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FATTY ACID PROFILE AND AFLATOXIN CONTAMINATION OF WALNUTS (*Juglans regia*)

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ABSTRACT

Samples of walnuts from three different districts of Pakistan; Swat (MS-18), Dir (Dir-2) and Gilgit (Serr), a composite sample from Afghanistan (Sulemani) and an imported variety Pecan from USA were artificially inoculated with spores of *Aspergillus flavus* and then analyzed for proximate composition, fatty acid profile and aflatoxins. Aflatoxin contents in the inoculated samples indicated the presence of B_1 (245.91 to 600.84 ng g^{-1}), B_2 (0.00 to 40.86 ng g^{-1}), G_1 (199.68 to 1068.48 ng g^{-1}) and G_2 (99.61 to 30.15 ng g^{-1}). A reduction in crude protein (6.8 to 17.93 %), crude fat (0.89 to 5.81 %) and Nitrogen Free Extract (9.5 to 24.58 %) was observed in inoculated samples. Ash and fiber were not affected ($r > 0.05$) while moisture increased (170.27 to 225.68 %) due to addition of spores in solution form. Various fatty acids like oleic acid, linoleic acid and linolenic acid were decreased while palmitic acid and stearic acid showed an increase over control (un-inoculated samples). Interaction of aflatoxin with proximate composition and fatty acids showed that B_1 was positively correlated ($r > 0.05$) with protein, ash and fiber while negatively correlated ($r < -0.05$) with fat and oleic acid. Aflatoxin B_2 showed positive relation with ash, NFE, palmitic acid and stearic acid. An inverse proportion of this toxin was observed with fat, oleic acid and linoleic acid. The response of G1 was positive towards fiber, NFE, palmitic acid and stearic acid. A negative effect of G_1 was noted on fat, linoleic acid and linolenic acid. Aflatoxin G_2 showed an inclining behavior towards protein, oleic acid and linolenic acid while a declining effect was observed with ash, palmitic acid, stearic acid and linoleic acid. It was concluded from all the data that growth of *Aspergillus flavus* and aflatoxin production had a deep effect on the chemical composition of substrate. Any supplementation of fatty acids and reduction of moisture may cause reduction in aflatoxin production which would be a possible way of risk management of aflatoxin.

Keywords: walnuts, aflatoxin contamination, fatty acid profile, *aspergillus flavus*, stearic acid, palmitic acid, oleic acid.

1. INTRODUCTION

Walnuts (genus *Juglans*) belong to the walnut family Juglandaceae. Walnuts are grown on deciduous trees, 10 to 40 meters tall. Male and female reproductive organs are borne in different, petalless flower clusters on the same tree. Flowering time of walnut tree is late spring and its fruit is collected in October. The Persian walnuts are also a favorite dry fruit in Pakistan. The trees of the specie are found in the northern and northwestern mountains of KPK (Khyber Pakhtoon Khwa) with a height of 1500 to 3000 meters. Walnut production in Pakistan is about 20,000 tons per year. The bulk is produced in KPK and Azad Kashmir. The total annual production of walnut is worth Rs.200 million, which are usually exported to Middle East countries (Iqbal, 1991).

Walnuts are famous for its oil content. Walnut oil contained poly unsaturated fatty acids like oleic acid, linoleic acid and linolenic acid. Among the saturated fatty acids, stearic acid and palmitic acid are worth mentioning (Aceites, 2005). In fact, the omega-3 fatty acid content of walnuts is 40 to 500 times greater than most other nuts (McKay and Sibley, 2007).

Like other nuts, which are susceptible to the attack of *Aspergillus flavus*, walnuts have also found to be contaminated with toxin related to *Aspergillus flavus*. Infection of walnuts, by various strains of the fungi *Aspergillus* Species may result in production and accumulation of aflatoxins, which are a threat to food safety and detrimental to quality (Mahoney *et al.*, 2003).

Aflatoxins are a group of chemically related mycotoxins, which are carcinogenic in nature. The aflatoxin-producing moulds occur widely, in temperate, sub-tropical and tropical climates, throughout the world; and the aflatoxins may be produced, both before and after harvest, on many foods and feeds especially oilseeds, edible nuts and cereals (Coker, 97).

