THE ESTIMATION OF AFLATOXIN LEVEL IN GRAINS AND PULSE ON THE BASIS OF THE HIGH RATE OF LIVER CANCER PATIENTS IN ARARIA DISTRICT (SIRSIAKALA VILLAGE) OF BIHAR, INDIA.

Abstract

The present study was conducted after a news article published on 12th November 2015. It was about the village of Araria known as "Cancer Tola" It was about the village of Araria known as "Cancer Tola" where mostly people suffered from liver cancer and the rate of death was very high. To find the reason behind it we conducted a study which included personal meetings with the patients and villagers to collect more information for the study and find the actual reason behind this disease. In personal interviews, we mainly collected information about the feeding behaviour of their day-to-day life of the patients and people of this village. From the gathered information, we assumed that the problem has arisen from their feeding habitats and it may be due to grains which they use as their main food. We collected grains pulse (*Phaseolus aureus*) moong from their homes for further study. After the study, we found that their grains and Pulse (moong) have been affected by the *Aspergillus flavus*, which produces aflatoxin B1 & B2. These aflatoxins greatly affect human health and causes. Aflatoxins have both carcinogenic and hepatotoxic actions, depending on the duration and level of exposure.

Keywords: Aflatoxin, Aspergillus, Cancer, Grain, Maize, Mycotoxin

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I. INTRODUCTION

A new paper Danik Jagran (On Date 12-11-2015) Bhagalpur, Bihar, India edition published breaking news. It is about the village of Araria district, "Block Bharagama', Sirshia kala Panchayat (Rahmaan Tola). In that village according to " Danik Jagran" many people survived from cancer. (Maximum liver cancer).

According to the news, this area is known as "Cancer Tola" and most people survived from liver cancer and the rate of death is high. So, we assume that this is the problem of food habitats and it may be due to grains which they use as their main food. For the study, we went to that place on the date of 25-02-2016 to study. We tried to meet with the patient to know their food habits and related queries as well and we collected food samples.

The following points were noted after meeting the study place. The living style of the village is not good or septic. The people generally used to take wheat, rice, moong and maize as the main course of food. The water is not potable and it is a very low land area. The patient who survives from cancer is the aged between 40-60 years which means they are infected slowly. Most of patients do not use tobacco, so it is not regarded as the main factor for cancer. Pulse of moong (*Phaseolus aureus*) is consumed highly and also used as food very faithfully. Maize is also harvested but not widely used as the main food by villagers. Soils and land area are highly moiled. After this information, we thought that this is the problem of food habitats or problems in food (Which is used in the main course). It has been assumed that, it may be due to the soil fungi *Aspergillus flavus.* The aflatoxins are synthesized by the filamentous fungus *Aspergillus flavus* and related aspergilli. These fungi affect a number of seeds and grains including peanuts, corn, cottonseed, a number of nuts. Aflatoxins are therefore relatively easy to manufacture, simply by practising poor grain. husbandry. Aflatoxins taken in the diet are further converted to the still dangerous aflatoxins M1 and M2 that are secreted in the milk. Aflatoxins are mycotoxins that are naturally produced by Aspergilus species, primarily *A. flavus*. As a result of the toxin's connection to A. flavus, it was given the name "aflatoxin" (Guo *et al*., 2008). The groundnut meal that had been fed to various farm animals contained this fungus. Notably Severe Cases of Aflatoxin Ingestion: In 2004 in Kenya 125 people died and nearly 200 others were treated after eating aflatoxin contaminated maize. The deaths were mainly associated with homegrown maize that had not been treated with fungicides or properly dried before storage. Due to food shortages at the time, farmers may have been harvesting maize earlier than normal to prevent thefts from their fields, so that the grain had not fully matured and was more susceptible to infection (Lewis *et al*., 2005). Aflatoxin is a crystalline chemical that dissolves to a concentration of 10–20 mg/l in water and is easily soluble in moderately polar solvents including chloroform, methanol, and dimethyl sulfoxide. A naturally occurring fungal metabolite called aflatoxin is a highly stable substance that may withstand routine food and feed processing techniques (PACA, 2013). Acute aflatoxicosis outbreaks caused by extremely contaminated food also have been reported in Kenya, India, and Thailand (Council for

Agriculture Science and Technology (CAST) 2003). Many agricultural commodities are commodities are vulnerable to attack by a group of fungi that are able to produce toxic metabolites called mycotoxins, aflatoxins have assumed significance due to their deleterious effects o human beings, poultry and livestock. When a sickness known as "Turkey 'X' Disease" caused a serious epidemic in the UK in 1960, killing over 100,000 turkey chicks,

the aflatoxin issue was first identified. Toxins found in peanut meal that had *Aspergillus flavus* infections were identified as the disease's cause and were known as aflatoxins. The primary objective of this study was to characterize the extent of aflatoxin contamination within the grains (which people in the study area consumed more i.e. Moong and Maize).

