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Research Article

Study of mycotoxins from wheat grains from the Thakur Village Market, Kandivali, Mumbai, India

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Abstract: Mycotoxins are fungal metabolites, which are toxic for the human beings, when consumed. They are usually found in various types of food grains invaded by the fungal pathogens. Aflatoxin is the most common among them and prime mycotoxin produced by various species of *Aspergillus* as well as some other fungi, which can be detected for their presence and can be extracted and estimated by different methods. Thakur Village market is one of the most crowded areas at Kandivali. The shops of small, local vendors situated by the sides of gutters and drainage systems are in unhealthy conditions. They still use gunny bags for storage of grains. Presence of such an unhealthy environment with warm temperature and moisture are the ideal places for growth of fungal pathogens and production of mycotoxins. Wheat is the main food grain purchased from such kind of vendors by the local people. Hence, in the present studies, authors have tried to work on extraction, estimation, and detection of different types of mycotoxins from wheat grains from the Thakur Village Market, Kandivali. Five samples from the said market were selected for screening, where all of them were invaded by the fungal pathogens and showed presence of different types of mycotoxins. *Aspergillus* was the most common fungus while aflatoxin and ochratoxin were the common mycotoxins. These mycotoxins were detected and confirmed by using TLC technique.

Keywords: *Aspergillus*; Aflatoxin; Ochratoxin; TLC

Introduction

Several mycotoxins in agricultural products, especially seeds can cause health hazards to people and animals, which leads to the economic problems (Knutsen *et al.*, 2017). It has been observed that the dangerous mycotoxins are always present in food, feeds, and surrounding environment as natural products. Pathologically, there are four main categories of mycotoxins as hepatotoxins, nephrotoxins, vomitoxin and neuro musculotoxin (Vanhoutte *et al.*, 2016). Some of these are potential carcinogenic and mutagenic agents. Among all the mycotoxins studied so far, Aflatoxin is one of the most effective and dangerous hepatocarcinogen and mutagen. Hence, we must test, identify, and prevent possible contamination of mycotoxins by designing various measures of prevention and control. Mycotoxins are of major concern in grain storage due to their existence because of the fungal development during previously existing conditions (Abass *et al.*, 2014). These conditions include various factors such as moisture content, temperature, storage period, contamination rate, broken grain and impurities, insect presence, oxygen rate, damages during harvest processing and grain and seed transport (Lazzari, 1997; Scussel, 2002; Antos, 2002; Garcia *et al.*, 2003; Scudamore, 2005). Aflatoxins, Ochratoxin A, fumonisins, Deoxynivalenol, T-2 toxin and Zearalenone are the most common mycotoxins.

Presence of aflatoxins is the main hazard to the world due to their rigorous occurrence and extreme toxicity. Various species of fungi especially of *Aspergillus* such as *Aspergillus*

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flavus and *Aspergillus parasiticus* mainly produce aflatoxins on large scale (Aycicek *et al.*, 2005). Presence of aflatoxins as natural components in most of the commodities, such as wheat, corn, soybean and peanut and other cereal crops, consumed on large scale by the human beings and animals (Luz *et al.*, 2017). Presence of high amount of moisture and temperature are the main factors that can cause the occurrence of mycotoxins at pre-harvest and post-harvest stages (Aycicek *et al.*, 2005).

Thakur Village market is one of the most crowded places at Kandivali, which is with mixed population. There are skyscrapers and malls on one side while there is even slum area on the other side. The slum area shows presence of local vendors with their shops situated on the sides of gutters and drainage systems in unhealthy conditions. They still use gunny bags to store various types of grains in their shops. Due to presence of unhealthy conditions, presence of moisture due to gutters and drainage systems along with warm temperature due to gunny bags; these places are ideal conditions for growth of the fungal pathogens in the food grains. In due course of time, these fungal pathogens produce the mycotoxins. Hence, these fungal pathogens and mycotoxins remain in the food grains and consumed by the people unknowingly. They are responsible for various health hazards in the human beings and even prove to be lethal. The most common food grains purchased by the local people from these vendors are the wheat grains, which is the staple food of them. Hence, screening of some samples of the wheat grains from the said local market was done to detect and confirm presence of the fungal pathogens as well as mycotoxins, if present.