Subsequent research revealed that there are a number of distinct, but structurally related aflatoxin compounds. The four most commonly seen being designated B_1, B_2 and G_1, G_2. The aflatoxin molecule contains a coumarin nucleus linked to bifuran and either a pentanone, as in AFB_1, and the dihydro derivative AFB_2, or a six-member lactone, as in AFG_1 and its corresponding derivative AFG_2. These four compounds are separated by the colour of their fluorescence under long wave ultraviolet illumination (B for Blue and G for Green). The subscripts relate to their relative chromatographic mobility. Of the four, B_1 is found in highest concentration followed by G_1 and G_2. *Aspergillus flavus* mainly produces B_1 and B_2 (Sanz *et al.*, 1989).

Analytical methods used for aflatoxin and fatty acid analysis are based on GLC (Gas Liquid Chromatography) and HPLC (High Performance Liquid Chromatography) (USDA, 1999).

Keeping in view the hazards of aflatoxin contamination in food products especially in tree nuts and

the wide spread use of walnuts, it was imperative to initiate this study to assess the nature of the problem and suggest ways and means to control aflatoxin contamination of food and nuts in KPK.

2. MATERIALS AND METHODS

2.1 Sample collection

Walnuts samples from three different districts of Pakistan (MS-18, Dir-2, Serr) were collected from local markets of the respective areas of Swat, Dir and Gilgit. A composite sample was collected from Sulemani and an imported variety Pecan of USA was obtained from Research System, KPK Agricultural University Peshawar. They were first deshelled and stored at 4 ^{0}C in refrigerator before being analyzed. Each sample was then subjected to the following analysis.

2.2 Proximate composition

Moisture, ash, crude fat, crude protein and crude fiber were determined by standard method of AOAC (2000). Nitrogen Free Extract (NFE) representing the total carbohydrates was calculated by subtracting the sum of the percentages of moisture, crude protein, crude fat, ash and crude fiber from 100. Nitrogen was determined by Kjeldhal analysis, multiplied by 5.4 and reported as protein.

2.3 Artificial Inoculation of walnut by *Aspergillus flavus*

The purpose of artificially inoculating the spores of *Aspergillus flavus* in to walnuts is to find that either the *A. flavus* strains of Pakistani environment are sterile or toxic, and also to find the effect of aflatoxin on proximate composition and fatty acid profile of different walnut varieties of different ecological zones of this region.

2.3.1 Preparation of media for fungal growth

Malt extract medium (MEA) was prepared by dissolving 50 g MEA in 1 liter distilled water. One gram *streptomycin* was added and was autoclaved (Prestige Medical 2075) at 121 °C for 20 minutes.

For the preparation of coconut media, 1 kg powdered coconut were taken and dissolved in 1 liter distilled water. Added 3 g agar and 1 g streptomycin to it and were sterilized at 121^{0}C for 20 minutes in an autoclave.

2.3.2 Frequency of isolation and total fungal counts in samples

Four seeds were randomly taken from samples of all the selected commodities and directly plated on Petri plates containing MEA and malt salt agar medium. All the inoculations were made in triplicate. The total fungal populations and dominant genera were isolated from the samples using either Malt Extract Agar (MEA) and Coconut Agar media (CAM) or Malt Salt Agar (MS) media (Christensen, 1957).

2.3.3 Isolation and purification of fungus

After a period of one week, the incubated Petri dishes were checked for *Aspergillus flavus*. Heads (conidia) observed under microscope were matched with the standard *A. flavus* colonized in one of the Petri dish. Spores from identified *A. flavus* colonies were transferred on coconut media in already filled Petri dishes. They were incubated for a period of one week at 23-25 °C. The incubated Petri dishes were examined under UV light for toxic strains of *A. flavus* which reflected blue fluorescence. Spores from purified culture of *A. flavus* were taken in 5 ml detergent solution. The detergent with spores was transferred to sterilized vials and stored in refrigerator at 4^o C for using as inoculum of samples.

2.3.4 Preparation of inoculums

Seven days old cultures of *A. flavus* used as a source of spores (conidia). The suspension was filtered and centrifuged twice for 5 min and re-suspended in sterile distilled water to remove germination inhibition factors that appear to be present in the conidial matrix (Railey *et al*., 1997).

