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EFFECT OF NEEM (*Melia azadirachta* L.) LEAF AND BISHKATALI (*Polygonum hydropiper* L.) ROOT POWDER FOR DECONTAMINATION AND CALCIUM HYDROXIDE FOR DETOXIFICATION OF AFLATOXIN IN RICE, MAIZE AND WHEAT

M. N. H. BHUIYAN[1]*, M. T. HASSAN[1], M. BEGUM[1], M. AHSAN[1] and M. RAHIM[1]

ABSTRACT

The study was conducted at the Laboratory of Food Toxicology Research Section, Institute of Food Science and Technology, Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh. Samples (Rice, maize and wheat) were collected from six Divisions namely Dhaka, Rajshahi, Chittagong, Sylhet, Khulna and Barisal of Bangladesh in July 2012. To elucidate the effect of Neem (*Melia azadirachta* L.) leaf and Bishkatali (*Polygonum hydropiper* L.) root powder for decontamination and Calcium Hydroxide for detoxification of aflatoxin in rice, maize and wheat was studied which can be useful for farmers and stockholders. Neem leaf and Bishkatali root powder treatment was found to be good decontamination method for Bangladeshi people because both of this plant are easily available in the country. After treatment with Neem leaf powder, no incidence of aflatoxin occurred after three and six months except in the maize. A 40 % incidence was found after six months but the level of contamination was very low, 0.8-3.2 ug/kg. Another treatment with Bishkatali root powder, after three months with a very low level (2.4 ug/kg) 10 % incidence was found only in maize. A 40 % incidence was also observed after six months in this commodity. Rice and wheat was found contamination free after three months but in the wheat after six months 20% incidence was observed. A cost effective method for chemical detoxification of aflatoxin in maize with 1% and 5% Calcium Hydroxide an average detoxification rate was found 58.3% and 68% respectively.

Keywords: Aflatoxin, Occurrence, Decontamination, Detoxification and *Azadirachta.*

INTRODUCTION

Aflatoxins are produced by many species of *Aspergillus*, a fungus, the most notable ones are *Aspergillus flavus* and *Aspergillus parasiticus*. Occupational exposures to aflatoxins in agricultural workers, people working in oil mills and granaries have been reported (Sorenson *et al.,* 1984) and European Union set action levels for food grains and feed stuffs (Commission regulation 466/2001). Aflatoxins are the most potent hepatocarcinogen and mutagen among mycotoxins (Hudler, 1998). After wide experimentation on many animal species like rats, rainbow trout's, aflatoxin especially aflatoxin B_1 is confirmed as a potential carcinogen (IARC, 1993). The main target organ in mammals is the liver (Machida and Gomi, 2010). Chronic exposure also leads to a high risk of developing liver cancer (Aguilar *et al.,* 1993). Chronic, subclinical exposures do not lead to symptoms as dramatic as acute aflatoxicosis. Children are particularly affected by aflatoxin exposure which leads to stunted growth and delayed development (Abbas, 2005). Low levels of aflatoxin exposure require continuous consumption for several weeks to months for signs of liver dysfunction to appear (Bingham *et al.,* 2003). The expression of aflatoxin-related diseases is influenced by factors such as species, age, nutrition, sex and the possibility of concurrent exposure to other toxins. Aflatoxins have been isolated from all major cereal crops. Due to the capacity of aflatoxins to cross the placental barrier, can cause genetic defects at foetal stages itself (Maxwell *et al.,* 1998). The staple commodities regularly contaminated with aflatoxins include maize, rice, wheat and a variety of spices intended for human or animal consumption (Rambo *et al.,* 1975; Stoloff, 1976; Qutel *et al.,* 1983; Pozzi *et al.,* 1995; Ewaduh, 1992; Adebajo *et al.,* 1994). When aflatoxin B1 is ingested some transformations occur and secondary new aflatoxin M_1 and M_2 having same acute toxicity as B_1 is produced which are generally found in cow's milk (Coker, 1979). When processed, aflatoxins get into the general food supply where they have been found in both pet and human foods, as well as in feed stocks for agricultural animals. International sources of commercial peanut butter, cooking oils (i.e. olive oil) and cosmetics have been identified as contaminated with aflatoxin. In many of these contaminated food products, the aflatoxin exceeded

