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Occurrence of aflatoxins in mahua (*Madhuca indica* Gmel.) seeds: Synergistic effect of plant extracts on inhibition of *Aspergillus flavus* growth and aflatoxin production

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ABSTRACT

Occurrence of aflatoxin in *Madhuca indica* Gmel. seeds was determined by competitive ELISA. Eighty percent of mahua seed samples were found to be contaminated with aflatoxin. Total aflatoxin content ranged from 115.35 to 400.54 ppb whereas the concentration of AFB_1 was in the range of 86.43 to 382.45 ppb. Mahua oil was extracted by cold press expeller and analysed for contamination of aflatoxin in both the oil and cake samples. Total aflatoxin and aflatoxin B_1 were 220.66 and 201.57 ppb in oil as compared to that in cake samples where it was 87.55 and 74.35 ppb, respectively. Various individual and combined plant extracts were evaluated for their efficacy against growth of *Aspergillus flavus* and aflatoxin production *in vitro*. Combination of botanicals were found to be more effective in controlling fungal growth and aflatoxin production than individual extracts. Results of the present study suggests that synergistic effect of plant extracts can be used for control of fungal growth and aflatoxin production. These natural plant products may successfully replace synthetic chemicals and provide an alternative method to protect mahua as well as other agricultural commodities of nutritional significance from toxigenic fungi such as *A. flavus* and aflatoxin production.

1. Introduction

Aflatoxins are secondary metabolites produced by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* are known to be potent carcinogens and hepatotoxic agents and pose serious hazards to human and animal health (Stoloff, 1977). Aflatoxin B_1 (AFB_1), the most potent of the four naturally occurring aflatoxins namely aflatoxin B_1, B_2, G_1 and G_2 is highly toxic and carcinogenic metabolite produced by *Aspergillus* species on agricultural commodities (Leontopoulos et al., 2003). Aflatoxins have been detected in various food commodities from many parts of the world (Smith and Moss, 1985) and are presently considered as one of the most dangerous contaminants of food and feed. Besides their effect on health of human and animals, aflatoxin also has an impact on the agricultural economy through the loss of crop production and the time and costs involved in monitoring and decontaminating efforts as FAO and WHO have imposed regulatory guidelines of 20 ppb of total aflatoxins as the maximum allowable limit in food or feed substrate. In some European countries aflatoxin levels are regulated below 5 ppb (Jiujiang et al., 2002). However, the potential for such toxicity is highest in countries like Kenya where consumption of contaminated maize, a staple food led to 317 cases of poisoning and 125 deaths in rural area (Lewis et al., 2005; Molyneux et al., 2007).

Kernels of green-colour egg size fruits of mahua (*Madhuca indica* Gmel.) contained 55–65% of soft yellow oil that is widely used locally for cooking and tallow (The Wealth of India, 1962). Refined mahua oil is utilized for cooking, confectionery and in chocolate making. The plant is economically important because of the role it plays in yielding country liquor, edible succulent corollas and oil from seeds (Roy and Bhattacharya, 1999). Cocoa butter extenders with temperature-resistant properties have been prepared by fractionation and blending of *M. indica* (mahua) and *Garcinia indica* (kokum) fats (Jeyarani and Reddy, 1999). Herbal nutritious chocolate composition has been formulated using extract of *M. indica* (mahua) in combination with other plant extracts claiming several nutritional properties (Pushpangadan et al., 2007). Mahua oil is used as edible oil by tribals and poor farmers in mahua growing rural areas of India. Oil is used for edible purposes by blending 5% of it in other edible oils as reported by the National Oilseed and Vegetable Oil Development Board of the Ministry of Agriculture, Govt. of India.

Harvesting period of mahua seeds is at the time of peak rainfall (July–August) when farmers store seeds with high moisture content that favour fungal infection and aflatoxin contamination.

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Literature survey revealed that no systematic work has been done on occurrence of aflatoxin in *M. indica* (mahua) seeds and inhibition of fungal growth and aflatoxin production using botanicals. The objective of the present study was to investigate the occurrence of aflatoxins in mahua seeds and evaluate efficacy of selected individual plant extracts or their combinations for inhibition of *A. flavus* growth and aflatoxin production.