About 50 g whole nuts were uniformly inoculated with diluted spore's solution. The inoculated nuts were transferred to sterilized brown bottles. The bottles were capped and were incubated at 30 °C for 15 days. The nuts were dried at room temperature and were tested for aflatoxin.

2.3.5 Walnuts inoculation with A*spergillus flavus*

About 50 g whole nuts were uniformly inoculated with diluted spore's solution. The inoculated nuts were transferred to sterilized brown bottles. The bottles were capped and were incubated at 30 ^{0}C for 15 days. After incubation period, nuts were taken out and cleaned with distilled water to remove spores and mycelia of fungus. The nuts were dried at room temperature and were tested for aflatoxin.

2.4 Aflatoxin determination

2.4.1 Standardization of HPLC

50 µl of each of aflatoxin standards (Sigma Aldrich, PECAN) containing 400 ng g^{-1} of AFB_1 and AFG_1 and 100 ng g^{-1} of AFB_2 and AFG_2 were run through HPLC system and a calibration curve was prepared.

2.4.2 Extraction and cleanup

Aflatoxins in the samples were determined by HPLC, using Altex Model 110 flow pump; Perkin-Elmer model LC 420 injector- autosampler (20µl loop) and Model 650-10LC fluorescence detector, (Excitation 365 nm, emission 440nm, slit width 12 nm) and the AOAC method 990.33 (AOAC, 2000). Briefly, 10 g grinded walnut sample were taken in 250 ml conical flask. 100 ml methylene chloride and 5 g cupric carbonate were added. The sample was shaken with wrest hand shaker for 30 minutes. The content of flask was filtered through whatman No. 2 filter paper and clear 50 ml filtrate was

collected. 30 ml of the filtrate was evaporated in china dish on water bath. The residue was redissolved in 4 ml acetonitrile and transferred to 250 ml separator. 25 ml petroleum ether was added to the separator which given rise to two layers. The lower layer was collected and transferred to another separator. Again 25 ml petroleum ether was added to it and the lower layer was collected. The petroleum ether layer was again heated to dryness for 30 minutes on water bath. The residue was redissolved immediately in 1 ml methylene chloride and evaporated to complete dryness over a water bath. 50 µl TFA (Trifloroacetate) and 4 ml HPLC grade solvent was added to the residue. HPLC solvent was the solution of acetonitrile, water and acetic acid in the ratio of (10:90:1). The extracted solution was then stored in black colour ependorff tubes for HPLC analysis.

2.4.2 HPLC analysis

During HPLC application, flow rate was set at 1 ml/ minute and the column was equilibrated for 30 minutes. A pressure of about 824 pa was developed. 50 µl of each aflatoxin mixed standard B1, G1, B2, and G2, standards containing G1 400 ppb, B1 400 ppb, G2 100 ppb, and B2 100 ppb concentrations were run through HPLC system and calibration curves were prepared. The extracted solution was injected through a loop of 20 µl. Chromatograms of aflatoxin G1, B1, G2, B2 were obtained. The peak height and area was automatically calculated by the CSW32 software.

2.5 Fatty acids determination

2.5.1 Lipid extraction

About 5 g walnuts were finely ground. The oil was extracted with 6 ml hexane: isopropanol (3:2, v/v) at room temperature under vigorous stirring for 1 hour in glass beakers to facilitate homogenization of the nuts. The walnut extract was filtered through a buchner funnel under vacuum, and the residues were washed twice with 4 ml hexane/ isopropanol solvent. Thereafter, 7 ml of 6.7 % sodium sulphate was added and the samples were vortexed for 1 minute and centrifuged at 2000 r.p.m. for 10 minutes. The solvent layer was removed and the pure oil was collected (Savage *et al.* 1997).

2.5.2 Preparation of fatty acid methyl esters

Fatty acid methyl esters (FAME) were prepared from extracted oil. Approximately 200 mg extracted oil was treated with 1 ml methanolic NaOH (40%) at 100 ^{0}C for 15 min. in ground glass stoppered tubes. The tubes were cooled on ice, 2 ml boron trifloride was added and the tubes were boiled for further 15 min. The tubes were again cooled on ice, then 1 ml iso-octane and 2 ml saturated sodium chloride was added, shaked vigorously and was left undisturbed for complete separation in two layers. The upper layer containing the FAME was transferred to a small tube and stored at -20 ^{0}C. The methylated fats were analyzed by capillary column gas liquid chromatography (GC).