[1]Food Toxicology Research Section, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research, Dhaka-1205, Bangladesh. *Corresponding author's Email: nhbmb@yahoo.com

FDA, or other regulatory agency, safe limits (McDaniel, 2012; Leong *et al.,* 2012; Mahoney *et al.,* 2010; Li *et al.,* 2009). Metabolism plays a major role in deciding the degree of toxicity (Eaton and Gallhager., 1994). Species susceptibility to aflatoxin mainly depends on its liver detoxification systems, genetic makeup, age and other nutritional factors (Howard and David, 1990). A study in West Africa showed a significant correlation among the aflatoxin exposure and stunted growth in children who are exposed to aflatoxin right for neonatal stages (Gong *et al.,* 2002). In Bangladesh, rice, maize and wheat are reported of aflatoxin contaminated but the present status is not known (Mustafa *et al.,*2000; Huq *et al.,* 1999; Dawlatana *et al.,* 2002). Moreover, any decontamination and detoxification procedure is not studied for farmers and businessmen to store crops as per Bangladesh aspect. Aflatoxin may be eliminated or reduced by applying physical, chemical and biological methods (Jalili *et al.,* 2010). Some of the reduction methods showed satisfactory results (Srivastava *et al.,* 2009; Kumar *et al.,* 2010); however, many of them are not suitable to be used as foods because the resultant products cannot be consumed by humen (Piva *et al.,* 1995; Rustom, 1997; Proctor *et al.,* 2004; Akbas and Ozdemir, 2006). Consequently, safer, more economical and more practical methods need to be found out. Neem, (*Azadirachta indica* A.) Juss (syn. *Melia azadirachta* L.) is a subtropical tree native to the drier areas of Asian and African countries. Neem components have reputed value for their medicinal, spermicidal, antiviral, antibacterial, antiprotozoal, insecticidal, insect repellent, antifungal and antinematode properties (Khan *et al.,* 1988 and SaiRam *et al.,* 2000). It is also reported that another plant Bishkatali (*Polygonum hydropiper* L.) root extract has antifungal activity (Hasan *et al.,* 2009). On the other hand, alkalis cause the hydrolysis of the lactone ring in Aflatoxin B_1 (Camou-Arriola and Price, 1989). Study shows that 0.5% calcium hydroxide decreased aflatoxin levels by 43% (Price and Jorgensen, 1985). The objective of this study was to elucidate the effect of Neem (*Melia azadirachta* L.) leaf and Bishkatali (*Polygonum hydropiper* L.) root powder for decontamination and Calcium Hydroxide for detoxification of aflatoxin in rice, maize and wheat which can be useful for farmers and stockholders.

MATERIALS AND METHODS

The study was conducted at the Laboratory of Food Toxicology Research Section, Institute of Food Science and Technology, Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh. Samples (Rice, maize and wheat) were collected from six Division namely Dhaka, Rajshahi, Chittagong, Sylhet, Khulna and Barisal of Bangladesh in July 2012.

Sampling: Rice, maize and wheat were collected and analyzed to carry out this work. Samples were collected from six divisions namely Dhaka, Rajshahi, Chittagong, Sylhet, Khulna and Barisal of Bangladesh. Numbers and weights of samples taken are outlined in table 1.

Table 1. Sample collected for rice, maize and wheat from six divisions (Dhaka, Rajshahi, Chittagong, Sylhet, Khulna and Barisal) in July 2012.