II. MATERIAL AND METHODS

Collection of Samples: We collected the sample of grains (Maize, Rice, Wheat and Moong Dal pulse). They are generally used to intake mostly people of that village about 5 to 8 samples collected from each grain from the patient's home. We also collected soil samples from their field and then packed all the samples for further study.

BGYF Test: Samples were grined and after grinding it was observed under uv very minutely. The sample which produces blue-green fluorescence was taken for further processing. It is assumed that the sample which produces a blue-green colour in the presence of UV light might affected by aflatoxin which was produced by a toxigenic strain of Aspergillus.

Plating of Sample: First of all, the samples were taken (Moong, Wheat and Maize). All seeds are dipped in distilled water and then autoclaved for 30 minutes. After that half of the seeds were dipped in NaOHCI for surface sterilization of the samples (used as treated samples). Half of the seeds were made non-treated without being dipped in NaOHCl After this process we plated all the samples in a humid environment. The sterilized filter paper was used to maintain humidity in the petri plate.

The treated and non-treated seeds were arranged in a circular shape and left for the first week for vigorous growth of fungi. The large seed was taken in a ratio of 1:8 and the small in a ratio of 1:8:16 and inoculated in BOD at 37^0 centigrade.

25 gm powdered sample was mixed in 62.5ml methanolic water mixture in a ratio of 6:4. The Mixture was filtered with the help of filter paper. 0.25ml n hexane 15 ml water and saturated NaCl were added to the filtered mixture. After that whole mixture is taken in a separating funnel, shaken vigorously and left it 20-30 minutes to form two separate layers. The lower level of the mixture was then taken and 25ml of chloroform was added in the separating funnel. Shaken vigorously and leave it for 20-30 minutes to form two separate layers. The lower level of the mixture was passed through Na_2SO_4 (250 mg.) for the normal of culture.

Again, the whole mixture was passed through $CuCO_3$ (Copper carbonate) for the removal of plant pigment. After that the mixture was dried in the water bath and after drying 1ml chloroform ($CHCl_3$) was added and stored in a vile at 40^0 centigrade. The quantity of toxin is determined after thin-layer chromatography then observation under ultraviolet ray.

Identification of Toxigenic Strain: SMKY medium has been used to identify toxigenic strains. For preparation of SMKY medium, 200gm Sucrose, 5mg $MgSO_4$, 3mg KnO_3, 7gm Yeast have been dissolved in 1000ml distilled water. Leave it at room temperature after autoclaving. All types of *Aspergillus* strains which were collected in pda slant were inoculated in different conical flask containing SMKY medium for proper growth of fungal strain. After 10 days each conical flask was filtered individually.

In the collected SMKY medium 25 ml of chloroform was added, shaken vigorously and left for 20-30 min. The lower layer of solution was collected and passed it through Na_2SO_4 and $CuCO_3$ and then dried it on water bath till it became fully dried. 1 ml chloroform was added and stored it at 4°c. This process is followed for each and every conical flask. All samples were loaded on TCL, isoamyl alcohol and methanol were taken in the ratio of 90:32:2. Observed under UV rays. Under UV light many spots produced Blue-Green colour which means the specific type of strain produced aflatoxin B1,B2, G1 and G2. The sample spots were scrubbed on paper and dissolved in 5ml methanol. Centrifuge it at 3000rpm, supernatant was taken and optical density was noted at 260nm.

