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MYCOTOXIN CONTAMINATION AND INDUCED BIOCHEMICAL CHANGES IN EDIBLE TREE NUTS

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Natural occurrence of mycotoxins investigated in edible tree nuts namely *Buchanania lanzan, Juglans regia* and *Prunus armeniaca* collected from Uttrakhand region under field and storage conditions. Out of 165 samples analyzed, 69 produced mycotoxins in varying concentrations. Majority of samples are infected with aflatoxin B1 and B2 in the range of 0.08 – 0.98 µg/g. Toxigenicity of *A. flavus* isolates was 25.7, 26 and 12 respectively and concentration of aflatoxin in the range of 0.2 to 31µg/ml. Results indicate significant reduction in level of nutritive content like protein, starch and fat in nut samples due to fungal infection and mycotoxin contamination.

KEY WORDS: Mycotoxins, Biochemical changes, Edible Tree Nuts

Mycotoxin contamination of various foodstuffs is a major problem in tropics and subtropics, where climatic conditions and storage practices are conducive to fungal growth and toxin production. Aflatoxins are the most toxic form of mycotoxins. Some types of food such as dried fruits, spices and nuts, show an increased risk of aflatoxin production due to fungal infection (Wang and Liu 2007, Soubra *et al.* 2009)

Mycotoxins contamination in agricultural commodities and other foodstuffs has been widely investigated from different parts of world, however, the commodities with the highest risk of aflatoxin contamination are corn, peanuts and cottonseed (Soubra *et al.* 2009; Kenjo *et al.* 2007; Wang and Liu 2007).Edible tree nuts (apricot, walnuts and chironji) are an exceptionally valuable crop, as these are widely used as dry fruits and confectionery items. These products can be contaminated with aflatoxins or ochratoxins, with the former being of special concern because of the strict regulatory levels (4 ppb total aflatoxins) applied by the European Community (EC). Aflatoxins have been associated with various diseases, such as aflatoxicoses, in livestocks, domestic animal and humans through out the world (Williams *et al.* 2004). The occurrence of aflatoxin is influenced by certain environmental factors and hence, the extent of contamination may vary with geographical locations (Mwanda *et al.* 2005), agricultural and agronomic practices and the susceptibility of commodities to fungal invasion during preharvest, storage and processing (Park 2002). Aflatoxins have received great attention because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans (Purchase 1974, Krishnamachari *et al.* 1977). Due to potential role of aflatoxins and other mycotoxins in causing cancer and other health hazards in human, detail investigations in edible commodities especially in tropical countries like India is necessary. The nutritive quality of these is also deteriorated due to fungal infection.

Numerous reports are available on the occurrence of mycotoxins. However, very few reports are available on potential occurrence of mycotoxins in edible tree nuts and this study attempts to fill that gap.

MATERIALS AND METHODS

Samples of edible tree nut under study (165 samples) were collected from different locations of Uttrakhand state of India under field and storage conditions. Each sample was placed in a sterile polyethylene bag, sealed and double-sealed with another bag for storage and further studies.

The incidence of mycoflora was studied and list of mycoflora associated was prepared. The

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mycotoxin producing fungi were isolated (ISTA 1999, Seo *et al.* 2008) and screened for their mycotoxin producing potential on specific liquid media (Diener and Davis 1966, Schwenk *et al.* 1958 and Davis *et al.* 1972). Natural contamination of mycotoxins in oilseed samples was carried out by standard methods. The change in the level of starch, protein and fat content of the edible nuts infected with three frequently occurring fungi i.e. *A. flavus, A. niger* and *P. citrinum* was investigated. Statistical analysis of the results were done to infer the effect of season and source on incidence of mycoflora and natural occurrence of mycotoxin contamination. ANOVA was carried out for analyzing the differences in the starch, protein and oil content for control and fungal infested samples of oilseeds using SPSS package(SPSS 1977, Youssef *et al.* 2008).

Extraction of Mycotoxins From Samples: The samples were observed under long wave UV light (360nm) for their probable contamination (Bright Greenish Yellow Fluorescence test or BGYF) and the samples giving characteristic fluorescence were extracted chemically for the presence of aflatoxins by the methods of Thomas *et al.* (1975) which also gave simultaneous detection of zearalenone in those samples. The samples, in which fungi producing other mycotoxins were associated, were extracted by the method of Roberts and Patterson (1975).