Sample collection area	Dhaka (No. and weight)	Rajshahi (No. and weight)	Chittagong (No. and weight)	Sylhet (No. and weight)	Khulna (No. and weight)	Barisal (No. and weight)	Total no. of samples
CSDs (Central storage depots) / mill sample	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	90
Markets	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	90
Farmer's stores	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	15x 5 kg	90

A total of 270 samples of each commodity were analyzed for total Aflatoxin B_1, B_2, G_1 and G_2. The moisture content was also measured and a total of 30 contamination free samples of each commodity were separated among the samples for decontamination which had less than 15% moisture. Ten maize samples were also separated for detoxification among the naturally contaminated samples found by analyzing all maize samples which were contaminated with aflatoxin above 40.0 ug/kg.

For bag storages (like Central Storage Depots, CSDs) each 5 kg sample was composed of fifty 100 grams increments. For markets ten stalls were randomly selected and 500 grams were purchased to

make one composite sample of 5 kg. From randomly selected ten villagers one composite 5 kg sample was purchased from village open market which was representative sample of farmer's store. The 5 kg was sub-divided in to 1 kg sub sample in a rotary Cascade sample divider (Pascall Engineering Co. Ltd., England) and powdered in a sub-sampling mill by Simplabelt Variable speed drive (Simplatroll Ltd., Bedford) to collect 200 gram representative sample. The plants (Neem leaf and Bishkatali root) for treatment were collected from the field of medicinal plants of BCSIR laboratories, Chittagong. The plant's leaves and roots were sun dried (moisture ~ 10.0) and made powder for this study.

Apparatus*:* Blender (Warring commercial, England), Mechanical shaker-flatbed, Denley Ins., SPE vacuum manifold, (Supelco Visiprep 5-7030). SPE reservoirs -70 ml and 30 mL. (Varian), SPE adaptors (Varian), SPE Cartridge -3mL, 500 mg PH packing, Bond-Elut (Varian), Sample concentrator, Techne, DB-3, Syringe filter cartridge -13 mm diameter x 0.45 micron, Disposable Syringes-3 mL, Volumetric flask - 250 mL and 10 mL. Vortex mixer, Remi Equip., CM101, Micro syringe capable of injection volumes up to 50 microliters and HPLC system (Agilent).

Reagents: Acetone, Methanol and Acetonitrile (HPLC grade), sodium sulfate anhydrous (heated for at least 2 hour at 550°C), Lead acetate, chloroform. All the solvents used for the analysis purchased from Merck, Germany. Aflatoxins standards were obtained from Sigma Chemicals, USA.

Sample preparation

Extraction: Extraction was carried out from 200 gram sub-sample by making slurry with water at 1:2 ratio of sample: water. An appropriate volume of acetone was added to 100 gram of slurry to produce acetone to water ratio 1:4 and shaked in a mechanical shaker for 30 minutes and then collected the filtrate through a Whatman no. 1 filter paper in a conical flask. 10 ml methanol and 1 ml lead acetate was added to 10 ml of the filtrate in a 250 ml measuring cylinder and was made up to 150 ml with distilled water (Huq *et al.,*1999).

Clean up: Clean up was done using SPE Cartridge -3 mL, 500 mg PH packing was attached to 75 ml reservoir and a vacuum manifold. The cartridge was conditioned by passing of 15 mL methanol followed by 15 ml water under vacuum after adding 1 gram methanol washed celite. Then 150 ml prepared sample solution was passed through cartridge under vacuum at the rate of 10 ml/ min. The cartridge was then washed with 10 ml of water. Any remaining water from the cartridge was removed by the passage of air for about 5 minutes. The 75 ml reservoir was replaced with a 25 ml reservoir and successively another reservoir (4 ml) containing anhydrous sodium sulphate (500 mg) and inserted between the cartridge and vacuum manifold. The aflatoxins were eluted using 4 ml of chloroform at the rate of 0.5 ml/min in a 7 ml vial (Huq *et al.,* 1999).

The vial was dried under the stream of nitrogen at 45°C in a sample concentrator and reconstituted with 1 ml methanol and water (1:1) for HPLC analysis.