2.6 Statistical analysis

Analysis of Variance (ANOVA), correlation and mean comparison was carried out by a statistical package, M-Stat-C. Means were compared by T-test and the interrelationships of different parameters were determined by correlation analysis. Analysis was carried out in triplicate and data were presented as a mean along with standard deviation.

3. RESULTS AND DISCUSSIONS

Proximate composition (Table-1) of control and inoculated samples indicated that moisture content was increased from 170.27 to 225.68 % due to inoculation with moist spores of *Aspergillus flavus*. Crude protein and crude fat were reduced during inoculated period by the values of 6.8 to 17.93 % and 0.89 to 5.81 % respectively. The effect of inoculation was negligible on ash and fiber content. The content of NFE was decreased by a range of 9.5 to 24.58 %. These values were in line with Saleem *et al*. (2006) and Parveen *et al*. (2004).

The fungal profile (Table-6) of walnut kernels from various locations showed the presence of *fusarium, Alternaria, Eurotium, Aspergillus niger, Pink yeast* and *Penicillium*. Among this fungus some were remained un-identified due to lake of facilities. *Aspergillus niger* was worth mentioning contaminating Afghan samples (sulemani) by 70×102 CFU/g of dry wt. followed by *Alternaria*. The presence of *Aspergillus flavus* was not found in the collected samples.

The fatty acids contents of control and inoculated samples (Table-2) were found to have similar fashion of increase and decrease. Among the fatty acids stearic acid and palmitic acid were increased while oleic acid, linoleic acid and linolenic acid showed % decrease. The present data agreed with Mahoney *et al.* (2004*)*. Mahoney *et al.* (2004) provide proof that gallic acids were to be essential for inhibiting aflatoxin production and their presence may cause disturbance in enzymatic pathways of aflatoxin production.

The toxin content (Table-3) of the inoculated samples showed that all the samples contained aflatoxins. Aflatoxin G_1 was found in range of 199.68 to 1068.48 ppb. Aflatoxin B_1 was ranged from 245.91 to 600.48 ppb. Aflatoxin G_2 content was 30.15 to 99.61 ppb. While aflatoxin B_2 was 0.00 to 40.86 ppb. Control samples of walnuts were also analyzed in which every sample showed zero level of aflatoxin. The data agreed with Magbanua *et al.* (2007), who studied that certain amino acids were to be essential for aflatoxin production and their absence or deletion may cause disturbance in enzymatic pathways of aflatoxin production. The Que-king *et al.* (1997) also reported similar data.

The correlation of toxin with proximate composition (Table-4) B_1 and G_2 were positively correlated ($r > 0.05$) with a decrease in protein while G_1 and B_2 were not significantly correlated. G_1, B_1 and B_2 had shown negative correlation ($r < -0.05$) with a decrease in fat content. G_2 was positively correlated which is almost non significant. B_1 and B_2 had shown positive correlation

($r > 0.05$) with a decrease in ash content. G_2 was negatively correlated ($r < -0.05$) while G_1 was nearly non significant. Similarly B_1 was positively correlated ($r > 0.05$) with a decrease in fiber content while G_1, G_2 and B_2 were not significantly correlated. G_1 and B_2 were positively ($r > 0.05$) with decrease in NFE content while B_1 and G_2 were not significantly correlated. The present data was in line with Saleem *et al*. (2006). Parveen *et al*. (2004) also reported similar data.

The correlation of toxin with fatty acids (Table-5) showed that G_1 and B_2 were positively correlated ($r > 0.05$) with an increase in palmitic acid contents. G_2 was negatively ($r<-0.05$) correlated while B_1 was not significantly correlated. Similarly G_1 and B_2 were positively correlated ($r > 0.05$) with an increase in stearic acid content. G_2 was negatively ($r < -0.05$) correlated while B_1 was found non-significant. In the same manner B_1 and B_2 were negative correlated ($r < -0.05$) with a decrease in oleic acid contents while G_2 was positively correlated ($r > 0.05$). In oleic acid case, G_1 was not significantly correlated. G_1 and B_2 were negatively correlated ($r < -0.05$) with a decrease in linoleic acid contents while B_1 and G_2 were not significantly correlated. In case of linolenic acid, G_1 was negatively correlated ($r < -0.05$) with a decrease in linolenic acid contents. B_1 and G_2 were positively ($r > 0.05$) correlated while B_2 was not significantly correlated. Davis and Diener, (1968) had showed that glucose, ribose, xylose and glycerol were considered excellent substrates for both growth and aflatoxin production by *A. parasiticus*, where as oleic and fumaric acids supported growth, but not aflatoxin production.

