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**RESEARCH ARTICLE**

# Detection of fusarial toxins from loose and packed red chilli powder marketed in Jammu and Kashmir state, India

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## *Abstract*

In India chilli powder is sold both loose and packed under different brand names. During storage the quality of red chilli powder is under a major threat of fungal contamination as they are hygroscopic in nature. Deteriorating effect of storage fungi is especially of great concern as many of their strains may produce toxic secondary metabolites. In view of this, an investigation was conducted on loose and packed red chilli powder marketed in Jammu and Kashmir state to determine the association of *Fusarium* species and their toxins.

Samples procured from different markets were found to be contaminated with only two species of *Fusarium* viz., *F. semitectum* and *F. verticillioides*. However, HPLC analysis conducted for estimating natural incidence of fusarial toxins revealed the presence of zearalenone (2.66 - 63.29 µg/g), zearalenol (12.35 - 33.80 µg/g) and deoxynivalenol (52.22 - 180.61 µg/g) in the samples. Detection of three fusarial toxins in more than permissible limits from investigated samples suggests that dried red chilli powder is a favourable substrate for their production and therefore not completely safe for human consumption. In addition, low abundance of fusarial species in the samples does not necessarily imply the absence of their toxins. It is possible that some toxigenic fusarial species may have invaded the chillies before harvesting, during drying and storage and contaminated them with their toxins before actually getting destroyed by some biotic/abiotic factors



## Introduction

Chillies are commonly referred to as hot peppers, bell peppers, red peppers, pod peppers, cayenne peppers, paprika, pimento and capsicum in different parts of the world. According to Food and Agriculture Organization (FAO), pungent fruits of all the cultivated species of *Capsicum* are collectively called as chillies (Berke and Shieh, 2001). The ripe chilli fruit has a deep red colour with smooth and shiny skin. Harvesting is done when the chilli pods are well ripened and have a moisture content of 65-80%, which must be brought down to 8-10% to prepare the dried spice and its powder. Consumption of chillies is increasing day by day as it is an important source of vitamins A, C and E for the world population (Bosland and Votava, 2000). Herbalists have promoted peppers for their health enhancing effects like clearing the lungs and sinuses, protecting the stomach by increasing flow of digestive juices, triggering the brain to release endomorphins (natural painkillers) and protecting the body against cancer through antioxidant activity (Andrews, 1995). Red chillies are famous for their pleasant aromatic flavour, pungency and high colouring substance. They are usually ground to release the natural flavour and ease their dispersion in curries. Chilli powder is obtained by grinding the pericarp and seeds of ripe fruits of various varieties of *Capsicum annuum*. In addition, it may also contain a variable proportion of other parts of the fruit, such as placenta, calyx and stalk. Chilli powder thus obtained is sold loose or packed under different brand names.

In India, red chillies from Kashmir valley have the reputation of being the best as there is no match of its quality. The red colour and fragrance of Kashmiri red chillies is even more than the best quality of Hungarian sweet paprika. Quality of red chillies powder is under a major threat during storage as it is hygroscopic in nature and may result in caking, discolouration, hydrolytic rancidity and mould growth. Deteriorating effect of storage fungi is especially of great concern as many of their strains may produce toxic secondary metabolites, collectively called as mycotoxins. All mycotoxins are low molecular weight natural products produced chiefly by filamentous fungi such as *Aspergillus, Penicillium, Fusarium* and *Alternaria* species under suitable conditions of temperature and humidity (Adebajo and Diyaolu, 2003; Zain, 2011). Among these, fusarial species are most important as they may invade the chillies both before and after harvesting (Sharma and Sumbali, 1993). The original source of *Fusarium* species in both circumstances is the field, which later proliferate in the storage if conditions permit. Infact *Fusarium* species are probably the most prevalent toxin-producing fungi of the northern temperate regions which can affect human health (Creepy, 2002). International Agency for Research on Cancer (1993) pointed out that among the five agriculturally important fungal toxins, zearalenone, deoxynivalenol and fumonisins constitute important *Fusarium* toxins and classified them as Group 2B carcinogens, potentially carcinogenic to human beings. In addition, zearalenone (ZEN) and zearalenol (ZOL) have strong estrogenic activities (Takemura *et al*., 2007), whereas deoxynivalenol (DON) is associated with nervous system disturbances, irritation of the gastrointestinal tract and hemorrhage in the consumers (Rotter *et al*.,1996).

