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# Influence of UV irradiation on the aflatoxin production capability of *Aspergillus spp.* isolated from contaminated *Arachis hypogea* L.

**Parul Bishnoi[1], Harish Chandra✉[2] and S. V. S. Chauhan[1]**

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## Abstract

**Aflatoxins are toxic secondary metabolite of fungi such as *Aspergillus flavus* and *A. parasiticus*. The fungi occur in a variety of foodstuffs and feed intended for both livestock and human consumption. Aflatoxins produced are potent mutagens and are suspected human carcinogen. During the past two decades several chromatographic and other methods have been developed for identification as well as quantitative determination of aflatoxins in agricultural and food products. An enzyme linked immunosorbant assay (ELISA) was used for the determination of total aflatoxin production in peanut sample and fungal strains after UV irradiation. *A. flavus* ITCC 1717 and isolated strains were irradiated with different interval of time standard strain and isolated *A. flavus* showed almost similar pattern of reduction, at 12 hr the maximum reduction 98.3% and 97% respectively was obtained.**

***Keywords***: *Aflatoxin, Aspergillus spp., ELISA, UV irradiation*

## Introduction

Aflatoxins are secondary metabolites produced by fungal species of genera *Aspergillus* specifically by *Aspergillus flavus* and *A. parasiticus*. It has been reported that out of the known strains of *A. flavus* and *A. parasiticus*, only about one-half produces toxin. There are 14 known aflatoxins but most of these are metabolites formed endogenously in animals administered by one major toxin, i.e. aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$. The letters B and G refer to the fluorescent colors blue and green respectively, displayed by the aflatoxins on absorption of long wave UV light and the subscripts 1 and 2 refer to their separation pattern in TLC plates. These toxins are usually found together with various foods and feeds in various proportions; however, aflatoxin $B_1$ is usually predominant and is the most toxic. When $B_1$ and $B_2$ are ingested by dairy cows, a portion of these aflatoxins is hydroxylated and appear in milk in lactating animals as $M_1$ and $M_2$ at the rate of 1.5% of ingested B aflatoxin (Frobish *et al*., 1986), which may be found in dairy milk. These metabolites are about 75% as toxic to ducklings as the parent compounds (Wogan, 1969).Groundnut (*Arachis hypogaea* L.) occupies an important position in the economy of developing nations. The major groundnut producing countries are India, China and the United States. Groundnut is nutrient dense agricultural product, which is very high in energy due to its high fat and protein content. The carbohydrate content of groundnut is relatively low, being under 30% of the whole nut. The nut has relatively high content of fiber. It is an industrial crop whose major utilization is a source of oil. The number and type of microbes present on the produce is important in deterioration and numerous molds may be involved, but most common are species of *Aspergillus*, *Penicillium* and *Fusarium*. Aflatoxins present in groundnuts are relatively heat stable and are not eliminated completely by ordinary cooking procedures. *A. flavus* invades groundnut seeds both at pre-harvest growth stages and at post-harvest drying/curing and storage, producing aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$.Due to widespread nature of fungi in the ecological system and the production of toxic metabolites by some fungal species, the presence of aflatoxins in foods

## Author's Address

[1]School of Life Science, Dr. B.R.Ambedkar University Agra (U.P) India
[2] Department of Biotechnology G.B.Pant Engineering College Ghurdauri, Pauri, Garhwal (Uttarakhand)
E-mail: hreesh5@gmail.com

and feeds is a great concern for human and animals health. Although the most effective way to control aflatoxins contamination is to prevent formation of the toxins in field and during storage. Technology does not yet exist for the complete prevention of toxin formation or the removal of aflatoxin from foods and feeds (Chu and Ueno, 1977).

