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Full Length Research Paper

Fungal incidence and co-contamination of aflatoxins & citrinin in raisins, pistachio nuts, walnuts and almonds marketed in Indo-Gangetic Plain of Bihar

Punam Jeswal, Dhiraj Kumar* & Manoj Kumar

P. G. Department of Biotechnology, A. N. College, Patna – 800013

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The aim of this study was conducted to assess the mycotoxigenic fungal association and co-occurrence of aflatoxins & citrinin contamination in raisins, pistachio nut, walnut and almonds from Indo-gangetic plain area of Bihar. 188 samples of raisins, pistachio nuts, walnuts and almonds were collected from gangetic area of Bihar state. Fungal associations were examined by Potato dextrose agar (PDA) media and Standard blotter paper method. 11 fungal species were isolated from these dried fruits & nuts samples in which *Aspergillus niger* and *Aspergillus flavus* was the most dominant species. Qualitative and quantitative examinations of mycotoxins were conducted by Enzyme Linked Immuno Sorbant Assay (ELISA). 76.3% of raisins and 82% of pistachio samples were contaminated with either aflatoxins or citrinin or both. 462 ppb of aflatoxins were detected from pistachio whereas only 174 ppb were detected in almonds samples. The detected citrinin level was lower in concentration than aflatoxins but the amount was sufficient to induce nephrotoxic effects. The results of this study suggest that raisins, pistachio, walnut and almonds are susceptible substrate for aflatoxigenic as well as citrinin producing fungi and further aflatoxins and citrinin productions.

Keywords: Mycotoxigenic fungi, aflatoxins, citrinin, ELISA, dried fruits

INTRODUCTION

Ganga is the largest and holy river of India and dense population resides in Gangetic plain area. 44900 square kilometer area of Bihar comes under Indo-Gangetic plain where about 103.8 million populations reside. This area is full of fertile soil, water, humidity and adequate temperature. All these factors affect the fungal growth and further mycotoxins production.

Raisins, Pistachio, walnut and almonds are commonly used dried fruits in Bihar. Raisins are widely used in cultural cooking especially in desert because of its sweet and delicate taste. It is dried grapes containing high energy contents and also used in the treatment of anemia and for weight gain in healthy way. Pistachios are naturally dense food and rich source of proteins, unsaturated fatty acids, dietary fibers, Vitamin B_6, calcium, riboflavin, Vitamin A and K (Slavin & Lloyd, 2012). The kernels of pistachio nut are often eaten whole, either fresh or roasted and salted. It controls the blood pressure and Foods & Drugs Administration, USA also approved that the pistachio seeds lowering the risk of heart disease. Almonds are

*Corresponding Author's Email: dhirajbiotech@yahoo.in

commonly used in Indian cuisine for garnishing sweets and eaten as raw and roasted. It is full of nutrition, Vitamin E and considered good for heart. Walnut is full of omega-3 fatty acids along with copper, manganese, molybdenum and biotin. It reduces incidence of coronary heart diseases and gallstones. It is also beneficial in hypertension, cancer and inflammation (Ros, 2010).

Mycotoxins are the toxic secondary metabolites of fungi generally produced on wide range of edible substances under diverse conditions. Most common fungal species that produces mycotoxins are generally belongs to *Aspergillus*, *Penicillium* and *Fusarium* genera (Perrone et. al., 2017). Aflatoxins are toxic, mutagenic, immunosuppressive and carcinogenic agents, generally produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Among 18 different aflatoxins, aflatoxins B_1, B_2, G_1 & G_2 are commonly found in consumable substrate in which aflatoxin B_1 is the most toxic and placed as grade-I carcinogen by International Agency of Cancer Research, Lyon, France (Eaton & Groopman, 2013). Citrinin is generally found as co-contaminant with other mycotoxins. It is nephrotoxic and generally produced by *Penicillium citrinum* and *Penicillium verrucosum* (Anninou et. al., 2014).

