BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2013. Vol. **10**(1), 01-07

Influence of *Aspergillus flavus* and *Aspergillus terreus* on the protein contents contaminated with Aflatoxins in Peanut seeds at Al-Bayda Governorate, Libya

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(Received: 28 March 2013; accepted: 03 May 2013)

Protein contents showed a big difference in peanut grains assessed by Kjeldahl's and Bailey's methods, the over all mean of protein content of the two peanut grains varieties are not differ significantly at P < 0.05. The protein content of peanut grains affected significantly (P < 0.01) by the time after contamination with *Aspergillus* species *Aspergillus flavus* and *Aspergillus terreus* (*A.f* Vs *A.t*), we suggested that it seems that the protein content of peanut increased from o day to 4 days after contamination, then decreased as the time after contamination increase until 20 days, the result from effect of contamination with *Aspergillus* that produce aflatoxin .The grains had been examined for aflatoxins's existence inside it shown that English peanut (*Arachis hypogea* L.), had been produced aflatoxins. The isolated fungi had been investigated for their capabilities to produce aflatoxins shown that only *Aspergillus flavus* had been produced aflatoxins.

Key words: *Aspergillus flavus* and *Aspergillus terreus*, Protein contents, Aflatoxins, Peanut seeds.

Mycotoxins are secondary metabolites produced by many filamentous fungi and contaminated various agricultural commodities in pre-harvest, harvest, post-harvest and in storage conditions (Kumar *et al*., 2008). Sreedhara and Subramanian, (1981) were found through protein isolation the majority of aflatoxins originally present in the peanut meal is precipitated with the protein fractions. Natural occurrence of sheep (20%25), cattle, and camel feedstuffs were found to be contaminated with various mixtures of aflatoxins AFB_1, AFB_2, AFG_1, and AFG_2 in Libya (El-Maraghy, 1996).

Aflatoxins are highly toxic and carcinogenic compounds (Monteiro and Prakash, 1994), it has been found to cause cancer in all species of animals tested and are among the most po-tent carcinogenic compounds yet identified. In-terestingly, aflatoxins show up not only in plant materials but also in animal-based foods we consume such as meat, eggs, and dairy products (Alexopoulos *et al*., 1996). Under suitable conditions, certain moulds, especially when exposed to dampness, will produce toxic, or otherwise deleterious, metabolites known as mycotoxins (Rodricks *et al*., 1977; Pohland and Wood, 1991).

Peanuts seed are the most important in the world that used feed and food source that are infected with many fungi lead to excrete mycotoxins occasionally under Conditions are favorable (Hesseltine, 1974). *A. flavus, A. fumigatus, A. niger, P. chrysogenum* and *F. oxysporum* were the most common fungal species on peanut (E1-Maghraby and EI-Maraghy, 1987,Janardhana *et al*., 1999; Chandra and Sarbhoy, 1997; Devi *et al*., 2001;

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Amadi and Adeniyi, 2009 and Reddy *et al.*, 2009). Thus, the present study was designed to detection of protein contaminated with aflatoxins in some peanut seeds (Arabic & English) in Al-Bayda Governorate, Libya.

MATERIALS AND METHODS

Samples collection

Commercial seeds from plant family, Fabaceae: peanut (*Arachis hypogea L.*), (Neergaard, 1983; Ali *et al*., 1989); have been collected at 2006 from some markets in Al-Bayda city.

Measured Peanut seed protein

The tested Arabic and English peanut seeds have been superficial sterilized by sodium hypochlorate 4% to 1mn, then washed by sterilized distilled water. The two varieties of grains have been artificial inoculated by the *Aspergillus flavus* and *Aspergillus terre*s separately on PDA media. The two varieties of grains have been incubated for 20 days at 25ºC. The two varieties of grains protein value have been measured before and after artificial inoculated of grains by the two fungi; which have been incubated for 20 days at 25ºC. The peanut grain protein values have been measured every 4 days; after removed the fungus from each grain using Bailey and Kjeldahl's methods.

Bailey s Method

0.1 g of sample (powder) was weighed and mix very well in 10 ml of 1N NaOH . The mixture was kept for 24 hr, then it centrifuged at 5000 rpm for 10 min, 1 ml from supernatant was taken all 0.2 ml Benedit reagent than 2.8 ml 1N NaOH, and mixed well (final vol. 4 ml). The mixture has been kept at room temperature for 15 min and reed at 330 nm. The results were compared with the standard carve of Albumin (Bailey, 1967).