For the past 20 years researchers have studied the immunological method to detect the presence of mold in foods (Shim *et al*., 2007; Kolosova *et al*., 2006; Yong and Cousin, 1995; Van der Horst *et al*., 1992). For aflatoxin detection, a great deal of research has been carried out to search for sensitive, specific and simple method. Among the many methods developed in recent years, immunological methods have been shown to be very promising because of their versatility, sensitivity and specificity. An overall recognition of the potentially wide application of new powerful detection techniques has not only led to simple protocols that allow accurate detection of small amount of aflatoxin in food and feeds but has also generated considerable interest in the preparation of commercial kits for aflatoxin analysis. Recently, kits for the detection and quantitation of aflatoxins have become available from several commercial sources. A number of methods have been investigated in connection with their ability to inactivate aflatoxins in contaminated food and feedstuffs. Chemical used as post harvest treatments causes ecological problems or are potentially harmful to humans, with increasing concern of consumers about residues on fruits and vegetables they have been progressively restricted in most countries. Therefore, safe alternative detoxification or control methods need to be developed (Artes, 1995).Irradiation is a physical method, in which ionizing radiations (e.g. X rays, gamma rays, ultra-violet rays) potential changes may occur in molecules of the irradiated objects and living organisms. Aflatoxins are sensitive to UV radiation. $AFB_1$ (Aflatoxin $B_1$) absorbs UV light at 222, 265 and 362 nm, with maximum absorption occurring at 362 nm, which may lead to the formation of up to 12 photodegradation products (Samarjeeeva *et al*., 1990). UV energy penetrates the outer cell membrane, passes through the cell body and disrupts its DNA preventing reproduction. The microbicidal activity of the UV light depends on the length of exposure; the longer the exposure the greater the cidal activity. It also depends on the wavelength of UV used. The efficiency of UV-C radiation against a wide variety of microorganisms already has been reported (Abshire and Dunton, 1981; Sommer *et al*., 1996).The present study was conducted to investigate the presence of aflatoxin in peanut samples collected from local market of New Delhi and efficacy of UV irradiation on aflatoxin detoxification.

## Materials and Methods

### *Sampling*

Samples of peanut (*Arachis hypogea*) were collected from local market of New Delhi, India. All samples were stored in sealed plastic bags and kept at room temperature in dark and dry place. 100 gm sample of peanuts was milled and immediately analyzed for fungal count and total aflatoxin.

### *Viable plate count of Mycoflora*

Fungal count of peanut was determined by using pour plate method as per IS protocol (IS 5403, 1999) in which 11 gm of finely ground sample were aseptically serially diluted in 99 mL of pre sterilized 0.85% of normal saline upto $10^{-6}$ dilution and 1 ml of each dilution was plated on PDA (Potato Dextrose Agar) media. Solidified plates were incubated in inverted position at 28 ± 1 $^{o}$C for 5 days. After five days fungal count was determined using the formula given below. Same dilution was plated on ADM (Aspergillus differential medium, HiMedia ltd) for detection of *A. flavus*.

$$\text{Total Fungal count in cfu/gm} = \frac{\Sigma c}{(n_1 + 0.1\, n_2) \times d}$$

Where Σ c is the sum of colonies counted on all the plates.

$n_1$ is the number of plate counted in the first dilution taken.

$n_2$ is the number of plate counted in the second dilution.

d is the dilution from which the first counts were obtained plate.

**Sample preparation and Immunoaffinity clean up**

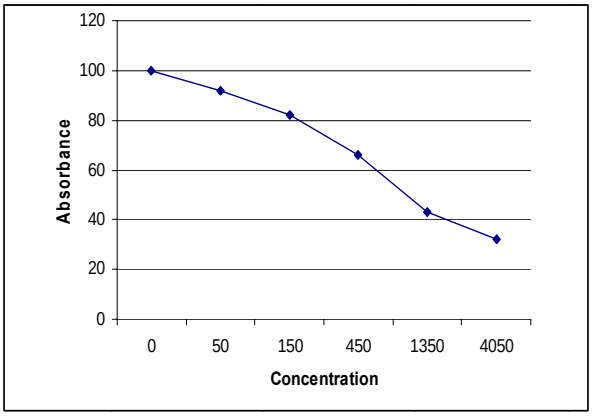
5g fine ground powder of peanut were extracted with 25 ml of 70% aqueous methanol using a laboratory homogenizer and filtered through Whatman No. 1 filter paper. 100µl of each filtrate