Table 1: Table sows molecular weight and molar extraction of aflatoxin (B1, B2 and G1)

Aflatoxin	Wavelength	Molecular Weight	Molar Extraction
B1	360	312	22000
B2	362	314	23400
G1	360	328	18700

III. RESULT AND DISCUSSION

In the present investigation, we extract aflatoxin from collected seed samples and also from different strains of *Aspergillas species* cultured by plating methods from all varieties of collected seeds and pulses (Maire Wheat, Moong). In the case of maize, we found the maximum aflatoxin B1, concentration is 1.64 µg/ml and the minimum in the case of wheat B1. In the case of moong, It was found 0.912µg/ml. In the case of moong, we found the maximum aflatoxinB2 concentration (0.456µg/ml) and the minimum concentration in the case of maize aflatoxin B2 was 0.82µg/ml (Table: 6) The intermediate concentration of toxin B2 in the case of wheat was found 0.163µg/ml. We collected approximately 20 types of A. strain from all seed samples. Among all, few strains were capable of producing toxins most were non-toxigenic strains. In the case of non-treated maize samples, we found *Aspergillus flavus*. It produced aflatoxin B2 (0.201µg/ml) and B1 (0.402 µg/ml) (Table: 2). The strain 2 produce aflatoxin B is 0.141µg/ml. In the case of the non-treated wheat sample, we found *Aspergillus parasiticus* strain because it produced only aflatoxin G1 and aflatoxin G2. The strain 1 produced a maximum, G1 concentration which is 4.61µg/ml and strain 2 produced G1 concentration is 0.114µg/ml (Table 2). In the case of G2 strain 1 produced a maximum of 2.30µg/ml and the minimum produced by strain 2 is 0.228µg/ml. In the case of the non-treated moong sample, we found *Aspergillus flavus* because it produced aflatoxin B1 and B2. The strain 1 produced aflatoxin B1 is maximum, which was 3.72 µg/ml and strain 2 produced B1 concentration is 0.170µg/ml (Table: 2)

Table 2: Concentration of aflatoxin from fungal Sample (Starin-1) in collected grain and pulses (non-treated,).

Fungal Strain of *A. flavus from* nontreated	B1 (µg/ml)	B2 (µg/ml)	G1 (µg/ml)	G2 (µg/ml)
Maize Nontreated	0.402	0.201	-	-
Moong Nontreated	3.72	-	-	-
Wheat Nontreated	-	-	4.61	2.30

We did not find any concentration of aflatoxin B1 and B2 in the case of non-treated wheat. In the case of the treated maize sample, we found *Aspergillus flavus* because of strain B1 and B2. in which strain 2 produced aflatoxin B1, which was 0.283µg/ml and strain 1 produced minimum aflatoxin B1 which is 0.0141µg/ml. The Strain 1 produced a maximum B2 was 0.141 µg/ml and strain 2 produced aflatoxin B2 is 0.070 µg/ml.

Table 3: Concentration of aflatoxin from fungal Sample (Starin-2) in collected grain and pulses (non-treated).

Fungal Strain *A. flavus* from Nontreated 2	**B1(µg/ml)**	**B2(µg/ml)**	**G1(µg/ml)**	**G2(µg/ml)**
Maize Nontreated 2	0.283	-	-	-
Maize Nontreated 2	0.170	-	-	-
Wheat Nontreated 2	-	-	0.114	0.228

In the case of the treated moong sample, we found *Aspergillus flavus* strain it produced only alfatoxin B1 and B2. Strain1 produced a maximum aflatoxin B1 concentration is 0.553 µg/ml and the minimum produced by strain 2 is 0.17 µg/ml and in strain 1 B2 concentration was 0.276 µg/ml and in strain 2 B2 concentration became 0.85 µg/ml. In the case of the treated wheat sample, we found *Aspergillus paracities* strain because it produced only G1 and G2 the strain I produce maximum G1 concentration which was 2.94 µg/ml and the minimum G2 concentration was 1.47 µg/ml.

Table 4: Concentration of aflatoxin from fungal Sample (Starin-1) in collected grain and pulses (treated).

Fungal Strain of *A. flavus* from treated sample1	**B1(µg/ml)**	**B2(µg/ml)**	**G1(µg/ml)**	**G2(µg/ml)**
Maize 1	0.0141	0.141	-	-
Moong1	0.553	0.276	-	-
Wheat1	-	-	2.94	1.47