Natural occurrence of fusarial toxins in spices has been reported from different countries of the world including India (Patel *et al*., 1996; Santos *et al*., 2011; Boonzaaijer *et al*., 2008). Realizing the importance of kashmiri red chilli powder as an important spice used by Indians, an attempt was made to determine the natural occurrence of *Fusarium* toxins viz., ZEN, ZOL and DON in Kashmiri red chilli powder samples sold loose and packed in the markets of Jammu and Kashmir state, India.

## Materials and Methods

During the investigation period, loose and packed Kashmiri red chilli powder samples were brought to the laboratory in pre-sterilized polythene bags and assessed for the associated *Fusarium* species and their toxins.

**Isolation of Fusarium species from chilli powder samples**

Isolation of *Fusarium* species from the samples was done by following the method of Harrigan (1998). In this method, 5 g of sample was taken in a 250 ml Erlenmeyer flask containing 45 ml sterilized distilled water and homogenized thoroughly on an electric shaker for 15 minutes. Ten fold serial dilutions were prepared and 1ml portion of suitable dilution was poured in petriplates by using a sterilized pipette. For the recovery of maximum *Fusarium* species from each sample, three different media – Czapek Dox agar (CDA), dichloran 18% glycerol agar (DG-18) and malt salt agar (MSA) were used and for each medium five replicates were maintained. The medium was poured by making gentle rotational movement of petriplates so as to ensure uniform spreading of the sample. Petriplates thus prepared were incubated at 28 $\pm$ 2ºC for 7 days. Finally, the colonies were counted and the results were expressed as average colony forming units in thousands per gram of sample using the following formula (Parikh and Shah, 2006).

$$\textbf{cfu/g} = \frac{\textbf{a x d}}{\textbf{s}}$$

**a** = average number of colonies on the petriplate
**d** = dilution factor
**s** = dry weight of sample taken

Percentage frequency of occurrence (%) was calculated by using formula:

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

**Extraction of mycotoxins from red chilli powder**

Samples of red chilli powder collected from Jammu and Kashmir divisions were analyzed for mycotoxic contaminants by using modified multimycotoxin method developed by Roberts and Patterson (1975). In this method, 25g of the sample was taken in an Erlenmeyer flask containing 100 ml mixture of acetonitrile and 4% potassium chloride (90:10v/v). Extraction was done by putting the flask on a horizontal mechanical shaker for 30 minutes. Thereafter, extract was filtered through Whatman no. 41 filter paper. The filtrate was taken in 250 ml separating funnel, defatted and extracted twice with 50 ml iso-octane. The upper iso-octane layer containing lipid was discarded and 12.5 ml distilled water was added to the lower acetonitrile layer. Basic mycotoxins were extracted from the lower acetonitrile layer, thrice by using 20 ml chloroform each time. Lower chloroform acetonitrile layer was collected in a conical flask and drained through Whatman no. 41 filter paper having a bed of anhydrous sodium sulphate. The extract was collected in a beaker and marked as extract I. The aqueous layer left in the separating funnel was acidified with 1ml of 1.0 N HCl and the acidic mycotoxins were extracted from it thrice by using 10 ml chloroform each time. Lower chloroform layers were combined, passed through anhydrous sodium sulphate bed, collected in a beaker and marked as extract II. Extracts I and II were combined and then evaporated to dryness on a water bath.

For separation of pigment from the extract, dried residue was re-dissolved in 1.25 ml of acetonitrile and transferred into a dialysis sac made from dialysis tubing, which was thoroughly washed with distilled water. The dialysis sac was equilibrated against 25 ml of acetone water mixture (30:70 v/v) in a stoppered conical flask for 16 hours by gentle shaking on a wrist action shaker. To improve recovery of mycotoxins, dialysis sac was again equilibrated for 6 hours against 25 ml of acetone water mixture (30:70 v/v). Aqueous acetone dilysates were combined and extracted with 15 ml of chloroform three times in a separating funnel. Methanol (3 ml) was added to it for the clear separation of layers. Chloroform extracts were combined, passed through anhydrous sodium sulphate bed and dried on a water bath. Dried residue was dissolved in 1 ml of chloroform and stored in a small screw cap vial for qualitative and quantitative analysis of mycotoxins.