Kjeldahl s Method

1 g of sample (powder) was taken into Kjeldahl's flask containing of 5 g of K_2SO_4, 2.5 g of $CaSO_4$ and 25 ml of conc. H_2SO_4 .The mixture were digested for 2.30 hr, cooled at room temperature and then added 200 ml of distilled water. 70 ml of NaOH (45%) were added to the mixture carefully, and then a piece of Zink. The flask has been conducted to distillate Kjeldahl's operate to 1.30 hr. Ammonia was received from the solution to a conical flask (250 ml) with 50 ml of Boric acid with 4 drops of Promo Phenol Blue indicator and titrated with H_2SO_4 solution. Blank's constant is subtracted from the number of titration then calculating the percentage of nitrogen and protein value.

Examining of aflatoxins existence inside some peanut seeds:

Some peanut seeds Arabic and English; has been tested by using the Alfa card total test (R.Biopharm Rhone LTD, 2005).

Aflacord total simplified procedure

50 g ground sample + 100 ml of 80% methanol have been placed into blender jar and blended for 2 min at high speed. The blended extract has been passed through filter paper and filtrate has been collected.2. 5 ml of filtrate have been poured through a solid phase column and passed through slowly by applying pressure with the plunger. The cleaned up filtrate has been collected in the filtrate collection tube.

The appropriate volume of cleaned filtrate and HPLC Grade methanol: water (80:20 V/V) has been add to one of the graduated dilution tubes and mixed by inverting several times. 1 ml of diluted filtrate has been removed and transferred to a vial containing 3 ml of sample diluents buffer and mixed by inversion. 500 µl of cleaned up filtrate have been applied to a port with a pipette and let passed through the membrane. 100 µl of the reconstituted conjugate have been applied to the port and let passed through the membrane. 100 µl of wash buffer (green label) have been applied and let run through the membrane.

Any excess fluid has been wiped away from the port with a paper tissue. 100 µl of substrate (blue label) have been applied to the membrane, and allow the colour to develop for 5 min (start timer when the substrate is added). 100 µl of stop solution (yellow label) have been applied and results have been read immediately after the stop solution has passed through the membrane. The control spot must develop a clearly visible purple colour in order to have a valid test result.

The sample should be considered as negative; when the sample and the control spot both have a clearly visible purple colour development. The sample should be considered to be positive; when the sample spot fails to develop a readily detectable colour.

Investigating the capability of some fungi to produce aflatoxins

Growth of fungi

Aspergillus flavus, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus ochraceus*, *Penicillium* sp, *Fusarium oxysporium*, *Fusarium gramenarium* and *Alternaria* spp have been used; by isolation from some pretested grains in this test after having been grown in potato dextrose broth (PDB) at 25±2ºC for 20 days. The test has been applied to the media after removed of fungus using Alfa card total test with modification (R. Biopharm Rhone LTD, 2005).

Statistical analysis

The data of total protein content have been statistically analyzed by applied ANOVA-Multiple-way with interactions, using Statistical Analyses Systems (SAS, 1998).

RESULTS

Arabic and English peanut grains had been tested to determine the influence of *Aspergillus flavus* and *Aspergillus terreus* in theire protein value by using Bailey's and Kjeldahl's methods (Table 1):

Bailey s Method

There is a significant effect due to the time after contamination with the two species of *Aspergillus* on protein content ($P < 0.01$) and all the interaction, except the interaction between variety of peanut and time after contamination. The highest values of protein content were obtained after 12, 20, and o8 days after contamination (16.13, 15.90 and 14.98%, respectively), Figures 1, 2 illustrated this relationship.

Table 1. Protein content of peanut as affected by variety, species of *Aspergillus*, time after contamination and their interaction

Item	Method of determination	
	Kjeldahl	Bailey
Overall mean	26.65	15.08
Peanut grain variety (V):		
Arabic	25.78	15.47
English	26.32	14.70
Level of significance	n.s	n.s.
Aspergillus species (S):		
Aspergillus flavus	26.12	15.09
Aspergillus terrteus	25.97	15.08
Level of significance	n.s.	n.s.
Time after contamination (T):		
0 day	24.72[c]	14.45[b]
04 day	34.09[a]	14.25[b]
08 day	28.92[b]	14.98[a]
12 day	24.20[c]	16.13[a]
16 day	23.46[c]	14.80[a]
20 day	20.91[d]	15.90[a]
Level of significance	**	**
Interactions between:V and S	n.s.	*
V and T	n.s.	n.s.
S and T	n.s.	*
V, S and T	n.s.	**

n.s. : not differ significantly at $P < 0.05$.
* : Differ significantly at $P < 0.05$.
**: Differ significantly at $P < 0.01$.
a, b, c, d: Means with the same letters are not differ significantly at $P < 0.05$

Table 2. Examining of aflatoxins existence inside some grains

Grain	Aflatoxin's existence
Arabic peanut (*Arachis hypogea L.*)	-
English peanut (*Arachis htpogea L.*)	+

+ Produced.
- None produced.