Sample analysis

Samples were analyzed using HPLC system-Agilent: Liquid chromatography consist of Agilent: Solvent delivery system (pumps) Series 1100, Agilent series 1100 Column oven Agilent 1200 series Flourosence detector, Manual injector and Cobra cell for post column derivatization. Software: Agilent ChemStation. HPLC Column was C18, 250mm (L) X 4.6mm (ID) 10µ (Grace). Mobile phase was 630 ml water, 220 ml methanol, 150 ml acetonitrile, 120 uL of concentrated Nitric acid and 100 mg potassium bromide in isocratic mode with 1 ml flow rate. Total run time was 15 Min and Injection volume was 20 uL. Colum oven temperature was 30°C and excitation wavelength 365 nm, emission wavelength 464 nm. Recovery was calculated for aflatoxins (B_1, B_2, G_1 and G_2) fortified at 2 ug/kg, 10 ug/kg, 20 ug/kg, 100 ug/kg and 200 ug/kg levels using peak area of chromatograms at concentrations ratio 5:1 (B_1, G_1 : B_2, G_2) of standards and was found 87-92 %. Suitable seven point calibration curve was done, preferably on matrix at 0.5, 2, 10, 25, 50, 100 and 250 ng/ml (ug/kg) level. Linear regression was 0.99. The calibration batch was prepared from the mixed working standard of 1000 ug/kg in methanol and water (1:1) which was prepared from stock standard of 20 ppm in acetonitrile. A control spiked samples of 2 ug/kg, 50 ug/kg and 200 ug/kg was run after every 10 samples followed by a

solvent as blank. The method was validated as per European commission decision (Commission decision, 2002). The limit of detection was 0.5 ug/kg, Decision Limit (CCα) was 4.34ug/kg and Detection Capability (CCβ) was 4.64 ug/kg.

Decontamination and Detoxification study

For decontamination, 1 kg of 30 samples of each commodity which were separated as contamination free and moisture level less than 15% were taken. Ten samples were used as common control sample (not treated), 10 samples for Neem leaf powder treatment and 10 samples for Bishkatali root powder treatment among 30 samples. Twenty (20) gram of Neem leaf powder was individually mixed with 1 kg of 10 samples of each commodity and 20 gram of Bishkatali root powder was individually mixed with 1 kg of another 10 samples of each commodity. The rest of the ten common control samples were not treated. All control and treated samples were stored in a traditional tin container for six months and were analyzed after three and six months. Results were shown in table 2a and 2b. For detoxification, each 2 kg of 10 naturally contaminated known samples of maize were taken and 1 kg of each samples were individually soaked in two litre of 1% of Calcium Hydroxide and boiled for 30 minutes. Another 1 kg of each samples were individually soaked in two litre of 5% of Calcium Hydroxide and boiled for 30 minutes. All the Calcium Hydroxide treated samples were analyzed for aflatoxins. Results were shown in table 3.

RESULTS AND DISCUSSION

Results

A total of about 270 samples of each commodity of Rice, Maize and Wheat were collected and all were analyzed for total aflatoxin (B_1, B_2, G_1 and G_2). Treatment with Neem leaf and Bishkatali root powder (Table 2a and 2b) was noted that after three and six months there was afltoxin contamination in the control samples of each commodity which was used as a common control for both treatments and the incidence rate was increased after six months, up to 80 % in maize. After treatment with Neem leaf powder (Table 2a), no incidence of aflatoxin occurred after three and six months but the maize. In the maize 40 % incidence was found after six months but the level of contamination was very low, 0.8-3.2 ug/kg. Another treatment with Bishkatali root powder (Table 2b), after three months with a very low level (2.4 ug/kg) of 10 % incidence was found only in maize. A 40% percent incidence was also observed after six months in this commodity and the level of contamination was fairly high (2.6-19.8 ug/kg).

Table 2a. Incidence rate of aflatoxin contamination in rice, maize and wheat treated with Neem powder. (Common ten samples of each commodity were taken as control (not treated) and ten as treated).