**Qualitative estimation of fusarial toxins**

For detection of ZEN, ZOL and DON, aliquots of sample extract (50 µl) were spotted on TLC plates along with the standards and developed in a solvent system consisting of toluene : ethyl acetate : formic acid (6:3:1, v/v/v). After drying, plates were observed under long wave UV light. ZEN spots were located as blue green fluorescent spots; ZOL spots were located as light blue fluorescent spots and that of DON as sky blue spots. Confirmation was done by spraying the plates with freshly prepared saturated solution of aluminium chloride in 95% ethanol and then heating it at 120°C for 10 min. The spots of ZEN, ZOL and DON became brighter in appearance.

**Quantitative estimation of fusarial toxins**

Quantitative analysis of ZEN, ZOL and DON was done through high performance liquid chromatography. The analytical equipment for HPLC (CLASS-LC10 SHIMADZU) consisted of a liquid chromatographic pump LC-10AT, an auto injection system SIL – 10A with 50 µl sample loop, and a variable wavelength absorbance UV – VIS detector SPD – 10 set at 365 nm. The analytical column was CLC – ODS (4.6 x 250 mm), filled with ODS (M), RP-18 material, 5 µm particle size (Merck).

Analysis of ZEN was done by using modified method of Scudamore and Patel (2000). For this, a variable wavelength absorbance fluorescent detector set at 274 nm excitation and 440 nm emission was used. The mobile phase consisted of acetonitrile : water (55:45, v/v) and was used at a flow rate of 0.5 ml/min. Injection volume for extract solution was 5 µl Analysis was performed at room temperature (25 - 30°C) and quantification of ZEN was done by comparison of the retention time (9.8 min) and peak area observed in the ZEN standard with those observed for samples.

For analysis of ZOL, a previously published method of James *et al.* (1982) was modified and used. By using a variable wavelength absorbance UV detector set at 236 nm estimation of ZOL was done. The mobile phase consisted of methanol : water (75:25, v/v)and was used at a flow rate of 1 ml/min. Injection volume for extract solution was 5 µl. Analysis was performed at room temperature (25- 30°C) and quantification was done by comparison of the retention time (5.8 min) and peak area observed in the ZOL standard with that observed in samples.

Quantitative analysis of deoxynivalenol (DON) was done by using a modified method of Golinski *et al.* (1996), and for this a variable wavelength absorbance UV detector set at 229 nm was used. The mobile phase

consisted of methanol : water (85:15) at a flow rate of 1 ml/min and the retention time was 2.9 min. Injection volume for extract solution was 5 µl. Analysis was performed at room temperature (25 - 30°C) and quantification of DON was done by comparing the retention time and peak area observed in the DON standard with that observed in samples.

## Results

During the present investigation, chilli powder samples (loose and packed) yielded only two *Fusarium* species, viz., *F. semitectum* and *F. verticillioides* (Table 1). In both the loose and packed chilli powder samples, *F. semitectum* was detected in 14.0 percent samples with a total colony count of $2.8\times10^2 - 1.0\times10^3$ cfu/g and $8.5\times10^2 - 1.7\times10^3$ cfu/g respectively. Similarly, *F. verticillioides* was also found to contaminate loose (10.0 percent frequency) and packed chilli powder samples (5.0 percent frequency) with a total count varying from $3.8\times 10^2 - 9.5\times 10^2$ cfu /g.

Perusal of data presented in table 2 shows that 57.0 percent of chilli powder samples sold loose in the markets were contaminated with zearalenone and range of toxicity varied from 11.81 – 63.29 µg/g. In case of packed chilli powder, lesser samples (48.0 percent) were positive for ZEN contamination and the amount detected was also less (2.66 – 57.35 µg/g). Earlier, Santos *et al*. (2011) reported 30.0 – 90.0 µg of ZEN/kg of chilli and paprika powder samples from Spain. Among the various fusarial toxins, zearalenone is significantly toxic to the reproductive system of animals (Milicevic *et al*., 2010).

Zearalenol (ZOL) is another naturally occurring fusarial toxin derived from zearalenone. Both the marketed forms of chilli powder (loose and packed) were also found to be contaminated with zearalenol (Table 2). About 48.0 percent samples of loose chilli powder were found to be positive for ZOL. Quantitative estimation by HPLC showed that the level of contamination in these samples was quite high (12.35 – 33.80 µg/g of the sample). However, among the packed chilli powder samples, only 29.0 percent samples were positive for ZOL contamination and the range varied from 12.37 – 29.89 µg/g of sample (Table 2). Earlier, Hashmi and Thrane (1990) also reported zearalenol and zearalenone production from some fusarial species isolated from *Capsicum annuum*.