Mycotoxins contamination in cereals, oil seeds and spices are reported by many researchers (Barbera et. al., 2017; Mmongoyo et. al., 2017; Jeswa; & Kumar, 2015) but very fragmentary reports are available regarding co-contamination of mycotoxins in dried fruits. However these are mainly confined to aflatoxins and ochrtoxin A contamination (Campone et. al., 2015; Janati et. al. 2012; Heshmati et. al., 2017). Presence of aflatoxins and citrinin contamination in raisins, pistachio, walnut and almond samples were directly associated with the health of consumers. The present study was conducted to assess the association of toxigenic fungi and co-occurrence of aflatoxins and citrinin in raisins, pistachio, walnut and almond samples of Bihar. This is the first report from Bihar regarding co-occurrence of aflatoxins and citrinin in these dried fruits. It has been observed that the pistachio and raisins samples are most susceptible substrate for fungal growth and further aflatoxins and citrinin production where as almond and walnut was a little bit resistant to citrinin producing fungi and its production.

MATERIAL AND METHODS

Survey and Sample collection

An extensive survey of different districts of Bihar located in Indo-Gangetic plain areas have been carried out. Buxar, Bhojpur, Patna, Chapra and Bhagalpur districts were selected for sample collection. These areas have been selected because they have dense population. Total 188 samples (55 raisins, 50 pistachios, 40 walnut and 43 almonds) were collected from different markets. 100 gm of each sample were kept into the sterile cellophane then into sterile brown envelop and kept it at 4°C to arrest any mycotoxins formation.

Screening of fungi

Samples were placed on Potato Dextrose Agar (PDA) media and Standard blotter paper and incubate at 28 °C for 5 – 7 days. Plates were examined visually and by binocular stereomicroscope and fungal counts were recorded. Identification of fungi was carried out by morphological characteristics and followed the taxonomic schemes of Maren (2002) for *Aspergillus*, Pitt (1988) for *Penicillium* and Dugan (2006) and Paul *et. al*. (1983) for other genera.

Qualitative and Quantitative analysis by TLC and ELISA

The Qualitative and quantitative detection for co-occurrence of aflatoxin and citrinin in raisin, pistachio, walnut and almond samples were analyzed by Thin Layer Chromatography (TLC) and enzyme linked immunosorbent assay (ELISA). Qualitative detection of samples was analyzed by the methods of Scott et. al. (1970).

Further positive samples were reconfirmed by ELISA and quantitative studies of samples were analyzed (Eslami et. al., 2015). AgraQuant total aflatoxin (COKAQ1000) was used for total aflatoxins from ROMER LAB (ASTRIA) and RIDASCREEN FAST citrinin Assay (6302) for Citrinin. 20 gm of sample were grinded and added 100 ml of 70% methanol blended for 3 minute. The solutions were filtered and supernatant was collected. 4ml of extract was transferred through cleanup columns. The presence of aflatoxins and citrinin was detected with specific ELISA kits (aflatoxins & citrinin kit) and the optical density was recorded by the ELISA reader (MERK mios mini) using a 450 nm filter with a differential filter of 630 nm. The minimum detectable amount of ELISA kit for aflatoxins was 4ppb and15ppb for citrinin. Standard curve was prepared with standard solution provided with ELISA kits. The optical densities of the samples were compared to the optical density of standards and interpretative results were determined using dilution factor.

RESULTS AND DISCUSSIONS

In our study, prevalence of mycoflora was associated with raisins, pistachio nut, walnut and almonds samples in which some were toxigenic (Figure. 1). A total of 6 different fungal genera belong to 21 species were isolated (Table 1). Fungi were identified on the basis of their culture and morphological characteristics, these were identified as

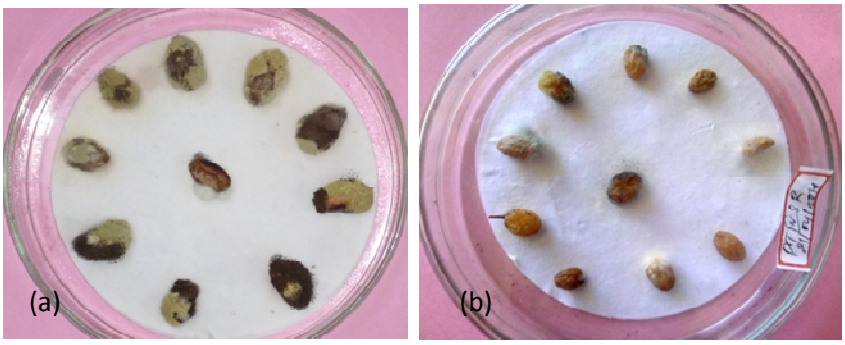

Figure 1: (a) Pistachio nut and (b) Raisins plated in Standard Blotter Paper showing vigorous growth of fungi