The samples of wheat grains were collected from retail vendors in the local market. The samples were collected in 4" x 5" zip – lock polythene bags, which were sterilized and preserved in the refrigerator at about 15 ^{o} to 16^{o} C till further use.

Materials and Methods

1. Collection of the wheat grain samples:

Sr. No.	Source	Code No.
1	Rajdeep Stores, Singh Estate, Kandivali (East)	SEKW1
2	Milan Brothers, Siddharth Nagar, Kandivali (East)	SNKW2
3	Palan & Pasu Co., Damu Nagar, Kandivali (East)	DNKW3
4	Nagrik Stores, Samata Nagar, Kandivali (East)	SNKW4
5	Janata Dhanya Bhabdar, Thakur Village, Kandivali (East)	TVKW5

2. Detection of fungal pathogens:

All the samples of wheat grains, which were collected from the local markets were infected by the potential fungal pathogens. The seeds were sown in a petri plate on moisten absorbent papers in sterilized distilled water. 5 seeds were kept equidistant, per plate. The seeds were incubated at 28^{o} C for 5 days. The method was first implemented by Doyer, (1938). The fungal pathogens were detected by blotters method.

3. Extraction of the mycotoxins:

The chemical analysis of any mycotoxin involves extraction of the test portion to separate the desired component from the bulk. Organic solvents to extract mycotoxins are chloroform, dichloromethane, acetonitrile, ethyl acetate, acetone, and methanol. The mycotoxin can be extracted from the seeds either by mixing the solvent with infected material for 1 to 3 minutes in a high-speed blender or shaken in a flask for time span of 30 minutes. Either extract the liquids in a separatory funnel or can be absorbed in a hydrophilic material which is (pre) packed in a column, after which extraction is accomplished by eluting the column with an extraction solvent. The later extraction

technique is used to determine aflatoxins in liquids, especially milk in which a laboratory prepared Celite column is used (Schuller, 1973; Betina, 1993; Trucksess & Pohland, 2000).

The choice of the solvent is dependent upon chemical properties of the mycotoxin to be extracted as well as on the properties of the material to be used. (Ismaeil, *et. al.* 2015). Usually, mixtures of solvents or solvents with small amounts of water and acids, are found to be most effective and efficient in the process of extraction. Many of the mycotoxins are sparingly soluble in water but aqueous solvents may be completely absorbed by the hydrophilic tissues, which leads to the most efficient extraction by organic solvents (Aiko and Alka Mehta, 2015).

4. Organic Solvent Extraction Method:

In the present studies, 100 gms of wheat grains were weighed and blended with 250 ml of methanol and water in proportion of (55:45) along with 100 ml of hexane. 4 gms of Sodium Chloride (NaCl) was also added. The mixture was blended at high speed. The solution was transferred to the centrifuge bottle and the mixture was centrifuged at 2000 rpm for 5 minutes. 25 ml of lower layer of methanol was pipetted in a separating funnel and 25 ml of chloroform was slowly added to it. The separating funnel was shaken vigorously after stoppering it. The chloroform layer at the lower level was separated into a beaker of 250 ml. The extract was dried on a water bath. The residue was dissolved in 200 µl mixture of benzene and acetonitrile with proportion of (98:2) (Tiana *et al.*, 2015).

The method which was implemented for the detection of mycotoxins in the present studies is given by Patterson, 1979, performed on the glass plates of 20 cm x 20 cm. For detection of mycotoxins, coated silica gel TLC plates were used, where Calcium sulphate ($CaSO_4$) was added as a binder of the silica gel to the glass plate. The mycotoxin extract @ 5 to 15 µl per plate was loaded.