2. Materials and methods

2.1. Collection of samples and fungal counts

Mahua seeds (10 kg each lot) were purchased from towns and villages of Lucknow during July–August, 2005. Fifty grams seeds from each lot were drawn in triplicate and dried in an oven at 35 °C till complete removal of moisture content. Dried seeds were ground to fine powder using laboratory grinder and analysed for their aflatoxin content. Fungal count (CFU g^{-1}) from seed samples were made by dilution method on potato dextrose agar using serial dilution. Ten grams of sample was finely ground in sterilized mortar pestle and aseptically transferred to 99 mL of pre sterilized of normal saline (0.85%). It was further diluted by transferring one mL of it to a tube containing 9 mL of 0.85% normal saline for making up to 10^{-6} dilution. One milliliter of diluted sample was poured aseptically along with 25 mL of sterilized media (PDA with Chloramphenicol supplement FD033) to each plate. After the media got solidified, the plates were incubated at 28 ± 1 °C for 5 days in inverted position.

2.2. Antifungal assay

Antifungal activity of various plant extracts was tested against aflatoxin producing strain (2799) of *A. flavus* obtained from IMTECH, Chandigarh using agar well diffusion method**.** The autoclaved PDA (potato dextrose agar) media was cooled to 40 °C. One thousand parts per million of each plant extract individually and in various combinations was mixed thoroughly and the media was poured in petri plates. After solidifying the media, three wells of 8 mm diameter were made in each petri plates and 40 μL of spore suspension contained 18×10^4 spores mL^{-1} of *A. flavus* were added to each well. The Petri plates were then incubated at 28 °C for 5 days. Control did not have any plant extract. Fungal growth of both the treated and untreated control plates was measured at every 24 h for 5 days following the method of Perea et al. (1990).

2.3. Estimation of aflatoxin

Five grams ground powder of mahua seeds was extracted with 25 mL of 70% aqueous methanol using a laboratory homogenizer and filtered through Whatman #1 filter paper. One hundred microliters of each filtrate was diluted with 600 μL of dilution buffer and 50 μL of diluted sample employed to immunoaffinity column for cleaning the samples. Aflatoxin fraction was finally eluted with 0.5 mL of HPLC grade methanol and total aflatoxin content and aflatoxin B_1 were determined using aflatoxin detection kit obtained from R-Biophram AG, Darmstadt, Germany.

Fifty microliters of standard solution of aflatoxin and eluted samples (in duplicate) were added to the wells of microtiter plate. Further, 50 μL of peroxidase enzyme conjugate and 50 μL of mouse monoclonal anti-aflatoxin antibodies were added to each well. The plates were incubated at room temperature in the dark for 30 min. After washing thoroughly with 250 μL distilled water three times, 50 μL of urea peroxidase (substrate) and 50 μL of tetramethylbenzidine (chromogen) were added to each well, mixed thoroughly and incubated for 30 min at room temperature in the dark. Reaction was stopped by adding 100 μL 1 M sulphuric acid (stop reagent) and the absorbance was measured at 450 nm using Bio-Rad ELISA microplate reader Model 680. There were three replicates for each seed lot investigated in the present study.