Table: 1 Mycofloral association and its count in different analyzed dried fruits samples

Name of Fungi	Fungal count (cfu/g)			
	Raisin	**Pistachio nut**	**Walnut**	**Almond**
Aspergillus parasiticus	2.1×10^4	3.1×10^4	-	1.2×10^2
Aspergillus oryzae	-	1.2 x 10	-	-
Aspergillus tamari	2.4 x 10	-	1.4 x 10	-
Aspergillus niger	5.4×10^3	7.2×10^4	4.6×10^3	6.4×10^3
Aspergillus flavus	6.4×10^3	8.5×10^4	2.8×10^3	3.5×10^2
Aspergillus ochraceus	4.8×10^2	5.1×10^3	6.1 x 10	2.2 x 10
Aspergillus versicolor	1.2 x 10	-	2.1×10^2	-
Aspergillus fumigatus	1.4×10^2	4.1×10^2	-	1.1 x 10
Aspergillus terreus	-	-	-	1.7 x 10
Aspergillus sydowi	-	1.5 x 10	-	1.4×10^2
Penicillium citrinum	4.8×10^2	3.4×10^3	2.6×10^3	2.1 x 10
Penicillium islandicum	0.4 x 10	-	-	1.2×10^2
Penicillium verrucosum	3.2×10^3	5.1×10^2	-	2.6×10^2
Penicillium purpurogenum	-	-	-	1.2 x 10
Penicillium cyclopium	1.4 x 10	-	-	2.8 x 10
Fusarium oxysporum	4.6×10^3	3.2×10^3	4.1×10^2	4.1×10^3
Fusarium moniliforme	3.8×10^2	4.6×10^2	-	3.4×10^2
Chaetomium globosum	-	-	1.1 x 10	0.6 x 10
Rhizopus nigricans	4.1×10^2	2.3×10^2	2.4×10^3	3.5×10^2
Rhizopus oryzae	-	3.1×10^3	-	-
Mucor hiemalis	4.8×10^3	6.4×10^3	-	5.2×10^3

Aspergillus paraciticus, A. oryzae, A. tamari, A. niger, A. flavus, A. ochraceus, A. versicolour, A. fumigates, A. terrus, A. sydowi, Penicillium citrinum, P. islandicum, P. verrucosum, P. cyclopium, Fusarium oxysporum, F. moliniforme, Chaetamium globosum, Rhizopus nigricans, R. oryzae and *Mucor hiemalis*. *Aspergillus flavus* and *Aspergillus niger* was the most dominant species and present in all dried fruits & nuts (Figure. 2). *A. flavus* contamination was highest in Pistachio nut (8.5×10^4) followed by raisin (6.4×10^3). Algahlibi *et. al.* (2004) isolates *A. flavus* and *A. niger* from the raisins samples in Yemen. Pistachio samples were also contaminated with another aflatoxigenic, *A. parasiticus* (3.1×10^4) fungi. Fernane *et. al.* (2010) has reported that pistachio nut of Spain had maximum aflatoxigenic fungal contamination and aflatoxin contamination level up to 1134.5 µg kg^{-1}. In present investigation, Raisins, walnut and almonds samples were also contaminated with mycotoxigenic fungi but lower than pistachio samples. Kashefi & Harati (2012) were reported that *Aspergillus* and *Penicillium* genera were the most prevalent in raisins samples and contaminated with mycotoxins. Citrinin producing *P. citrinum* was highly