The development of plate in the first direction was done with a mixture of diethyl ether, methanol, and water in ratio of (94:4.5:1.5). The drying of the plate was carried out and then is turned at the angle of 90° and developed in the second direction with a solvent mixture of chloroform and acetone in proportion of (9:1). We carried out detection and quantification under longwave UV light (365 nm) depending upon the colour of fluorescence and Rf values. (G. M. Ware, 1986; BARI, 1988).

Results

As per Table No. 1, five samples of wheat, which were collected from the local market showed presence of 9 types of the fungal pathogens. They were *Fusarium equiseti, Aspergillus niger, Curvularia geniculata, Drechslera maydis, Aspergillus flavus, Aspergillus nidulans, Alternaria brassisicola, Penicillium chrysogenium* and *Helminthosproium* sps. Sample No. SEKW1 showed presence of 4 fungal pathogens i.e. *Fusarium equiseti, Aspergillus niger, Curvularia geniculate* and *Drechslera maydis*, while Sample No. SNKW2 showed presence of only 2 fungal pathogens as *Aspergillus flavus* and *Drechslera maydis*. Sample No. DNKW3 also showed presence of 2 fungal pathogens, *Alternaria brassisicola* and *Aspergillus flavus*. Sample No. SNKW4 showed presence of 2 organisms, *Aspergillus niger* and *Aspergillus nidulans and* the last sample, sample No. TVKW5 showed presence of *Penicillium chrysogenium* and *Helminthosproium* sps.

Table No. 2 gives us information about the types of mycotoxins detected in various samples. As per that all the 5 samples of wheat showed presence of 2 types of mycotoxins predominantly i.e. Aflatoxin B and Ochratoxin A. Aflatoxin A was observed as blue fluorescent

spot while Ochratoxin A was observed as green fluorescent spot under UV radiations. The standard Rf values of Aflatoxin B and Ochratoxin A are 0.81 and 0.55 respectively but the values varied in different samples due to various factors.

The Rf values of Aflatoxin B were 0.81, 0.80, 0.79, 0.80 and 0.81 respectively in the samples SEKW1, SNKW2, DNKW3, SNKW4 and TVKW5 respectively. The Rf values of Ochratoxin A were 0.55, 0.54, 0.56, 0.57 and 0.55 respectively in the samples SEKW1, SNKW2, DNKW3, SNKW4 and TVKW5 respectively.

Table 1: Identification of Fungal Pathogens

Sr. No.	Code No.	Mycelial characters	Conidiophores and conidia	Pathogen
1	SEKW1	Branched, septate, whitish pink fluffy mycelium on the seeds	Macroconodia abundant, typically falcate with foot cell, tapering at both the ends, 4 septate	*Fusarium equisetii* (Uday Prakash, 2004)
		Cottony, black, circular colonies with powdery mass; hyaline, septate, unbranched	Conidia circular, 3.3µ in diameter; arranged in a chain on a single layer of sterigmata	*Aspergillus niger* (Raper and Fennell, 1965)
		Profusely branched, septate, brown coloured	Septate, erect, unbranched, dark brown, nodulose; Conidia curved, 4 – septate, about 23 x 12µ, 3rd cell from the base is larger & darker; terminal cell hyaline, slightly curved	*Curvularia geniculata* Ellis (1966)
		Brownish, black, cottony	Conidia arising in a group; long & slender, brown; curved. Tapering, 4 to 12 septate, measuring 72 x 11.2µ	*Dreschlera maydis* (Subram. & Jain, 1966); (Nishik & Miyake, 1926)
2	SNKW2	Yellowish green colonies with powdery masses	Same coloured conidia, double layer o bottle shaped sterigmata all over the vesicle	*Aspergillus flavus* (Raper and Fennell, 1965;Udaya Prakash, 2004)
		Brownish, black, cottony	Conidia arising in a group; long & slender, brown; curved. Tapering, 4 to 12 septate, measuring 72 x 11.2µ	*Dreschlera maydis* (Subram & Jain, 1966) (Nishik & Miyake, 1926),
3	DNKW3	Hyphae branched, septate, hyaline at first & then turning brown or olivaceous	Olivaceous, rarely branched, straight & upright, 20 – 50 x 7 – 8µ; conidia in chains, oblong. Tapering towards apex, basal cell rounded, apical cell cuboidal; 66 x 12µ	*Alternaria brassicicola* Ellis (1966).

