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DETECTION AND ESTIMATION OF AFLATOXINS USING BOTH CHEMICAL AND BIOLOGICAL TECHNIQUES

BY

M.K. REFAI*, M.E. HATEM*, EVA SHARABY*
AND M.M. SAAD**

Faculty of Veterinary Medicine, Cairo University
and Mycotoxins Lab., National Reasearch Centre,
Dokki, Cairo, Egypt.

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INTRODUCTION

Aflatoxins occupy the most important position among mycotoxins because of their carcinogenic nature for both man and animals. Moreover, aflatoxins occur in high frequency under natural conditions causing more or less tremendous economic losses resulting from contaminated foods and feedstuffs. Losses are difficult to estimate in animals and birds because they come about in many ways, mainly as a result of high mortality rate, low productivity, lower feed conversion rates besides the losses due to costs of control of fungal growth during storage of foods and feeds.

Detection and estimation of aflatoxins were mainly carried out using offical analytical techniques. Such techniques need either thin Layer Chromatograaphy (TLC) or High Performance Liquid Chromatography (HPLC). However, these methods are more or less expensive and sometimes need very expensive equipments as (HPLC). This has stimulated some investigators to try biological methods for detection and determination of aflatoxins in different materials. Generally, the biological methods are much cheaper than chemical ones.

The present work was planned to investigate the antibacterial activity of crude aflatoxins on different standard bacteria as well as on *Candida albicans*, with the aim of adopting a microbial inhibition test for detection and possibly estimation of aflatoxins in foods and feeds.

MATERIAL AND METHODS

Production of crude aflatoxins:

Production of crude aflatoxins was conducted using local isolate of *A. flavus* from the Dept. of Microbiology, Fac. of Vet. Med., Cairo, Univesity. After the identification of the isolate as recommended by Ajello et al. (1963), the prepared spore suspension of the inoculum was added to natural substrates (rice and corn) as described by Shotwell et al. (1966) and Yeast Extract Sucrose (YES) broth as recommended by Davis et al. (1966). Extraction of crude aflatoxins was carried out according to AOAC (1980). The obtained extracts were divided into two unequal parts, the smaller one was examined chemically for qualitation and quantitation of aflatoxins B_1, B_2, G_1 and G_2, while the 2nd part was kept for bioassay.

Chemical analysis:

Standard solutions of aflatoxins B_1, B_2, G_1 and G_2 were prepared by dissolving the crystals of each, separately in benzene-acetonitril mixture (98.2 v/v) then checked according to AOAC (1980). Separation and qualitation had been carried out by TLC. Aflatoxins B_1, B_2, G_1 and G_2 were detected visually using UV lamp with long (366) and short (254) nm as described by Pryzbylski (1975) and/or two dimentional TLC as described by Stubblefield (1983). The quantities of aflatoxins in the extracts were determined

by comparing the densitometer reading of fluorescence of standard spots with the intensity of sample spots in the linear correlation of standard curve.

Biological assay:

Standard strains of *B. megaterium* (a sporogenous strain KM), *B. subtilis* (strain 60015) and *P. denitirificans* (gram negative soil strain with as electron transport chain similar to mitochondria of higher cells) were obtained from Dept. Microbiology and Public Health of Michigan State Univ. Strain (No. 12228) of *S. epidermidis* and (No. 19433) of *S. faecalis* were from the American Type Culture Collection (ATCC) Maryland, USA. All these standard isolates as well as a field strain of *Candida albicans* were used for screening the antibacterial effect of crude aflatoxins. The concentrations of 40, 30, 20, 15, 10 and 5 µg crude aflatoxins per ml of (5% acetone water) were applied using gutter technique as described by Cooper and Woodman (1964).

Field application:

Fifty samples of peanuts, corn, rice, wheat and poultry rations were collected randomly from the markets and examined for aflatoxins biologically and chemically just to testify the validity of such bioassay.