were diluted with 600µl of dilution buffer and 50µl of diluted sample employed to immunoaffinity column (R-Biopharm Ag, Darmstadt, Germany) for cleaning the samples. The basis of the test is the antigen-antibody reaction. The column contains gel suspension to which monoclonal antibodies were attached covalently. The antibodies are specific for the Aflatoxin $B_1$, $B_2$, $G_1$, $G_2$ and $M_1$. Total aflatoxin content finally eluted with 0.5 ml of HPLC grade methanol and analyzed through ELISA microplate Reader Model 680 (Bio-Rad).

**Quantification of Total aflatoxin content**

Quantitative analysis of total aflatoxin was performed by competitive ELISA using Ridascreen total aflatoxin kit, Darmstadt, Germany. 50µl of standard solution of Aflatoxin and cleaned eluted sample in duplicate added to the wells of microtiter plate. After that 50 µl of peroxidase enzyme conjugate and 50µlof mouse monoclonal anti-aflatoxin antibodies were added to each well and incubated at room temperature in the dark for 30 minutes. After washing thoroughly with 250 µl distilled water three times, 50µl of urea peroxidase (substrate) and 50µl of tetra-methyl-benzidine (chromogen) were added to each well, mixed thoroughly and incubated for 30 minute at room temperature in the dark. Reaction was stopped by adding 100µl 1M sulphuric acid (stop reagent) and the absorbance was measured at 450nm using ELISA microplate reader Model 680 (Bio-Rad). A calibration curve was drawn using a wide range of total aflatoxin standards with concentration of 0 ppt to 4050 ppt. (Fig.1).

**Fig 1**. **Standard curve of different concentration of total aflatoxin**




**Fungal strains and detection of aflatoxin producing ability of selected strains**

The selected strains included *A. flavus* ITCC-1717 and one strain isolated from peanut kernels have been used for the study. Each isolated strain which gives typical characteristic on ADM (Aspergillus Differentiation Medium) was inoculated at the center of solidified agar medium (PDA) in 9 cm glass petriplate and incubated at 25°C. To observe the color change of colony reverse after incubation, dishes was placed upside down and a drop (0.2 ml) of 25% ammonia solution was put into the lid of Petridish. Immediately after the ammonia solution was put into Petridish, the colony reverse of aflatoxin producing strains become pink and no color change was observed with non-aflatoxin producing strains. The color change was restricted to the colony reverse. The surrounding agar did not show any color change (Saito and Machida, 1999).

*Detection of Aflatoxin from A. flavus.*Yeast malt broth was used as growth media for mycelia growth. 10 ml of reconstitute media in 50 ml centrifuge tube were autoclaved. Each tube was inoculated with a loopful of conidia from all fungal strain cultures and incubated at 25$^0$C for 9 days to obtain aflatoxin in detectable yield and the mycelium had fully grown on the broth surface (Davis *et al*., 1966). The mycelia clumps were harvested by centrifugation (8,000x g for 1 min at 20 $^0$C) and supernatant broth was subjected to aflatoxin detection.For aflatoxin detection, 5 ml of broth was withdrawn into 20 ml centrifuge tube, mixed with 6 ml of chloroform, and vortex for 5 min. Then 5 ml of chloroform was removed to glass tube and flushed with nitrogen to dryness under a hood. The residue was dissolved in 100 µl of methanol out of which 50 µl was used as sample in test procedure of Ridascreen aflatoxin total kit (R-Biopharm, Germany).The basis of the test is the antigen-antibody reaction. The wells in the microtiter strips are coated with capture antibodies directed against anti-aflatoxin antibodies. Standards or the sample solutions, aflatoxin-enzyme conjugate and anti-aflatoxin antibodies are added. Free and enzyme conjugate aflatoxins compete for the aflatoxin antibody binding sites (competitive enzyme immunoassay).