		Yellowish green colonies with powdery masses	Same coloured conidia, double layer of bottle shaped sterigmata all over the vesicle	*Aspergillus flavus* (Raper and Fennell, 1965; Udaya Prakash, 2004)
4	SNKW4	Cottony, black, circular colonies with powdery mass; hyaline, septate, unbranched	Conidia circular, 3.3µ in diameter; arranged in a chain on a single layer of sterigmata	*Aspergillus niger* (Raper and Fennell, 1965)
		Bluish green. Circular powdery colonies	Same coloured conidia on single layered sterigmata forming crown on top of the vesicle	*Aspergillus nidulans* (Raper and Fennell, 1965)
5	TVKW5	Profusely branched, septate, hyaline, greenish yellow	Long with broom like branching, flask shaped sterigmata, globose conidia in chains	*Penicillium chrysogenium* (Thom, 1910)
		Olive brown, proliferating cottony colony with beaded appearance, septate	6 – celled conidia, very tapering at the tip	*Helminthosporium* spp. (Nishik and Miyake, 1926)

Table 2: Detection of Mycotoxins

Sr. No.	Sample No.	Colour of Spot under UV	Rf Value	Mycotoxin identified
1	SEKW1	Blue Fluorescent	0.81	Aflatoxin B
		Green Fluorescent	0.55	Ochratoxin A
2	SNKW2	Blue Fluorescent	0.80	Aflatoxin B
		Green Fluorescent	0.54	Ochratoxin A
3	DNKW3	Blue Fluorescent	0.79	Aflatoxin B
		Green Fluorescent	0.56	Ochratoxin A
4	SNKW4	Blue Fluorescent	0.82	Aflatoxin B
		Green Fluorescent	0.57	Ochratoxin A
5	TVKW5	Blue Fluorescent	0.81	Aflatoxin B
		Green Fluorescent	0.55	Ochratoxin A

Discussion:

Five, randomly collected, samples of wheat grains from the local vendors at Thakur Village Market, Kandivali showed presence of 9 fungal pathogens and 2 types of mycotoxins, Ochratoxin A and Aflatoxin B. Accumulation of these mycotoxins in the cereal grains of wheat can prove to be dangerous and lethal, in extreme conditions, to the local people of the area of Thakur Village.

Aflatoxins are responsible for causing aflatoxicosis, which can be marked with extremely varied clinical signs. The disease shows various symptoms like depression, nervousness, abdominal pain, diarrhea, and death, in extreme conditions (Herrman, 2002). Ochratoxins are the mycotoxins produced by species of Aspergillus and Penicillium, which mainly occur in cereal grains and coffee beans. It is a carcinogenic agent carcinogenic agent and causes acute toxicity in human kidneys. It is also a carrier of alleles for phenylketonuria responsible for severe mental retardation in the foetus (Woolf, 1986).

As these toxins are unavoidable contaminants in the food, the Food and Drug Administration

(FDA) of USA has established an action 732 level for the total amount of aflatoxins which is decided to be at 20 ppb for all foods, including animal feeds (Munkvold *et al.*, 2004). TLC is common method of detection of extracted mycotoxins. Two-dimensional TLC was introduced to mycotoxin research by Kiermeier, 1970. Mycotoxin such as Zearalenone was determined by application of two-dimensional TLC procedures (Jemmali, 1977). The other mycotoxin determined by two-dimensional TLC procedure was sterigmatocystin (Van Egmond, 1980 and Paulsch, 1982) while ochratoxin A was determined by multimycotoxin method (Patterson, 1979).