The correlation data showed that the toxins were tied to proximate and fatty acid contents. Aflatoxin G1 adopted a decreasing response towards fats including linoleic and linolenic fatty acids ($r < -0.05$) while it had an inclined pattern with NFE, palmitic acid and stearic acid ($r > 0.05$). Aflatoxin B1 was mostly positively related ($r > 0.05$) with protein, ash, fiber and linolenic acid while it showed a negative trend ($r < -0.05$) in the case of fats.

Aflatoxin G2 was positively correlated ($r > 0.05$) with protein, oleic acid and linolenic acid while it had showed negative correlation ($r < -0.05$) with palmitic acid and stearic acid. The aflatoxin B2 was affected positively ($r > 0.05$) by ash, NFE and two fatty acids; palmitic and stearic acids, while fat was proved a decreasing agent where oleic acid and linoleic acid were noteworthy. Davis and Diener (1968) also proved a relation of fatty acids with toxin production by *Aspergillus*. Mahoney *et al.* (2004) also provide proof that gallic acid was an inhibititing agent for toxin while some showed the effect of phenolic compounds.

This showed that the toxin production efficiency of *Aspergillus flavus* was dependent on biochemical composition especially on fatty acid contents. There may be some constituents who showed repelling effect towards *Aspergillus flavus*, while some might have attractive nature for this mold; some reduce the production of toxin while some may have increasing effect on toxins production.

The data indicated that there was correlation of toxin with proximate and fatty acids which is a proof of this hypothesis that aflatoxin effect the chemical composition of walnuts. It requires further conformational research. If successed, it would be a preventive measure for *Aspergillus flavus* which produce toxin of extremely dangerous nature.

It was concluded from the experiment that walnuts were infested with various molds, and it was also observed that the *Aspergillus* species were toxigenic which were isolated from peanut and corn of local farm. The *Aspergillus flavus* can grow and produces aflatoxin in walnuts. The *Aspergillus* growth and its toxin producing capacity were dependent on seed composition and certain nutrients enhanced the toxin production while some have negative impact on toxin production.

Seeds should be tested for moisture and mold contamination because the presence of these may endanger high risk of aflatoxin production. The toxin content should be monitored in walnuts either *Aspergillus* is present or absent. Further research should be conducted to change composition chemically or genetically for toxin risk management. As some fatty acids have declining capabilities so they should be added during storage to minimize the risk of toxin production.

Table-1. Proximate composition of controlled and inoculated walnuts of five different locations (on dry weight basis).

Proximate composition	Swat (MS-18)	Dir (Dir-2)	Gilgit (Serr)	Afghanistan (Sulemani)	USA (Pecan)	Mean	SD ±
Moisture (%)							
Controlled	3.33	2.57	3.63	2.90	2.83	3.05a	
Inoculated	9.00	8.37	10.10	8.80	8.94	9.04b	
Crude protein (%)							
Controlled	14.45	12.46	14.00	10.61	12.78	12.86a	
Inoculated	13.65	10.74	11.49	9.87	11.91	11.53b	
Crude fat (%)							
Controlled	62.83	67.33	63.62	68.13	73.40	67.06a	
Inoculated	60.67	66.73	61.16	65.20	69.13	64.58b	
Ash (%)							
Controlled	1.67	1.83	1.87	1.56	1.63	1.71a	
Inoculated	1.66	1.77	1.85	1.54	1.60	1.69b	
Crude fibre (%)							
Controlled	1.93	2.01	1.92	2.00	1.87	1.95a	
Inoculated	1.92	1.99	1.86	2.00	1.86	1.92a	
NFE (%)							
Controlled	15.79	13.79	14.96	14.79	7.49	13.37a	
Inoculated	13.10	10.40	13.54	12.60	6.56	11.24b	

(Controlled)* Walnut samples without inoculation of *Aspergillus flavus* spores
(Inoculated)* Walnut samples infected with *Aspergillus flavus* spores
The mean values of control and inoculated followed by similar laters have no significant differences
The mean values of control and inoculated followed by different later have significant differences
Std±: Standard Deviation of means

Table-2. Fatty acid contents of controlled and inoculated walnuts of five different locations (on dry weight basis).

