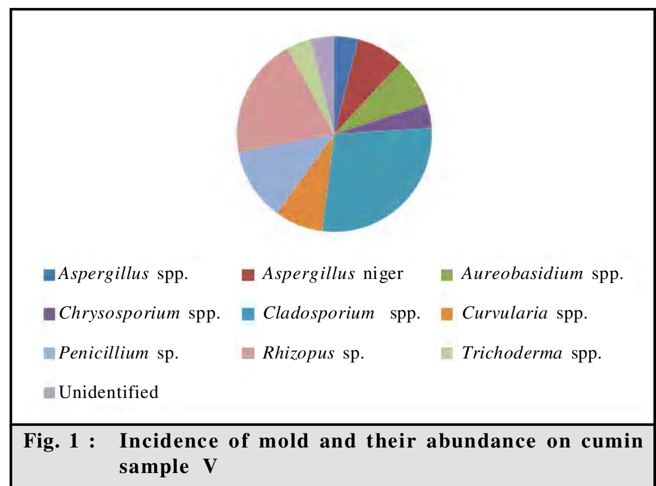

Fig. 1 : Incidence of mold and their abundance on cumin sample V

Determination of moisture estimation and pH:

Moisture content and hydrogen ionic strength (pH) for all spice samples are shown in Table 4. The average percentages moisture content of chilli, black pepper and cumin were 9.03, 10.63 and 8.82, respectively.

The hydrogen ion concentrations of spice samples fall in the magnitude of acidic. The lowest mean pH level in spice: water solutions were detected. The samples such as chilli, black pepper and cumin showed low pH level (Table 4).

Table 4 : Determination of moisture estimation and pH

Sr. No.	Name of the sample	Moisture content (%)	pH
1.	Chilli-I	8.95	5.16
2.	Chilli-II	9.46	4.32
3.	Chilli-III	10.14	5.52
4.	Chilli-IV	8.08	4.89
5.	Chilli-V	8.54	4.62
6.	Black pepper-I	10.32	6.7
7.	Black pepper-II	10.24	6.43
8.	Black pepper-III	9.62	6.52
9.	Black pepper-IV	12.16	6.61
10.	Black pepper-V	11.03	6.48
11.	Cumin-I	8.65	6.34
12.	Cumin-II	8.2	5.93
13.	Cumin-III	7.78	6.03
14.	Cumin-IV	9.25	6.28

Level of aflatoxin in different spices:

The level of aflatoxin Bl and B2 were found in different spices which were compared with standard (Table 5 and Fig. 2).

Red chilli and chilli powder prepared from dried fruits could be naturally contaminated with aflatoxin B1 (Reddy *et al.*, 2001). In the present investigation, chilli sample-III and V were contaminated with aflatoxin B1 but sample I, II and IV did not show aflatoxin B1 and B2 toxin were not exhibited. The present result confirmed the statement of Ravikiran *et al.* (2005) who have also observed similar result. In the present

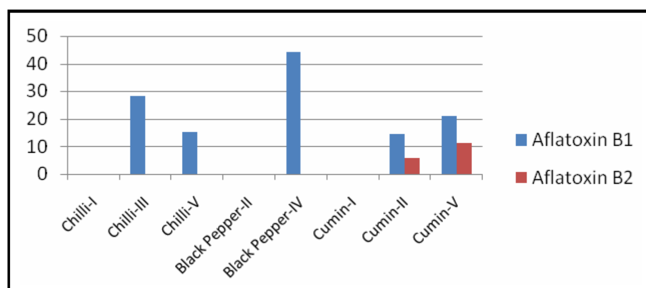

Fig. 2 : Occurrence of Aflatoxins in different sample spices

Table 5 : Occurrence of aflatoxins in different sample spices

Sr. No.	Name of the sample	Aflatoxin B1 Mean ± S.D.	Aflatoxin B2 Mean ± S.D.
1.	Chilli-I	ND	ND
2.	Chilli-II	ND	ND
3.	Chilli-III	28.18 ± 0.258	ND
4.	Chilli-IV	ND	ND
5.	Chilli-V	15.31 ± 0.424	ND
6.	Black pepper-I	6.70 ± 0.326	ND
7.	Black pepper-II	ND	ND
8.	Black pepper-III	ND	ND
9.	Black pepper-IV	44.38 ± 0.382	ND
10.	Black pepper-V	ND	ND
11.	Cumin-I	ND	ND
12.	Cumin-II	14.65 ± 0.329	5.85 ± 0.279
13.	Cumin-III	ND	ND
14.	Cumin-IV	ND	ND
15.	Cumin-V	20.94 ± 0.477	11.16 ± 0.360

ND – Not detected

study, the cumin sample II and V had contaminated with both aflatoxin B1 and aflatoxin B2. The sample-V showed the highest concentration of aflatoxinB1 (20.94ppb), and B2 (11.16 ppb), followed by sample-II.

Conclusion:

This study provided useful information on the health standards used for human consumption and hopes to raise the awareness of the public and decision makers to the health hazards of food contamination. Setting standards for microorganisms and aflatoxin in spices will reduce the risk of aflatoxin hazards. More surveys are needed for the study of the contamination of spices by microorganisms in India where thousand of tons of spices are consumed annually.

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