Qualitative And Quantitative Detection of Mycotoxins: Qualitative and quantitative estimation of mycotoxins were carried out using Thin Layer Chromatography (TLC). Silica Gel - G (with 13 % $CaSO_4$ as binder) was used as stationery phase for the TLC. 50 µl of chloroform extract was spotted along with the spots of mycotoxin standards obtained from Sigma, USA on TLC plates. The spotted chromoplate was developed in the solvent system comprising Toluene: isoamyl alcohol: methanol (90:32:2, v/v/v). After developing, the plates were air-dried and were observed under long (360nm) and short (260nm) wavelengths UV- light for the detection of mycotoxins.

Chemical confirmation of aflatoxin was done by Trifluoroacetic acid (TFA) as suggested by Stack and Pohland (1975). Presence of Ochratoxin on TLC plates was confirmed with ammonia fumes, which changed blue green spot to a deep blue color (Davis *et al.* 1969). Confirmation of citrinin was done by spraying TLC plates with freshly prepared mixture of 0.5 ml p- anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of conc. H_2SO_4 and then by heating the plate at 130º C for 10 minutes. This changed yellow streak of citrinin to yellowish green under long wave UV- light (Scott *et al.* 1970). Zearalenone was also confirmed by spraying TLC plates with acidic p- anisaldehyde solution (Scott *et al.* 1970) by which greenish blue fluorescence turned faint brown (in visible light) and faint yellow in long wave UV- light. Quantity of mycotoxin was estimated with the help of UV-Spectrophotometer (Nabney and Nesbitt 1965).

Biochemical Changes Due To Fungal Infection: To study the impact of fungal infection, tree nut kernels were surface sterilized for 10 minutes with 2% sodium hypochlorite solution, washed thoroughly with sterile distilled water and inoculated with spore suspension (250-300 spores/ml) of *Aspergillus flavus, A. niger and Penicillium citrinum.* The inoculated seed kernels were incubated for 11 days, powdered and used for estimation of protein, starch and extraction of fatty oil .

Method of Snell *et al.* (1961) was followed for estimation of starch. 200 mg of powdered sample was homogenized with 25 ml of 80% ethanol. After 5 minutes it was centrifuged at 3000 rpm and was evaporated to dryness in an evaporating disc. The residue was dissolved in 5 ml distilled water and from this stock solution starch was estimated. To dissolve starch 6.5 ml perchloric acid was added and stirred constantly for 10 minutes. After 15 minutes 20 ml distilled water was added and repeated for three times and the volume of the combined supernatant was raised to 100ml with

distilled water. To 1 ml of stock solution 10 ml of 0.1 Anthrone reagents was added. The bluish green solution was cooled at room temperature and its optical density was read at 630nm against the blank prepared in distilled water with the similar procedure.

Quantitative estimation of protein was done by the method of Lowry *et al.* (1951). 100 mg of powdered sample homogenised with 100 ml of acetate buffer (pH 4.8) was centrifuged at 3000 rpm for 15 minutes. 2 ml of test solution was taken from the supernatant. To this 10 ml of alkaline reagent (prepared by mixing 50 ml of 2% Na_2CO_3 solution in 0.1N NaOH solution and 1 ml of 0.5%$CuSO_4$ in 1% Na- K- tartarate solution) was mixed thoroughly and was allowed to stand at room temperature for 10 minutes. 1 ml of diluted Folin-ciocalteaue reagent (1:3 in distilled water) was added. After 10 minutes the optical density was read against the blank prepared by casein.

For Isolation and estimation of fat content edible tree nuts were crushed and extracted with petroleum ether (60 –80^0 C) in a soxhlet apparatus. The extract was dried over anhydrous sodium sulphate. It was then filtered and freed from the solvent (AOAC 1984).

RESULTS

Fifty five samples of *B. lanzan* were analyzed for the natural contamination of mycotoxins, out of which 23 were found to be contaminated with aflatoxins and zearalenone. Out of 58 samples of *J. regia*, 27 were contaminated with aflatoxins and citrinin. In case of *P. armeniaca*, 52 samples were analyzed out of which 19 produced aflatoxins and ochratoxins. In *B. lanzan, J. regia,* and *P. armeniaca,* the level of naturally contaminated aflatoxins were upto 0.48, 0.54 and 0.93 ppm respectively. Zearalenone was detected in four samples of B. lanzan at the concentration upto 0.04 ppm. Ochratoxins were detected in only five samples of *P. armenica* and the concentration was upto 0.06 ppm while citrinin (0.14 ppm) was detected in six samples of *J. regia* only (Table 1).