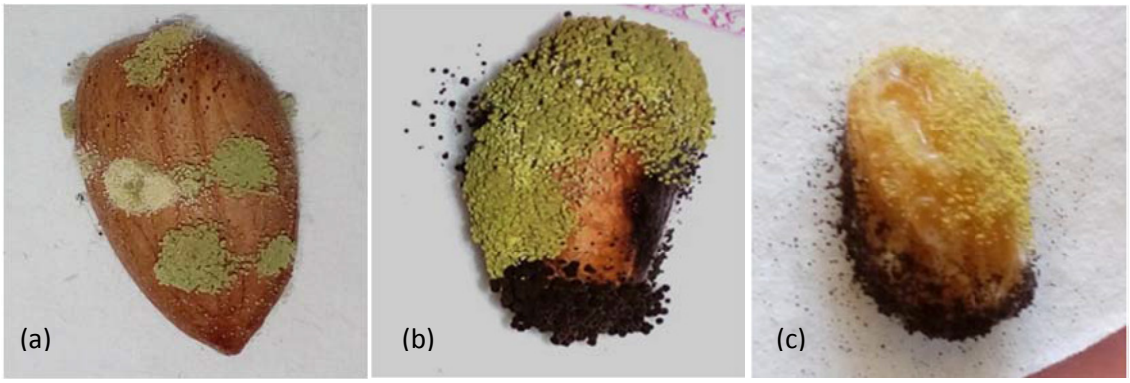

Figure 2: (a) Almond (b) Pistachio nut and (c) Raisin sample contaminated with *Aspergillus flavus and Aspergillus niger*

Table 2: Percent contamination of aflatoxins and citrinin in different samples

Samples	No. of sample analyzed/Positive samples	% contamination	Aflatoxins /(%)	Citrinin/(%)	Aflatoxins + Citrinin/(%)
Raisin	55/42	76.3	35 /(64)	2 (3.6)	5/ (9)
Pistachio nut	50/41	82.0	34 /(68)	0	7/ (14)
Walnut	40/29	72.5	21(52.5)	4 (10)	4 (10)
Almond	43/30	69.7	28/ (65)	0	2/ (4.6)

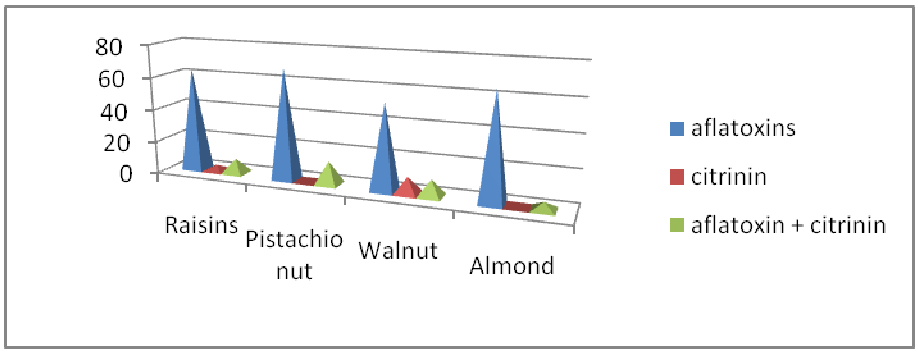

Figure 3: Aflatoxins and citrinin and co-occurrence in dried fruits samples and maximum contamination of aflatoxins in pistachio nut samples.

isolated from raisins (4.8 x 10^2 cfu/g) and least in almonds (2.2 x 10 cfu/g). Another citrinin producing fungi, *P. verrucosum* was also isolated from all substrates except walnut samples.

Co – occurrence of aflatoxins and citrinin

Aflatoxins and citrinin both were present in raisins, pistachio, walnut and almond samples. 82 % of pistachio nuts sample were contaminated with either aflatoxins or citrinin or both where as only 76.3 % of raisins, 72.5 % of walnut and 69.7 % of almond samples were contaminated. Table 2 shows that out of 82 % of contamination in pistachio nut, 68 % were contaminated with only aflatoxins and 14 % with aflatoxins and citrinin both but none of the sample was only citrinin contaminated. 76% of raisins samples were contaminated in which 64 % was only aflatoxin contaminated and 9 % was co-contaminated with aflatoxin & citrinin both (Figure. 3). Generally citrinin is present in nature as co-contaminant with other mycotoxins but 2 samples of raisin were contaminated with only citrinin. Almond samples were least contaminated and only