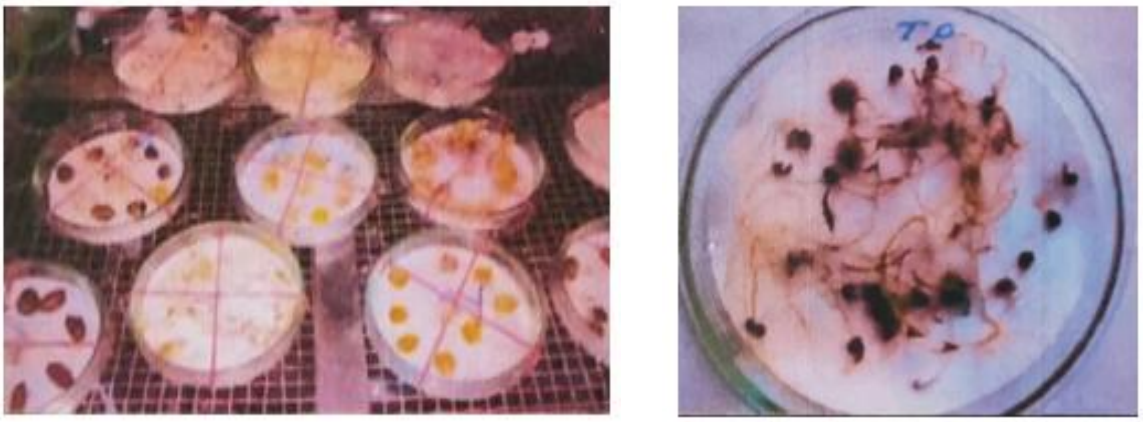
Table 5: Concentration of aflatoxin from fungal Sample (Starin-2) in collected grain and pulses (treated).

Fungal Strain of *A. flavus* from treated sample2	**B1(µg/ml)**	**B2(µg/ml)**	**G1(µg/ml)**	**G2(µg/ml)**
Maize 2	0.283	0.070	-	-
Moong 2	0.170	0.85	-	-

Table 6: Concentration of aflatoxin from fungal Sample in the collected seeds of Maize and pulses

Seed	B1	B2
Maize	0.326 µg/ml	0.82 µg/ml
Pulse	0.912 µg/ml	0.456 µg/ml

From the results, it was very clear that the gains and pulses of study area was greatly infected from aflatoxins of *Aspergillus flavus.* Aflatoxin production is strongly correlated with the growth of aflatoxigenic fungus, therefore conditions favourable for their growth are advantageous for aflatoxin formation. The moisture content (more specifically, the water activity) and temperature of the commodity are the main variables affecting fungal growth in stored food products. Food grains are typically harvested when they have a higher moisture content, which is then dried to reduce it to a safe level before storage. Delaying drying to safe moisture levels thereby increases the likelihood of mould growth and mycotoxin formation. (Chulze, 2010). Crops become infected with aflatoxin both before and after harvest and when the grain is stored. It can be created when developing maize is exposed to drought, pest stress, and extended hot weather conditions. If crop drying is postponed, contamination after harvest could happen. If crucial moisture levels are permitted to be exceeded when the crop is being stored, it may also happen (Herrman, 2006). According to Payne (1992), aflatoxin contamination of maize prior to harvest has been linked to insect damage, drought, high temperatures, and high humidity. Schmale (1998) asserts that because aflatoxins are produced by moulds under a variety of circumstances, challenges should constantly be taken into account while dealing with plant stress, harvest stress, storage stress, and feed out issues.



A **B**

Figure 1: A: Incubation of collected Seed sample for testing of Fungal species (*Aspergillus species*), **B:** Aspergillus strain developed in collected pulse (moong) from study site after platting method.

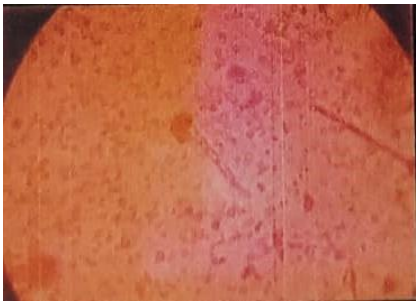


Figure 2: Image of developed Aspergillus species under compound microscope at 40X

The soil type, the genotype of the crop planted, the lowest and highest daily temperatures and the daily net evaporation are some important variables that determine aflatoxin contamination (Strosnider *et al*., 2006). In addition, crop stress or damage from insect activity, poor harvest timing, strong rains during and after harvest, and insufficient drying of the crop before storage all contribute to aflatoxin contamination (Lizarraga-Paulin *et al*., 2011). According to Carvajal and Castillo (2007), the development of aflatoxin in grain can occur in the field under storage settings between 20 and 40^0C with 10-20% humidity and 70–90% relative humidity in the air. Compared to other storage fungi, *A. flavus* has comparatively high moisture requirements (Amare *et al*., 2006). So, excessive seed moisture makes aflatoxin infection of grains worse. Between 40°N and 40°S of the equator, aflatoxin contamination is a recurring threat (PACA, 2013).