At the same time, the aflatoxin-antibodies are also bound by the immobilized capture antibodies. Any

unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylebenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromate into blue product. The addition of the stop solution stops Ag-Ab reaction due to which colour changes from blue to yellow. The measurement was made photometrically at 450 nm by ELISA reader (Bio-Rad 680).

**Effect of UV treatment against A. flavus**

Two strains of the *A. flavus* were identified for the production of aflatoxin, *A. flavus* ITCC 1717 and isolates from peanut. The aflatoxigenic strains were exposed with UV rays at different interval of time for the effects on aflatoxin inactivation or production. Fresh cultures of the aflatoxigenic strains on Potato Dextrose media plates were exposed directly to UV-C radiation (265 nm, germicidal) of 9 watt at 12 cm distance for different time periods (2 hr, 5 hr, 8 hr, & 12 hr) in triplicates. *A. flavus* isolate and *A. flavus* ITCC 1717 were screened for aflatoxin production using a procedure described by Davis *et al* (1966). Flasks (500 ml) containing 100 ml sterile YES medium (2% yeast extract and 20% sucrose) were inoculated with loopful conidium obtained from each isolate and incubated statically for 7–10 day in the dark at room temperature (21$^{o}$C).Following incubation, the flask contents were filtered (Whatman Filter No. 1), dried at 70$^{o}$C for 24 hrs in hot air oven and weighed. Individual filtrates (50 µl) were freezedried, dissolved in chloroform (5 ml), mixed vigorously, and then quantified for total aflatoxin using an ELISA kit (Ridascreens, R-Biopharm AG, Darmstadt, Germany).

## Results and Discussion

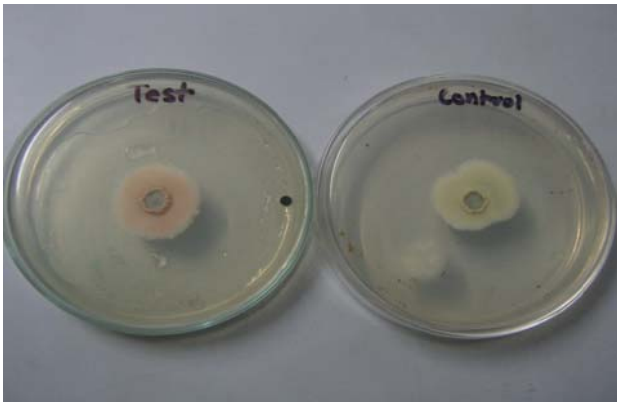
The Food and Drug Administration (FDA) has established an ”Action Level” of 20 ppb (Part Per Billion) for aflatoxin in peanut in interstate commerce (Table 1).In India the regulatory level are set at 30 ppb for all foods. This is the action at which federal agencies may take action including seizure of the peanut or prohibition of its sale. Elevators do not accept peanut with 20 ppb or more of aflatoxin unless they have a known alternative use. Aflatoxins are very potent compounds that cause variety of human and animal health problems. On rare occasions, livestock can die from ingesting aflatoxin contaminating feed. Most commonly aflatoxin reduces the feed efficiency and reproductivity of livestock. It can suppress the immune system of animals, leading to more frequent occurrence of infectious diseases. In the United State alone, the economic loss from mycotoxin is estimated to be $932 million (CAST, 2003). The result of Aflatoxin analysis of peanut samples collected from the local market of New Delhi was presented in Table 2. Viable count of peanut mycoflora varies from 2.9 x $10^{1}$ to 4.2 x $10^{6}$ cfu/gm. A viable plate count method for detecting mould contamination is used to determine the mycological quality of foods and agricultural commodities (Liewen and Bullerman, 1992). A high mould count indicates the possibility of aflatoxin contamination. Same dilution was also plated in ADM for the isolation of *A. flavus* which was merely used for the identification or detection of *A. flavus*. ADM (Aspergillus differential media) is a selective medium for *A. flavus* group of fungi which develop characteristic reverse orange color after 42 hrs incubation at 30$^{o}$C (Pitt *et al*., 1983). Aflatoxin contamination in peanut were determined by using competitive ELISA prior to immunoaffinity column clean up and found that out of 25 samples of peanut tested 19 (76%) samples were found positive for total aflatoxin and out of 19 samples, 13 samples (52%) were exceeding the maximum limit set by FDA (Table1). *A. flavus* was isolated in 8 samples and only 1 isolate are able to produce Aflatoxin (Fig. 2). In Gujarat, state of India 42% of the feed samples was found to be contaminated with aflatoxin (Flounder and Sheila, 1977). A survey of food grains in some village of Mathura District of U.P affected by the floods showed that out of 36 feed samples, 18 were positive for Aflatoxins (Mishra and Singh, 1978). Detection of mould using ELISA and viable plate count, the result shows positive correlation, the ELISA reading correlate the plate count and aflatoxin levels however no absolute pattern, similar type of finding were also reported by Yong and Cousin (2001). The effect of UV radiation on the aflatoxin producing ability of *A. flavus* ITCC 1717 and isolated strain were presented in Fig. 3 and Fig. 4 respectively.Effect of UV radiation on aflatoxin production of *A. flavus* ITCC 1717 and isolated strains shows almost similar pattern of reduction, at 12 hr the maximum reduction of about