Table 3. Capability of some isolated fungi to produce aflatoxins

Fungus	Capability to produc aflatoxin
Aspergillus flavus (1)	+
Aspergillus flavus (2)	+
Aspergillus niger (1)	-
Aspergillus niger (2)	-
Aspergilus terreus (1)	-
Aspergilus terreus (2)	-
Aspergillus ochraceus (1)	-
Aspergillus ochraceus (2)	-
Penicillium canescens (1)	-
Penicillium canescens (2)	-
Alternaria tenuissima (1)	-
Alternaria tenuissima (2)	-
Fusarium graminarium	-
Fusarium oxysporium	-
Many genera of fungi	-

+ Produced.
6 None produced

Kjeldahl s Method

Table (1) shows that the protein content of peanut grains affected significantly ($P > 0.01$) by time after contamination with *Aspergillus* species, while peanut variety (Arabic & English), *Aspergillus* species (*A.f* Vs *A.t*) and possible interactions had no significant effects ($P > 0.05$). Fig. (3, 4) illustrated the relationship between species of *Aspergillus* and time after contamination. It seems that the protein content of peanut increase from 24.72 at 0 day to 34.09 at 4 days after contamination, then decreased as the time after contamination increase until 20 days.

Examining of aflatoxins existence inside some grains

The grains had been examined for afltoxins's existence inside it by using Afla card total test; shown that English peanut (*Arachis hypogea L.*), had been produced aflatoxins, while other grains had not been produced aflatoxins, (Table 2,).

Investigating the capability of some isolated fungi to produce aflatoxins

The isolated fungi had been investigated for their capabilities to produce aflatoxins; by using Afla card total test with modification shown that only *Aspergillus flavus* had been produced aflatoxins, while other fungi had not been produced aflatoxins (Table 3, Fig. 5).

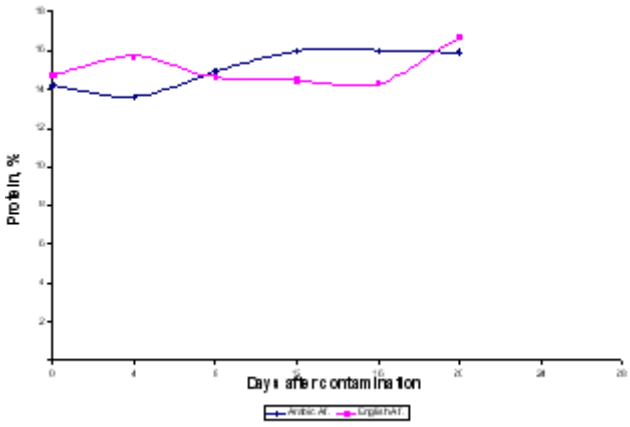

Fig. 1. Effect of time (day) after contamination with *Aspergillus flavus* on peanut protein content (Bailey's method)

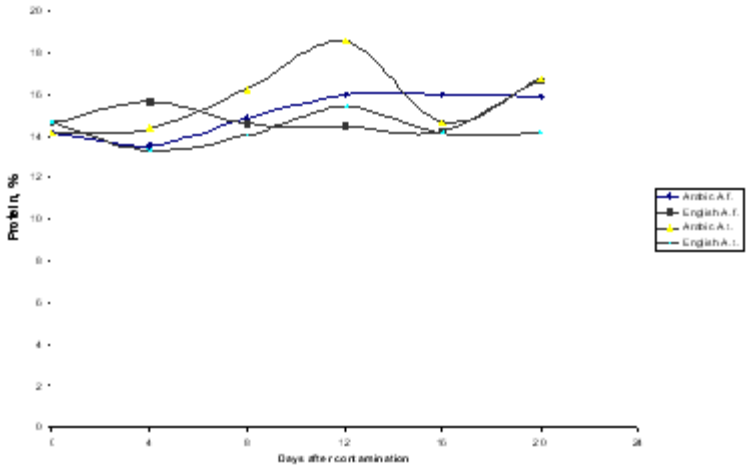

Fig. 2. Effect of time (day) after fungal contamination on peanut protein content (Bailey's method)