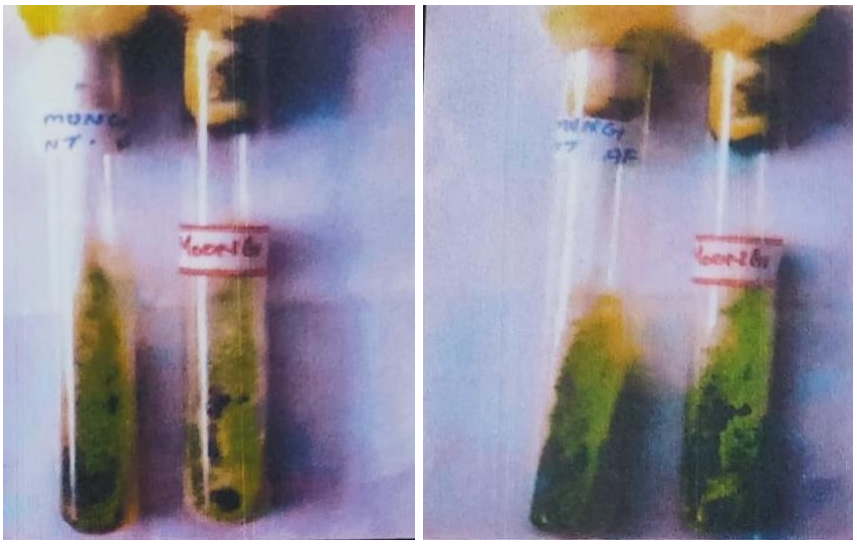


Figure 3: Culture of Aspergillus strain from treated and non-treated Samples in PDA

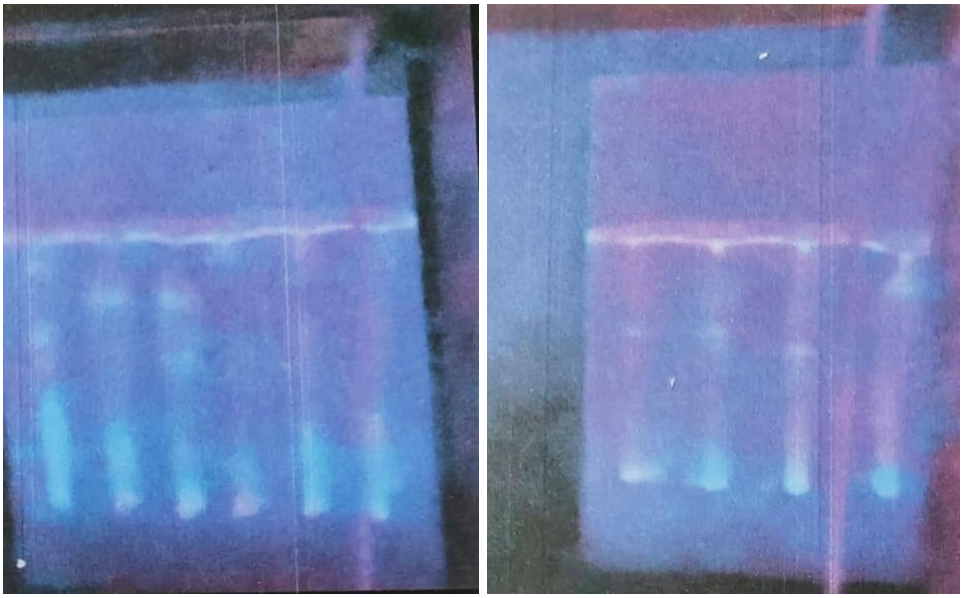
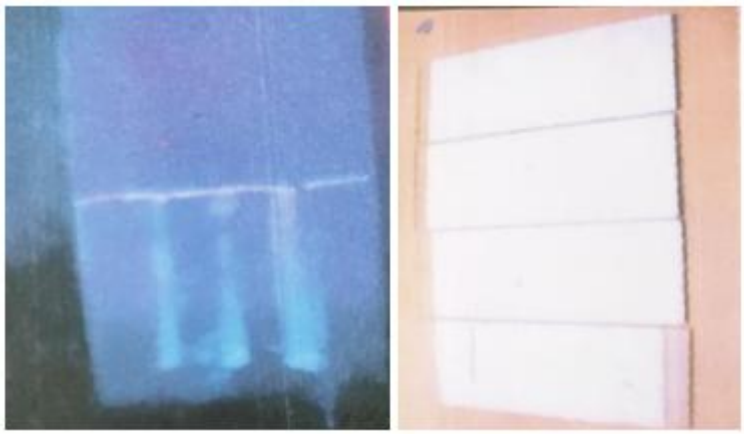