The 9 fungal pathogens detected were identified based on colony characters along with microscopic observations of the mycelium and the spores (Table No. 1). These fungal pathogens were responsible for production of Ochratoxin A and Aflatoxin B (Table No. 2). These mycotoxins were extracted by using organic solvent method (Tiana *et al.*, 2015) and subjected to the two-dimensional TLC for detection of Rf values (Patternson, 1979). The two-dimensional TLC showed presence of the green fluorescent spots with Rf values of 0.55, 0.54, 0.56, 0.57 and 0. 55 resp. and blue fluorescent spots with Rf values of 0.81, 0.80, 0.79, 0.82 and 0.81 resp. under UV Radiations. As per the reference, the mycotoxin with green fluorescent spot and standard Rf value of 0.55 is Ochratoxin A while the mycotoxin with blue fluorescent spot and standard value of 0.81 is Aflatoxin B. Hence, the spots of mycotoxins with Rf values equivalent to 0.55 (± 0.02) were detected and confirmed as Ochratoxin A while the spots of mycotoxins with Rf values equivalent to 0.81 (± 0.02) were detected and confirmed as Aflatoxin. Figure 1, 2 represents TLC of Ochratoxins and Aflatoxins.

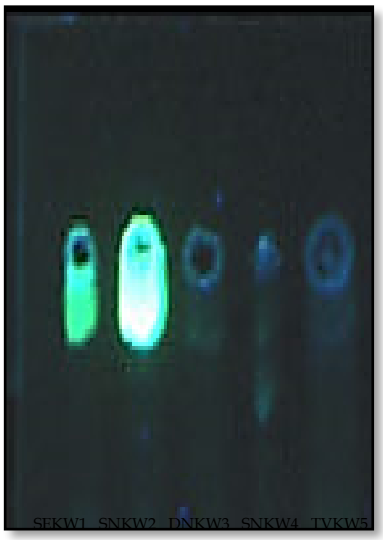

Figure: 1 : TLC - Ochratoxins

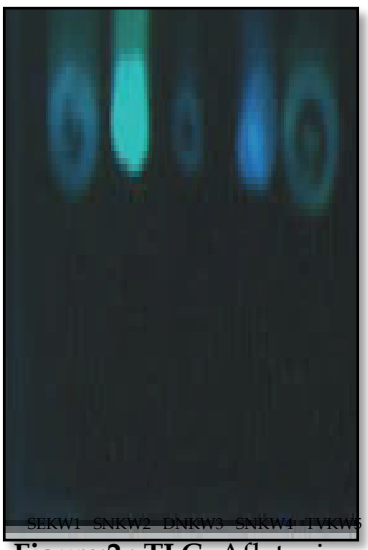

Figure:2 : TLC- Aflatoxins

Conclusion

In our observation, the grain samples of wheat collected from different kinds of retail vendors of Thakur Village Market, Kandivali (SEKW1, SNKW2, DNKW3, SNKW4 and TVKW5) were contaminated with the fungal pathogens (Table No. 1). It was found due to presence of conducive atmosphere *i.e.* presence of darkness, temperature of about 25° to 30° C and average moisture availability of about 60 to 70%, as the samples were kept into gunny bags in very unhealthy conditions. All these samples showed presence of various types of fungal pathogens but presence of different species of *Aspergillus* was detected very prominently, which were

responsible for production of the mycotoxins, especially Ochratoxin A and Aflatoxin B. There is a possibility that the other food commodities are also invaded by the fungal pathogens and contaminated with presence of the mycotoxins. When people are consuming such kind of contaminated food, they are liable for various kinds of health hazards and several problems. There are several ways of prevention and control of hazardous fungi and dangerous mycotoxins produced by them. The safe physical handling and various chemical treatments can be the main methods to control the fungal pathogens. Implementation of Pre and post-harvest proper drying of the grains, and storage of the grains in metal or plastic containers instead of gunny bags can be economical and effective, but sometimes it is not suitable during rainy season or wet condition. Chemical treatments such as alkalinisation and ammonisation are also highly effective and well-recognized.

Hence, we report and suggest that the applications of chemicals, which are toxic for and effective against fungal pathogens for the process of detoxification of mycotoxins should be developed and implemented. At the same time, local vendors and consumers should also be educated regarding this matter to spread awareness among them.

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