Fatty acids	**Swat (MS-18)**	**Dir (Dir-2)**	**Gilgit (Serr)**	**Afghanistan (Sulemani)**	**USA (Pecan)**	**Mean**	**SD ±**
Palmitic acid (%)							
Controlled	5.55	6.24	5.24	6.81	6.77	6.12a	
Inoculated	7.63	6.39	7.21	8.32	8.78	7.67b	
Stearic acid (%)							
Controlled	2.96	2.94	2.83	2.21	2.31	2.65a	
Inoculated	3.71	3.19	3.64	3.79	3.89	3.64b	
Oleic acid (%)							
Controlled	25.81	24.26	24.15	22.77	61.40	31.68a	
Inoculated	24.89	24.15	23.78	21.9	59.10	30.76a	
Linoleic acid (%)							
Controlled	56.68	55.04	56.86	56.51	28.01	50.62a	
Inoculated	55.08	54.78	55.91	55.11	27.11	49.60b	
Linolenic acid (%)							
Controlled	9.00	11.52	10.92	11.70	1.51	8.93a	
Inoculated	8.29	11.08	9.43	10.33	1.01	8.03b	

(Controlled)* Walnut samples without inoculation of *Aspergillus flavus* spores
(Inoculated)* Walnut samples infected with *Aspergillus flavus* spores
The mean values of control and inoculated followed by similar laters have no significant differences
The mean values of control and inoculated followed by different laters have significant differences
Std±: Standard Deviation of means

Table-3. Aflatoxin contents in walnuts of five different locations (Inoculated).

	Aflatoxin (ppb)				
	G_1	**B_1**	**G_2**	**B_2**	
Swat MS-18	741.86	515.22	45.40	40.86	1343.33
Dir Dir-2	199.68	292.79	99.61	0.00	592.081
Gilgit Serr	793.94	245.91	65.92	27.90	1133.68
Afghan Sulemani	1068.48	260.58	30.15	21.37	1380.59
USA pecan	761.38	600.84	73.72	40.67	1476.60
Mean	**713.068**	**383.068**	**62.960**	**26.160**	**1185.256**

Table-4. Correlation of aflatoxin with proximate composition of walnut samples.

	Moisture	Protein	Fat	Ash	Fibre	NFE
G_1	0.255	0.361	-0.716	0.418	0.151	0.844
B_1	-0.330	0.584	-0.526	0.787	0.757	0.070
G_2	0.190	0.558	0.384	-0.083	-0.105	-0.474
B_2	0.155	0.380	-0.763	0.778	0.037	0.643

Table-5. Correlation of aflatoxin with fatty acid contents of walnut samples.

	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
G_1	0.757	0.817	-0.411	-0.808	-0.682
B_1	0.457	0.274	-0.819	0.287	0.648
G_2	-0.612	-0.591	0.944	-0.459	0.597
B_2	0.949	0.545	-0.722	-0.773	0.228

Table-6. Percent molds isolated from selected walnut samples.

Fungus	Swat (MS-18)	Dir (Dir-2)	Gilgit (Serr)	Afghanistan (Sulemani)	USA (Pecan)	Total
Aspergillus flavus	–	–	–	–	–	–
Fusarium	22	19	20	19.3	17.0	97.3
Alternaria	26	27	25	17.1	12.7	107.8
Eurotium	11	13	12.4	-	14.8	51.2
Aspergillus Niger	24.7	25	25.2	25.8	22.3	123
Pink Yeast	16.1	15.8	16.2	21.4	10.6	80.1
Penicillium	-	-	-	16.2	22.4	38.6
Total	**99.8**	**99.8**	**99.8**	**99.8**	**99.8**	

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