Isolates of *Aspergillus flavus, A. ochraceus, Fusarium verticillioides* and *Penicillium citrinum* were screened under laboratory conditions for their ability to produce different mycotoxins (Table 2). Out of 35, 37 and 25 isolates of *A. flavus* obtained from stored samples of *B. lanzan, J. regia* and *P. armeniaca* respectively, 25.7%, 26% and 12% were found to be toxigenic which produced different combinations of aflatoxins in different concentrations (upto 26 ppm in *B. lanzan*, 19 ppm in *J. regia* and 4 ppm in *P. armeniaca*).

The percentage toxigenicity was comparatively lower in the isolates of other mycotoxigenic fungi from all samples. Seven isolates of A. ochraceus from *J. regia* (out of nineteen screened) and three from *P. armeniaca* (out of 12 screened) elaborated ochratoxin in the range of 0.2 – 3.5 ppm. Out of 19 and 21 isolates of *F. verticillioides* obtained from *B. lanzan* and *P. armeniaca* samples respectively, only 2 and 4 were toxigenic and produced zearalenone up to 4 ppm. Citrinin was elaborated by 7 isolates of *P. citrinum* obtained from *B. lanzan* (23 screened), 9 from *J. regia* (33 screened) and 2 from *P. armeniaca* (11 screened) and the range varied from 0.2 – 6 ppm (Table 2).

Fungal infection significantly affected the

Table 1. Natural contamination of Mycotoxins in Edible Tree Nut samples

Edible Tree Nut	No. of samples screened	No. of Positive samples	Mycotoxin produced	Range of toxin Production (µg/g)
B. lanzan	55	19 4	Aflatoxin Zearalenone	0.8-0.48 traces –0.04
J. regia	58	21 6	Aflatoxin Citrinin	0.4-0.54 traces-0.14
P. armeniaca	52	14 5	Aflatoxin Ochratoxin	0.8-0.98 traces-0.06

Table 2. Screening of fungi for Mycotoxin producing potential

Edible Tree Nut	Fungi screened	No. of isolates screened	% toxigenicity	Mycotoxin produced	Range of toxin Production **(µg/ml)**
B. lanzan	*A.. flavus*	35	25.7	Aflatoxins	0.6 –31
	F. verticillioides	19	10.5	Zearalenone	1. 8 – 2.2
	P. citrinum	23	30.4	Citrinin	3.2 - 4.0
J. regia	*A. flavus*	37	26.0	Aflatoxin	0.2 –24
	A. ochraceus	19	36.8	Ochratoxin	1.6– 3.5
	P. citrinum	33	27.2	Citrinin	2.4 – 6.0
P. armeniaca	*A. flavus*	25	12.0	Aflatoxin	0.2 –11
	A. ochraceus	12	25.0	Ochratoxin	0.2 – 1.4
	F. verticillioides	21	19.0	Zearalenone	3.0 – 3.8
	P. citrinum	11	18.2	Citrinin	0.76 – 1.4

Table-3 Changes in protein content due to fungal infestation

Treatment	Protein content(g/100gms)		
	B.lanzan	*P.armeniaca*	*J.regia*
Control	19.0 ± 0.35	31.4 ± 0.10	15.5 ± 0.23
P.citrinum	18.5 ± 0.15	31.0 ± 0.20	15.2 ± 0.35
A.flavus	18.4 ± 0.15	31.5 ± 0.25	15.6 ± 0.43
A.niger	18.5 ± 0.00	31.6 ± 0.33	15.1 ± 0.40
SEM ±	0.12	0.17	0.15
$CD_{(0.05)}$	0.54	0.77	0.69

Table-4 Changes in starch content due to fungal infestation

Treatment	Starch content(g/100gms)		
	B.lanzan	*P.armeniaca*	*J.regia*
Control	13.06 ± 0.29	7.80 ± 0.12	10.99 ± 0.11
P.citrinum	11.92 ± 0.34	6.58 ± 0.33	10.4 ± 0.12
A.flavus	11.22 ± 0.48	6.37 ± 0.16	9.74 ± 0.51
A.niger	11.50 ± 0.44	6.49 ± 0.49	10.69 ± 0.22
SEM ±	0.35	0.59	0.23
$CD_{(0.05)}$	1.57	2.66	1.03