RESULTS AND DISCUSSION

Table (1) shows both quality and quantity of aflatoxins produced by *A. flavus*. Data exhibited that the appropriate strain of *A. flavus* was capable to produce four types of aflatoxins namely, B_1, B_2 G_1 and G_2. The highest concentrations of yielded

aflatoxins were of G_1, while the lowest concentrations obtained were of aflatoxin B_2. Meanwhile, both aflatoxins B_1 and G_2 occupied an intermediate position.

Table (1): Quality and quantity of aflatoxins produced on natural substrates (rice and corn) and yeast extract sucrose (YES) broth.

Substrate/Broth	Concentrations of aflatoxins (ug/50 gm media or 100 ml broth)			
	B_1	B_2	G_1	G_2
Rice medium (50 gm)	52.0	11.5	64.7	28.9
Corn medium (50 gm)	40.3	17.9	45.0	40.3
(YES) broth (100 gm)	39.6	17.5	58.0	39.5

Shotwell et al. (1966) obtained the 4 types of natutally occuring aflatoxins B_1, B_2, G_1 and G_2 using standard strains of *A. flavus* (NRRL, 2999) when grown on rice medium. They could detect amounts of 634, 100, 160 and 20 ug aflatoxins B_1, B_2, G_1 and G_2, respectively from each gram of rice medium. These higher values of aflatoxins which exceed our corresponding figures more than 500 folds might be mainly due to the difference in the strain of *A. flavus* used as well as incubation temperature, time and aeriation (El-Bazza et al., 1982 and Lin et al., 1980).

Regarding aflatoxins quality, data exhibited that the concentration of aflatoxin G_1 was superior to all types of aflatoxins yielded on rice, corn and

Table 2: Mean values of zones of inhibition induced by crude aflatoxins.

Density of bacterial suspension(Count/ml)	Aflatoxin concentrations / ml.				
	5 ug	10 ug	20 ug	30 ug	40 ug
	B. megaterium (Sporogenous strain KM)				
5×10^4	7.0	7.5	11.0	12.0	14.0
5×10^3	10.0	11.0	12.0	14.0	15.5
5×10^2	10.0	11.5	14.5	16.0	17.0
5×10^1	14.0	15.0	16.0	20.0	20.5
	Staphylococcus epidermidis (ATCC, N0.12228)				
4.8×10^4	5.0	6.5	8.0	9.5	12.0
4.8×10^3	5.5	7.0	8.5	9.5	13.0
4.8×10^2	7.0	7.5	8.5	11.0	14.0
4.8×10^1	8.0	10.0	10.5	11.5	14.5
	Streptococcus faecalis (ATCC, N0.19433)				
4.2×10^4	4.5	6.0	7.5	8.5	11.0
4.2×10^3	4.5	6.5	7.5	9.0	12.0
4.2×10^2	6.0	7.0	8.0	9.5	12.5
4.2×10^1	7.0	8.2	9.0	10.5	13.0
	Bacillus subtilis (Strain 60015)				
4.5×10^4	3.0	4.0	5.0	6.0	8.0
4.5×10^3	4.0	5.5	7.0	7.5	10.0
4.5×10^2	5.0	6.0	7.0	8.0	10.5
4.5×10^1	6.5	8.0	8.5	10.5	12.0
	Paracoccus denitrificans				
3.2×10^4	--	1.5	2.0	2.5	4.0
3.2×10^3	--	3.0	4.5	5.5	8.0
3.2×10^2	--	5.0	5.5	7.0	9.0
3.2×10^1	--	6.5	7.5	9.5	11.5

YES media **(Table 1)**. Contrary, Shotwell et al. (1966) recorded that the highest amounts yielded on rice medium were of aflatoxin B_1 "the parent compound". Such difference in aflatoxins quality might by mainly due to incubation temperature besides the fungus strain as mentioned by Rabie and Smalley (1965). They reported that the most important factor controlling the proportion of aflatoxins B_1 to G_1 produced by *A. flavus* was temperature. They added that the optimum temperature for producing aflatoxin B_1 was 24°C, while the optimum for G_1 was 30°C. It could be explained referring that the major toxin B_1 had a molecular weight of 312 and its formula is $C_{17}H_{12}O_6$ while the molecular conposition of aflatoxin G_1 was found to be C_{17} $H_{12}O_7$ (Arae et al., 1967). However, many reports recorded that aflatoxins B_1,B_2, G_1 and G_2 were the naturally occurring forms obtained as a result of *A. flavus* activity (Stollof, 1977 and Wogan, 1977).