Table 3: Quantitative values of mycotoxins in different dried fruits

Samples	Mycotoxins (ppb)	
	Total aflatoxins (Mean ± SE)	**Citrinin (Mean ± SE)**
Raisin	248 ± 24.5	225 ± 35.2
Pistachio nut	462 ± 16.2	115 ± 28.5
Walnut	210 ± 24.0	130 ± 24.6
Almond	174 ± 21.8	95 ± 25.4

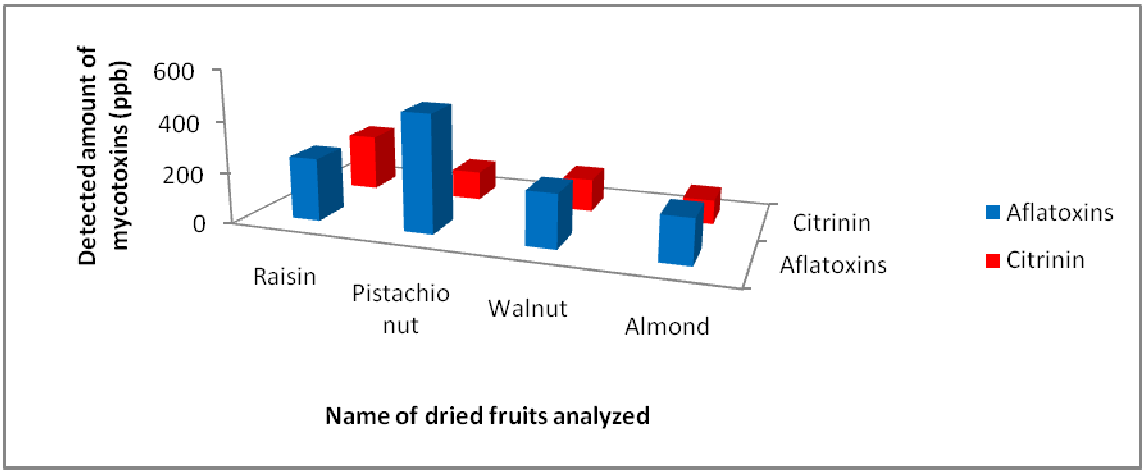

Figure: 4 Showing aflatoxins and citrinin level much higher than EU limit (4ppb for aflatoxins and 15 ppb for citrinin)

69.7% of samples were contaminated in which 65 % were only aflatoxins contaminated and 4.6 % was co-contaminated with aflatoxins and citrinin. Masood *et. al.* (2015) has also reported aflatoxins contamination in pistachio nut from Pakistan and the detected level was 7.89 µg/kg.

Natural occurrence of mycotoxins and risk assessment

Aflatoxins are carcinogenic in nature and there are many reports regarding carcinogenicity by aflatoxins exposure (Ostry et. al., 2017; Wogen, 1999). Citrinin is nephrotoxic and generally present as co-contaminant with other mycotoxins (Jeswal, 1995). Both of the mycotoxins are hazardous for the health of consumers. The EU set the maximum limit of 4 ppb for total aflatoxins and 15 ppb for citrinin in dried fruits and nuts. In our study, pistachio nut has maximum aflatoxins contamination (462 ppb) followed by raisins and Walnut (Table 3). Ferana et. al. (2010) was also detected the aflatoxins level up to 1134.5 µg kg^{-1} in pistachio nut from Spain. Citrinin was also detected from few of the dried fruits samples. The detected level of citrinin was highest in raisins (225 ppb) which was much higher than permissible limit. Whereas almond samples was least contaminated sample either by aflatoxins or citrinin or both (Figure 4).

CONCLUSION

On the basis of the present study, it may be concluded that raisins, pistachio, walnut and almond from gangetic plain area are susceptible substrate for fungal growth and further mycotoxins productions. All 4 types of dried fruits and nuts were contaminated with only aflatoxins or citrinin or both. Pistachio was the most common dried fruits used globally as nuts and is of highly nutritional values, had highest mycotoxin concentration. Almond was least contamination and the amount of aflatoxins & citrinin was lowest than other but it was sufficiently high to induce toxicity. Aflatoxins are carcinogenic whereas citrinin is nephrotoxic and presence of these two mycotoxins in dried fruits and nuts are hazardous and directly concern with the health of consumers. So, it is very important to care in processing, handling, transportation and storage system in gangetic

plain area to reduce the production of hazardous mycotoxins.

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