Commodity (10 as control and 10 as treated of each commodity)	Incidence rate (%) after three months			Incidence rate (%) after six months		
	Control sample	Treated sample	Level of contamination in treated sample (ug/kg)	Control sample	Treated sample	Level of contamination in treated sample (ug/kg)
Rice	20	-	-	40	-	-
Maize	40	-	-	80	40	0.8- 3.2
Wheat	20	-	-	40	-	-

Table 2b. Incidence rate of aflatoxin contamination in rice, maize and wheat treated with Bishkatali roots powder. (Common ten samples of each commodity were taken as control and ten as treated).

Commodity (10 as control and 10 as treated of each commodity)	Incidence rate (%) after three months			Incidence rate (%) after six months		
	Control sample	Treated sample	Level of contamination in treated sample (ug/kg)	Control sample	Treated sample	Level of contamination in treated sample (ug/kg)
Rice	20	-	-	40	-	-
Maize	40	10	2.4	80	40	2.6 - 19.8
Wheat	20	-	-	60	20	1.2 - 16.7

Table 3. Detoxification rate of aflatoxin in contaminated maize treated with 1% and 5 % of Calcium Hydroxide with 30 min boiling.

Sample code	Amount of aflatoxin naturally contaminated sample (ug/kg)	1% of Calcium hydroxide treated maize		5 % of Calcium hydroxide treated maize	
		Amount of aflatoxin after treatment (ug/kg)	Rate (%) of detoxification (%) (Average = 58.3)	Amount of aflatoxin after treatment (ug/kg)	Rate (%) of detoxification (Average = 68)
1	43.6	19.2	56	13.6	69
2	87.5	42.0	52	36.8	58
3	123.2	53.0	57	44.4	64
4	98.6	40.5	59	30.6	69
5	67.4	25.0	63	21.6	68
6	57.3	24.1	58	16.7	71
7	82.4	37.9	54	30.5	63
8	89.9	36.9	59	28.8	68
9	94.7	34.1	64	20.9	78
10	92.8	36.2	61	25.99	72

Rice and wheat was found contamination free after three months but 20% incidence was observed in the wheat with a fairly high level of 1.2-16.7 ug/kg after six months. Results of chemical detoxification of aflatoxin in maize with 1% and 5% Calcium Hydroxide in table 6, was found average 58.3% and 68% was achieved by using 1% and 5% Calcium Hydroxide respectively with 30 minutes boiling.

Discussion

Emphasize on the simplest and most practical way to prevent mycotoxin contamination in Bangladesh is necessary. From the results depicted in table 2a, table 2b and table 3, Neem leaf and Bishkatali root powder treatment is a good decontamination method for Bangladeshi people because both of this plant are easily available in the country and detoxification of the highly contaminated maize can be achieved by 1- 5% Calcium Hydroxide with 30 minutes boiling as maize is used in the cattle and poultry feed. Further study is needed to detoxify human consumable food but Neem and Bishkatali can be used easily in human consumable rice wheat and maize because it can be easily separated by a simple sieve just before it is ready to be consumed. It has been reported that decreased amount of aflatoxin synthesis by the aflatoxin producing fungi is accompanied by morphological changes (Torres *et al.,* 1980). Nonvolatile neem leaf constituents are known to potentially inhibit aflatoxin biosynthesis in *Aspergillus parasiticus* without affecting fungal growth (Bhatnagar and McCormic, 1988) whereas extracts obtained from leaf and seed extract inhibit both aflatoxin biosynthesis and retard fungal growth (Abyaneh *et al.,* 2005; Allameh *et al.,* 2002).

This study was carried out under the research and development programme of Bangladesh Council of Scientific and Industrial Research (BCSIR) to find out the effect of Neem (*Melia azadirachta* L.) leaf and Bishkatali (*Polygonum hydropiper* L.) root powder for decontamination and Calcium Hydroxide for detoxification of aflatoxin in rice, maize and wheat which can be useful for farmers and stockholders.

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