**Table 1. FDA regulatory levels for total aflatoxinsin livestock feeds and human food**

| Commodity | Concentration ppb |
|---|---|
| All products, except milk, designated for humans | 20 |
| Corn for immature animals and dairy cattle | 20 |
| Corn and peanut products for breeding beef cattle, swine, and mature poultry | 100 |
| Corn and peanut products for finishing swine | 200 |
| Corn and peanut products for finishing beef cattle | 300 |
| Cottonseed meal (as a feed ingredient) | 300 |
| All other feed stuffs | 20 |
| Milk | 0.5 |

Source: Park and liang [1993] and Park and Njapau [1989]

**Fig 2. Aflatoxin producing potential of isolate after exposing to Ammonia vapour. (A) Characteristic pink color indicative of aflatoxin production (Isolate). (B) Negative control (Non Aflatoxin producer)**



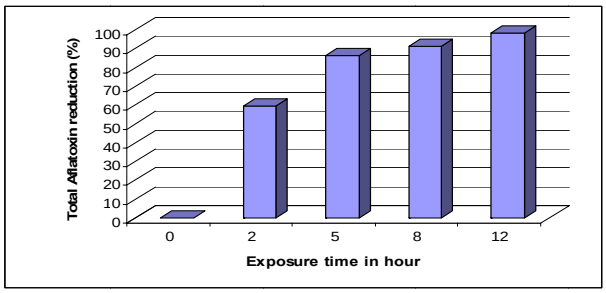




**Fig.3. Effect of UV radiation on Aflatoxin producing ability of Isolated *A. flavus.***

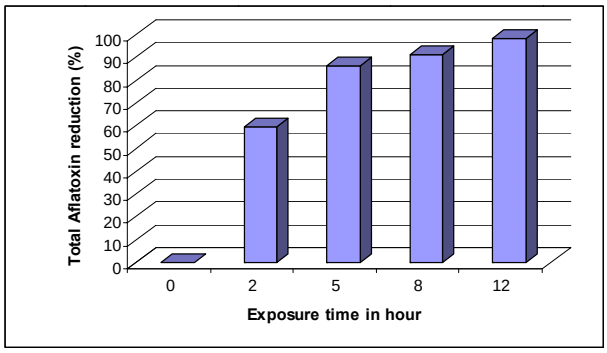



**Fig.4. Effect of UV radiation on Aflatoxin producing ability of *A. flavus* ITCC 1717.**

98.3% and 97% respectively was obtained. The detoxification of aflatoxin in fungi by UV could be due to two mechanisms: the direct inactivation of *A. flavus* conidia by the radiation or a change in the aflatoxin biosynthesis. UV-C is germicidal and, hence can inactivate fungal spores. The irradiation of fruits with UV-C was effective in the curative control of brown rot (*Monilinia fructicola* ) and soft rot (*Rhizopus stolonifer)*, and the best results were observed with an exposure time of 10 minutes (Bassetto, 2007). Green *et al*. (2004), reported that dose of 35 mJ/cm$^2$ ultraviolet germicidal irradiation can cause 90% inactivation of *A. flavus* on an agar surface. UV-C radiation was reported to significantly reduce the concentrations of *P. corylophilum*, *A. versicolor* and *Cladosporium* spp. UV-C light can significantly reduce the mouldpopulation on shells of eggs after 15 min exposure (Kuo, 1997). Begum *et al*. (2009) also studied the effect of ultraviolet irradiation on *A. flavus*, *A. niger*, *Penicillium corylophilum* and *Eurotium rubrum* and found 80–99% reduction of viable spores for all species except *A. niger*, for which the reduction was only 62%. Treatment of peanut oil with UV light for 2 hrs destroyed 40-45% of aflatoxins initially present in the oil (Shantha