Table-5 Changes in fat content due to fungal infestation

Treatment	Fat content (g/100gms)		
	B.lanzan	*P.armeniaca*	*J.regia*
Control	42.15 ± 0.61	48.96 ± 0.26	57.08 ± 0.42
P.citrinum	41.03 ± 0.40	48.44 ± 0.14	56.28 ± 0.15
A.flavus	39.06 ± 0.61	47.30 ± 0.09	55.25 ± 0.00
A.niger	41.27 ± 0.13	47.39 ± 0.45	56.55 ± 0.33
± SE	0.56	0.35	0.33
$CD_{(0.05)}$	2.53	1.57	1.49

nutritive quality of the edible nuts under study. In all samples protein content reduced significantly under infection by three test fungi i.e. *A. flavus, A. niger* and *P. citrinum.* The amount of protein in healthy edible nuts of *B. lanzan, J. regia,* and *P. armeniaca* was 18.9%, 15%, 31. and 41%, respectively, whereas the amount of protein in *P. citrinum* infected samples was 18.49, 15.21, and 30.95 %; in *A. flavus* infected samples 18.35, 14.68, and 30.56 % and in *A. niger* infected samples 18.49, 15.12, and 30.60 % in *B. lanzan, J. regia* and *P. armeniaca* respectively (Table 3).

The level of starch in healthy kernels of *B. lanzan, J. regia* and *P. armeniaca* was 13.05, 10.98, and 7.80 respectively where as the starch content in *P. citrinum* infected samples was 11.91, 10.41, and 6.58, in *A. flavus* infected samples 11.21, 9.73, and 6.36 and in *A. niger* infected samples 11.50, 10.68, and 6.49 ppm, respectively in *B. lanzan, J. regia, P. armeniaca* (Table 4).

In healthy edible nuts of *B. lanzan, J. regia*, and *P. armeniaca* fat content was 42.15, 57.08, and 48.95% respectively. The test fungi significantly reduced the yield of fat content and the yield was 41.03, 56.28, and 48.43% in *P. citrinum* infected samples; 39.06, 55.24, and 47.30% in *A. flavus* infected samples and 41.27, 56.55, and 47.38% in *A. niger* infected samples of *B. lanzan, J.regia* and *P. armeniaca* respectively (Table 5).

DISCUSSION:

The results of the study reveal that a number of fungi are associated with seed kernels of edible nuts. *Aspergillus flavus, A. niger* and *P. citrinum* were the most common fungi appearing on the samples. *A. flavus* has earlier been reported as most dominant fungus associated with nuts and oilseeds (Arya 2003). Jimenez *et al.* (1991) reported moulds and mycotoxins in edible nuts and oilseeds and detected mycotoxins upto 95 ppb in natural occurrence. The predominant fungi present in the samples were *A. flavus, A. niger, A. glaucus* and *Penicillium* spp. Isolates of different species were able to produce aflatoxins (B1B2G1G2), ochratoxin and citrinin. Abdel-Hafez and Saber (1993) detected mycotoxins in 90% of hazelnut samples and 75% of walnut samples.

Mycotoxin induced biochemical changes in edible seeds of forest origin, i.e., *Pinus gerardiana, Buchanania lanzan* and *Prunus amygdalus* have earlier been reported by Singh (2004), significant reduction in the level of starch, protein and nucleic acid content have been reported due to infection with aflatoxin B1. Earlier reports indicate food and feed commodities containing several mycotoxins as natural contaminants (Abramson *et al.* 1987, Zohari *et al.* 1987, Reichert 1988, Ranjan and Sinha 1991, Abdel Hafez and Sabah 1993, Khan and Singh 2000 and Singh *et al.* 2001), moreover, limited studies on tree nuts also indicate mycotoxins as natural contaminant (Lohani *et al.*1995 Singh and Shukla 2005a, b). It is important to note that in all cases, most of the contaminated samples contained aflatoxin above 20 ppb, the tolerance level fixed by WHO for human consumption.

Level of total mycotoxins above the regulatory limits in the tree nut kernels is a matter of great concern due to extensive and wide use of these dry fruits. *Buchanania lanzan* (chironji) and *J. regia* (walnut) kernels are valued as a dessert and are extensively used in confectioneries and highly esteemed after frying, salting. *P. armeniaca* (apricot) is used as a table fruit and the oil extracted from dry kernels is used as cooking and also in pharmaceutical and cosmetic industries.

It has been established that the nutritive quality of these nuts is also deteriorated due to fungal infection and mycotoxin production. Mycotoxins are genotoxic carcinogens, they can cause cancer by damaging genetic material. Since these edible nuts of tree origin are infected with mycotoxin producing fungi and the concentration of mycotoxins are quite high in natural occurrence, there is a risk of mycotoxicoses in the consumers. It is recommended that the nut kernels should be

screened and graded before being supplied for consumption purposes. The level of mycotoxins should be reduced to levels as low as technologically achievable. Improvisation in the harvesting, storage and handling of raw materials can minimize mould growth and reduce the risk of mycotoxin contamination in edible food products.

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