2.4. Extraction of plant materials

Seeds of *Pongamia pinnata* (karanj), *Azadirachta indica* (neem), plants of *Cymbopogon nardus* (Citronella), and rhizome of *Curcuma longa* (turmeric) were collected from Biomass Research Station, Banthra of National Botanical Research Institute, Lucknow, India. Roots of *Citrullus colocynthis* (Indian wild gourd or bitter cucumber) and gum-resin of *Commiphora wightii* (guggul) were collected from Rajasthan, India. Seeds of *P. pinnata, A. indica* and rhizome of turmeric were dried in an oven at 40 °C for 30 days to remove moisture content and ground to fine powder using laboratory grinder. Ground samples were defatted three times with hexane using Polytron Homogenizer (Kinematica 6100, Switzerland) at 6000 r.p.m. for 5 min. Residue thus obtained was air dried and extracted three times with methanol. The combined methanolic extract of each sample thus obtained was filtered and concentrated under reduced pressure. Guggul gum-resin was extracted 3 times with ethylacetate in a manner similar to that of methanolic extract using Polytron Homogenizer. Essential oil from *C. nardus* (Citronella) was extracted by Clevenger apparatus. One thousand parts per million concentration of each extract was prepared in DMSO and tested for their efficacy for growth inhibition of *A. flavus* and aflatoxin production *in vitro*. Seeds of mahua were extracted by cold press expeller (Komet, IB6 Monforts, Germany) to determine the total aflatoxin and aflatoxin B_1 contamination in mahua seed, cake and oil samples.

2.5. Mahua sample preparation for evaluation of aflatoxin production

Five kilograms of healthy, dried mahua seeds were ground to fine powder and 250 g each was placed in sterilized conical flasks. One milliliter of each plant extract individually and in combimations was added to each conical flask. Control did not have plant extracts. All the flasks were inoculated with 1 mL of conidial suspension (1×10^7 mL^{-1}) of *A. flavus* and mixed thoroughly by vigorous shaking following the method of Oyebangi and Efiuvwevwere (1999). All treated conical flasks were incubated at 28 ± 2 °C for 30 days.

3. Results and discussion

3.1. Quantitation of aflatoxins

Quantitative analysis of total aflatoxin and aflatoxin B_1 (AFB_1) was performed by competitive ELISA Microplate reader using total aflatoxin and aflatoxin B_1 test kit (RIDASCREEN, Dermstadt, Germany). Aflatoxins were separated and purified by immunoaffinity columns and purified fractions were analysed for total aflatoxin content and AFB_1 by antigen-antibody reactions using ELISA. A calibration curve was drawn using a wide range of total aflatoxin standards with concentration of 0–4050 ppt and a calibration curve for aflatoxin B_1 (AFB_1) was drawn using a range of standards with concentration of 250–4000 ppt. A plot in between the percentage absorbance and concentration of both the total aflatoxin and AFB_1 for a set of standard indicated a linear relationship. Several methods have been reported for the determination of aflatoxins in a number of food commodities (Gilbert and Anklam, 2002). Methods based on thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA) are mainly used in routine analysis. Nowadays, these methods involve immunoaffinity column clean-up procedures, which offer the extraction of aflatoxins from most food matrices with simple aqueous solvent mixtures (Stroka et al., 2000).

3.2. Occurrence of aflatoxin in mahua seeds

Results of mahua seed samples analysed for occurrence of total aflatoxin and AFB_1 content are presented in Table 1. Out of fifteen seed lots screened for their aflatoxin content, twelve were found to be contaminated with more than 100 ppb of total aflatoxin content. Total aflatoxin content ranged from 115.35 to 395.54 ppb, whereas AFB_1 ranged from 86.43 to 322.45 ppb. Twelve of the fifteen seed lots examined were found to be associated with fungi. Fungal count ranged from 2×10^2 to 2×10^5 C.F.U. g^{-1}. Cold press expelled oil, cake as well as seed samples were evaluated for total aflatoxin and AFB_1 content and the results are shown in Table 2. The concentration of total aflatoxin content was 315.51 ppb in seed, 87.55 ppb in cake and 220.66 ppb in oil whereas the concentration of AFB_1 was 282.36, 74.35 and 201.57 ppb in seed, cake and oil, respectively. The ratio of aflatoxin content in oil and cake was 7:3. Aflatoxins contamination in peanut products, cotton seed cake and other nuts and oilseeds have been reported by Loosmore et al. (1964) and Wagon (1965). A number of survey and monitoring programs have been carried out in several countries attempting to obtain a general pattern of the extent of food contamination (Yndestad and Underdal, 1975; Girgis et al., 1977; Tabata and Kamimura, 1988).

Tree seeds are subjected to infection by a variety of microorganisms that can induce spoilage or produce metabolites that are toxic to human, animals and birds. Significant correlation between