Deoxynivalenol is the most widely distributed fusarial toxin reported from various commodities by workers around the globe (Golinski *et al*., 1996; Koul and Sumbali, 2008; Sodhi and Sumbali, 2012). During the present investigation, 20.0 percent samples of chilli powder sold loose in the markets were detected to be contaminated with DON (level ranging from 85.79 – 180.61 µg/g). However, only 11.0 percent samples of packed/branded chilli powder were positive for this fusarial toxin and the amount varied from 52.82 – 99.01 µg/g (Table 2). Few other reports about contamination of spices with DON exist in the literature. For example, Patel *et al.* (1996) detected low concentration of DON (8 µg/kg) in capsicum powder samples from U.K. However, Santos *et al.* (2011) found a much higher level of DON (upto 269 µg/kg) in capsicum powder samples from Spain.

**Table 1: Frequency percent and total colony count of *Fusarium* species recovered from from loose and packed samples of red chilli powder.**

| Fusarium species recovered | Loose samples | | | | Packed samples | | | |
|---|---|---|---|---|---|---|---|---|
| | % Freq | cfu/g | | | % Freq | cfu/g | | |
| | | CDA | DG-18 | MSA | | CDA | DG-18 | MSA |
| *F. verticillioides* | 10.0 | - | $7.6\times 10^2$ | $9.5\times 10^2$ | 5.0 | - | - | $3.8\times 10^2$ |
| *F. semitectum* | 14.0 | $2.8\times 10^2$ | - | $1.0\times 10^3$ | 14.0 | $1.1\times 10^3$ | $8.5\times 10^2$ | $1.7\times 10^3$ |

**-, not detected**

**Table 2: Zearalenone (ZEN), zearalenol (ZOL) and deoxynivalenol (DON) contamination detected from loose and packed samples of red chilli powder.**

| Loose chilli powder | | | | Packed chilli powder | | | |
|---|---|---|---|---|---|---|---|
| **Sample Code No.** | **Fusarial contaminants detected** | | | **Sample Code No.** | **Fusarial contaminants detected** | | |
| | **ZEN µg/g** | **ZOL µg/g** | **DON µg/g** | | **ZEN µg/g** | **ZOL µg/g** | **DON µg/g** |
| L-1 | 36.57 | - | 85.79 | P-1 | - | 24.14 | - |
| L-2 | 24.44 | - | - | P-2 | - | - | - |
| L-3 | 31.92 | - | - | P-3 | 51.48 | - | 99.01 |
| L-4 | 50.11 | 12.54 | 180.61 | P-4 | - | - | - |
| L-5 | 36.92 | 18.8 | - | P-5 | 2.66 | 21.95 | - |
| L-6 | 40.33 | 12.35 | - | P-6 | 57.35 | - | - |
| L-7 | - | 18.01 | - | P-7 | - | - | - |
| L-8 | 40.88 | - | - | P-8 | - | 16.72 | - |
| L-9 | - | - | - | P-9 | 7.40 | - | - |
| L-10 | 49.07 | - | 125.37 | P-10 | 11.44 | - | - |
| L-11 | 63.29 | 15.73 | | P-11 | 24.65 | - | - |
| L-12 | - | 13.55 | - | P-12 | 45.1 | 29.89 | 52.82 |
| L-13 | - | 20.81 | - | P-13 | - | - | - |
| L-14 | - | - | - | P-14 | - | - | - |
| L-15 | 16.95 | - | - | P-15 | - | - | - |
| L-16 | - | - | - | P-16 | - | - | - |
| L-17 | 11.81 | 18.29 | - | P-17 | 43.42 | 29.89 | - |
| L-18 | 12.54 | 33.80 | - | P-18 | 34.54 | 12.37 | - |
| L-19 | - | - | - | P-19 | - | - | - |
| L-20 | - | - | - | P-20 | - | - | - |
| L-21 | - | 18.22 | 175.58 | P-21 | 13.59 | - | - |
| **Number of positive samples** | 12 | 10 | 4 | **Number of positive samples** | 10 | 6 | 2 |
| **Percent of positive samples** | 57.0 | 48.0 | 20.0 | **Percent of positive samples** | 48.0 | 29.0 | 11.0 |