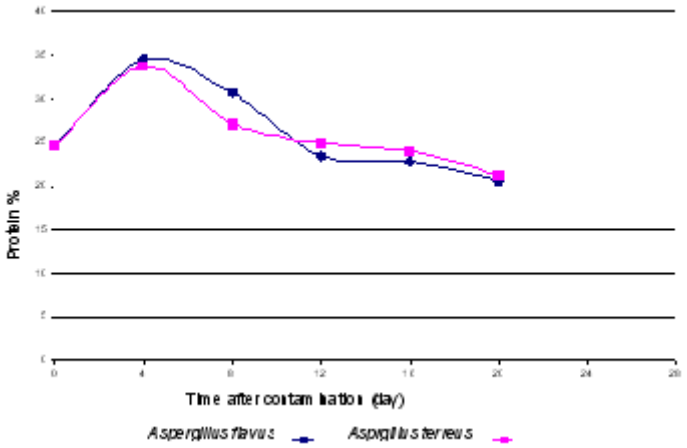

Fig. 3. Effect of time (day) after contamination on peanut protein content (Kjeldahl's method)

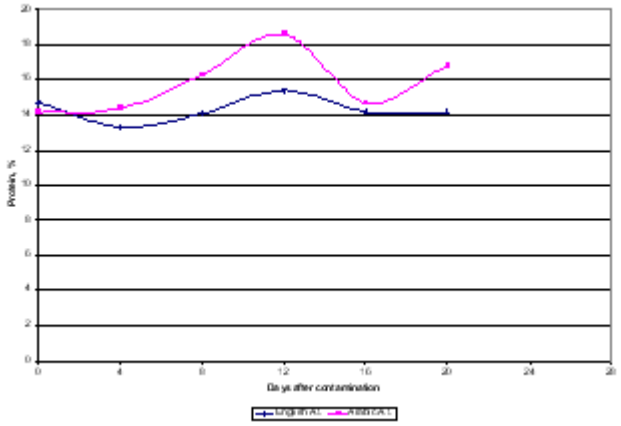

Fig. 4. Effect of time (day) after contamination with *Aspergillus terreus* on peanut protein content (Kjeldahl's method)

Fig. 5. Positive result for *Aspergillus flavus* sample

DISCUSSION

Protein experiment showed a big difference in protein content of peanut grains assessed by Kjeldahl's and Bailey's methods (26.65 Vc. 15.08%). This difference can be explained by the fact that the Bailey's method determined only the content of Albumin, on contrast the Kjeldahl's method determined the total crude protein (total nitrogen 6.25). That result which has been achieved by using Kjeldahl's method (26.65); it is in agreement with Chatfield and Adams (1940), Navia, *et al.,* (1957), Chughtai and Khan (1960) and Bredon and Marshall (1962). While that result which has been achieved by using Bailey's methods (15.08%); is in agreement with Bergeret and Masseyeff (1957) and Platt (1962), these results had been published by Adrian and Jacquot (1968). From Kjeldahl's and Bailey's method the results suggested that the over all mean of protein content of the two peanut grains varieties are not differ significantly at $P < 0.05$. And from Kjeldahl's and Bailey's method results that illustrated to the protein content of peanut grains affected significantly ($P < 0.01$) by the time after contamination with *Aspergillus* species (*A.f* Vs *A.t*), we suggested that it seems that the protein content of peanut increased from o day to 4 days after contamination, then decreased as the time after contamination increase until 20 days, the result from effect of contamination with *Aspergillus* that produce aflatoxin , Monteiro and Prakash, (1994) found aflatoxin affect on protein through addition of aflatoxin B1 to protein fractions and denatured hemoglobin reduced the extent and rate of hydrolysis.

The grains had been examined for aflatoxins's existence inside it shown that English peanut (*Arachis hypogea* L.), had been produced aflatoxins, this result is disagreement with (McDonald, *et al*., 2005) who found that the corn grains are the most of that in USA.

The isolated fungi had been investigated for their capabilities to produce aflatoxins shown that only *Aspergillus flavus* had been produced aflatoxins, *Aspergillus flavus* that fungus which is considered the most detrimental of mycotoxins-producing fungi, for its production of aflatoxins and its the most detrimental aflatoxin B1, that which had been examined in this study by using Afla card total test (qualitative enzyme immunoassay). The using enzyme immunoassay is a good method for the detection of aflatoxins, which applied in many research e.g. Ammida (2005) who worked on barley. This screening test is intended to serve as an indicator of the presence of total aflatoxin at levels of 4ppb, 5ppb, and 10ppb 15ppb 20ppb or 30ppb according to European and U.S. legislation. In additional Piermarini, *et al*., (2007) published a paper for aflatoxin B1 detection in corn, by using electrochemical immunoassay with new modification.

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