Figure 4: Identification of toxigenic strain of Aspergillus species on TLC



A **B**

Figure 5 A: Aflatoxin observation under UV of Seed sample on TLC. **B:** Extraction sample of Seeds and fungal Strain of *A. flavus* on TLC

Similar to this present study, Habtamu *et al.* (2001) have detected aflatoxin from maize, wheat, barley, teff, millet, sorghum, groundnut, faba bean, pea and pepper from Ethiopia, which are the major staple crops for the country. The more powerful toxins, AFB1 and AFG1, were found in this study. 90% of the sample in this study has more than 20 µg /kg of aflatoxins from legume crops.

In a further investigation conducted in Addis Abeba by Fufa and Urga (2007), aflatoxins (AFB1) at concentrations between 100 and 525 ppb were detected in 13.33% of samples of crushed red pepper and 8.33% of samples of shiro. According to an investigation of aflatoxin from southwest Ethiopia, 3.33% of the samples tested were positive at quantities of 92.59 µg/kg (Chemed *et al*., 2016). AFB1, AFG1, AFB2, and AFG2 were each found in this

experiment at concentrations of 41.08, 38.79, 7.56, and 5.16 µ/kg, respectively. In Ethiopia, groundnuts have been linked to 36–100% of aflatoxigenic fungus incidence, according to Abdi and Alemayehu (2014). Similar to this, south Ethiopian maize has a 22.72 µg/kg AFB1 contamination rate (Alemu *et al*., 2008).

According to Lewis et al. (2005), the greatest incident of aflatoxin poisoning occurred in Kenya in 2004, where 125 individuals died and over 200 others required medical attention after consuming tainted corn. The deaths were mainly associated with homegrown maize that had not been treated with fungicides or properly dried before storage. Aflatoxicosis is a general term used to describe illnesses brought on by aflatoxin exposure. Chronic aflatoxicosis causes cancer, immunological suppression, and other "slow" pathological problems in addition to acute aflatoxicosis' fatal outcome (Herrman, 2006). As we know in this study, we found people suffered from liver cancer. It may be due to the effect of aflatoxicosis. According to estimates from the World Health Organisation (WHO), aflatoxins may be the direct cause of up to 30% of liver cancer cases worldwide each year. As mutagens, hepatocarcinogens, and teratogens, aflatoxin links to DNA, RNA, and proteins can damage every living thing, from viruses to people, causing acute or chronic symptoms. Aflatoxin also forms adducts with DNA that serve as biomarkers of illness risk (Castillo and Carvajal 2007). According to Lizárraga-Pauln et al. (2011), AFB1 is the most common aflatoxin that is typically discovered in cases of aflatoxicosis and is responsible for acute toxicity, chronic toxicity, carcinogenicity, teratogenicity, genotoxicity, and immunotoxicity.

IV.CONCLUSION

Aflatoxins are poisonous byproducts of the Aspergillus fungus that can contaminate different foods and animal feeds. The most prevalent and most dangerous mycotoxins are aflatoxins. Aflatoxins are frequently linked to foods grown in the tropics and subtropics, including maize, rice, sorghum, barley, rye, wheat, groundnuts, soybeans, and cottonseed. Study say that aflatoxin B1 and B2 is very harmful for health, especially for liver (Liver cirrhosis, Liver carcinoma, etc.). In this investigation, we tested the grains (Maize and wheat) and pulse (Moong) and found the maximum concentration of aflatoxin B1 and B2 in the case of moong. However, aflatoxin has not been previously reported in moong. It has been also found that the villagers consumed moong very frequently in various forms of food throughout the year. So, this indication shows that there may be moong is responsible for liver carcinoma in the village Sirsiakala of district Araria, Bihar, India.

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