**-, not detected**

## Discussion

During the present investigation, only two species of *Fusarium* viz., *F. verticillioides* and *F. semitectum* were recovered from red chilli powder samples and that too with low abundance. However, these samples were detected to have high concentration of three fusarial toxins viz., ZEN, ZOL and DON. It is possible that before sun-drying, fusarial species may have existed in abundance and contaminated the red chillies with their toxic metabolites before actually getting destroyed by some biotic or abiotic factors. Therefore, low abundance of intact moulds does not necessarily imply the absence of mycotoxins. Among the chilli powder samples that were sold loose in the markets, majority were found to be detected with zearalenone which is a non-steroidal, oestrogenic, resorcyclic lactone produced primarily by some *Fusarium* species (Schwarzer, 2009). In humans, dietary exposure to ZEN has been associated with precocious pubertal development (Deng *et al*., 2012). While evaluating the carcinogenicity of zearalenone, International Agency for Research on Cancer (1993) found it to be a possible carcinogen. FAO (1997) fixed a limit between 60 and 200 µg/kg for this oestrogenic compound in raw and finished products sold in several countries. The tolerable daily intake (TDI) of ZEN established by the Panel on Contaminants in the Food Chain in Europe is 0.25 µg/kg body weight (EFSA, 2011). Therefore, in view of the reported toxicity, detection of very high amounts of ZEN in the market samples of red chilli powder is of great concern.

Zearalenol (ZOL) is also known to show oestrogenic properties but it is three to four times more oestrogenic than ZEN and may contribute to hyperoestrogenism (Bottalico *et al*., 1985). Presence of this mycotoxin in high concentration among a large number of chilli powder samples suggests that chillies are favourable substrate for ZOL production. It has been found that ZOL has two diastereomers designated as alpha-zearalenol and beta-zearalenol, which are both cytotoxic as they inhibit cell proliferation, total protein and DNA synthesis (Othmen *et al*. 2008). However, alpha-zearalenol is three to four times more active than beta-zearalenone (Othmen *et al.*, 2008).

DON is the most widely distributed fusarial toxin reported from various dried agricultural commodities (Gouze *et al.*, 2006; Sobrova *et al.*, 2010; Sodhi and Sumbali, 2012). Earlier, DON was implicated in large scale human toxicoses in Kashmir valley (Bhat *et al*., 1989). Symptoms in humans include anorexia, nausea, vomiting, headache, abdominal pain, diarrhoea, chills, giddiness and convulsions (Yoshizawa, 1983). Its occurrence in food and feed is also a potential marker of the occurrence of other mycotoxins (Sobrova *et al*., 2010). It is stable in storage and can withstand cooking (Eriksen and Alexander, 1998). DON is known to have a variety of immunological effects at very low exposures in laboratory animals that lead to increased susceptibility to bacteria, viral and fungal diseases (Pestka and Bondy, 1994). IARC (1993) classified DON in category 3 i.e., not carcinogenic to humans, although their co-occurrence with aflatoxins may synergize the carcinogenicity of aflatoxins (Ueno *et al*., 1992).

Detection of ZEN, ZOL and DON in more than permissible limits from investigated samples suggests that red chilli powder is a favourable substrate for mycotoxin production and therefore not completely safe for human consumption. Although dried red chillies and their powder are used in small amounts as a spice, yet their ingestion may lead to deleterious effects on human health. Moreover, as the developed countries have adopted stringent laws with respect to mycotoxin contaminants, the export rejected consignment of dried red chillies are usually thrown in the domestic markets, which is again of great concern. Therefore, modern storage practices that improve quality and decrease the probability of mycoflora and mycotoxin contamination need to be implemented. Recently, the Rapid Alert System for Food and Feed (RASFF, 2011) reported 66 notifications related to the unacceptable presence of mycotoxins in spices and most of these notifications were for capsicums (36 cases, 55%). Out of the 36 notifications of mycotoxins in capsicums, 28 notifications were classified as rejections at the border, 6 as altert and 2 as information notification (RASFF, 2011)

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## Conclusion

In the present investigation, detection of high concentration of fusarial toxins in the market samples of red chilli powder sold loose and packed is of great concern for the health of the people who consume them frequently. Creating awareness among consumers as well as developing new methods for drying and storage are of great importance for improving the quality and decreasing the probability of fusarial toxin contamination.

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