and Murthy, 1977). Exposure of artificially contaminated milk to UV light inactivated 3.6-100% of $AFM_1$ in the milk, depending on exposure time (2-60 min). Also, addition of hydrogen peroxide (1%) to the UV-irradiated milk (10 min) completely (100%) destroyed $AFM_1$ (Yousef and Marth, 1985). The photodegradation products were less toxic to chick embryo than the parent toxins (Andrello *et al*.,

1967). UV radiation (30 min) of dried figs artificially contaminated with AF-$B_1$ (250 µg/Kg) reduced the aflatoxin level by 45.7% (Altug *et al*., 1990). Chun *et al*. (2010) evaluated the inactivation of food borne pathogens on chicken breasts by different dose UV-C radiation and found that at 5 $kJ/m^2$ reduce the microbial population. Reduction in count of inoculated *Salmonella enterica* subsp. Enterica Ser. Enteritidis was also reported by de Souza and Fernandez (2011). UV radiation does not require heat or chemical and inexpensive. It is more advantageous than other existing sanitation method. FDA already approved the use of UV-C (wavelength of 220-300 nm with 90% of emission at 253.7 nm) on food products to control microorganism (Park and Liang, 1993).

**Table2. Total aflatoxin and fungal count in Peanut sample**

| Sample No. | Fungal count (cfu/gm) | TAF (ppb) | Growth on ADM |
|---|---|---|---|
| 1 | $2.8 \times 10^5$ | 141 | + |
| 2 | $1.4 \times 10^4$ | 46 | + |
| 3 | $2.4 \times 10^2$ | 27 | + |
| 4 | $2.9 \times 10^1$ | 16 | - |
| 5 | $4.6 \times 10^2$ | 2 | - |
| 6 | $9.6 \times 10^4$ | 38 | + |
| 7 | $1.2 \times 10^3$ | 28 | + |
| 8 | $2.5 \times 10^5$ | 144 | + |
| 9 | $3.8 \times 10^2$ | ND | - |
| 10 | $4.2 \times 10^6$ | 126 | + |
| 11 | $9.2 \times 10^2$ | 34 | + |
| 12 | $1.8 \times 10^2$ | 46 | + |
| 13 | $4.4 \times 10^3$ | 54 | + |
| 14 | $5.2 \times 10^2$ | ND | + |
| 15 | $8.0 \times 10^2$ | ND | + |
| 16 | $6.2 \times 10^2$ | ND | - |
| 17 | $2.0 \times 10^4$ | 86 | + |
| 18 | $1.1 \times 10^2$ | ND | - |
| 19 | $2.0 \times 10^2$ | 24 | + |
| 20 | $1.4 \times 10^3$ | 14 | - |
| 21 | $1.6 \times 10^2$ | 25 | + |
| 22 | $3.1 \times 10^2$ | 75 | + |
| 23 | $1.9 \times 10^4$ | 19 | + |
| 24 | $2.7 \times 10^2$ | 28 | + |
| 25 | $2.3 \times 10^2$ | ND | - |

TAF- Total Aflatoxin, ND- Not Detected, + sign indicate growth which shows typical characteristics of *A. flavus*

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