Regarding antibacterial activity of the crude aflatoxins, data showed that *B. megaterium, S. epidermidis, S. faecalis* and *B. subtilis* were widely inhibited by aflatoxins concentrations ranging from 5-40 ug/ml. While, *P. denitrificans* was not inhibited by concentration of 5 ug/ml and inhibition occurred only with concentrations of 10 ug/ml crude afaltoxins or more **(Table 2)**. Data also, revealed an approximate linear relationship between concentrations of aflatoxins used and the density of bacterial counts tested against each concentration of crude aflatoxins. On the other hand, *Candida albicans* was not inhibited at any concentration used. Many workers studied the antibacterial activity of aflatoxins on *B. megaterium*(Burmeister & Hesseltine, 1966, Lillehoj & Ciegler, 1968, Buckelew et al., 1972 and Tiwari et al., 1985). They demonstrated that the concentration of 5-30 ug aflatoxins/ml had an inhibition effect on *B. megaterium* . Such

Table 3 : Mean values of inhibition zones (mm) obtained from the 6 (ve^+) samples of contaminated wheat and corn

Standard Bacterial strains	Inhibition zones (mm)					
	Wheat			Corn		
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Bacillus megaterium	12.0	12.0	10.5	11.5	13.0	10.0
Svaphylococcus epidermidis (ATCC, No.12228)	8.0	8.0	7.2	7.5	7.0	6.0
Streptococcus faecalis (ATCC No. 19433)	7.5	7.5	6.5	7.0	6.0	5.5
Bacillus subtilis (60015)	6.5	6.5	5.5	6.0	5.0	4.5

The mean values of inhibition zones obtained from 24 hours broth culture diluted with sterilized normal saline to bacterial number of 5×10^2, 4.8×10^2, 4.2×10^2 and 4.5×10^2, respectively.

wide difference might be due to *B. megaterium* strains as well as the ratios between the four types of aflatoxins as reported by Wogan (1977) who demonstrated that the potency of aflatoxin B_1 is roughly double folds and 4 folds of aflatoxin B_2 and G_2, respectively. Data also, showed that the concentrations of crude aflatoxins are parallel to the density of bacterial suspension. These findings are in accordance with those obtained by Buckelew et al. (1972) and Tiwari et al. (1985) who found that the effect of aflatoxins on cells of *B. megaterium* was concentration dependent with a linear relationship in the death curve. They added that there was a bacteriostatic action of aflatoxin B_1 at lower concentration (8-16 ug/ml), while higher concentration (32 ug/ml) caused an irreversible bactericidal action on *B. megaterium*.

Similarly, *S. epidermidis*, *S. faecalis* and *B. subtilis* showed inhibition zones starting from '3.8 mm) to (8-14.5 mm) corresponding to crude aflatoxins concentrations of 5 and 40 ug/ml, respectively. Such data are in disagreement with the previous findings of Burmeister & Hesseltine (1966) who recorded that members of family Micrococaseae (including Staph.) were not inhibited by as high as 30 ug/ml of crude aflatoxins.

P. denitrificans was not inhibited at a concentration of 5 ug/ml in any of the bacterial dilutions used, but it was inhibited only at 10 ug/ml and higher concentrations up to 40 ug/ml. It was used for assaying the toxicity of aflatoxins as a model organism for the similarity of its electron transport chain to that of the mammalian cell mitochondria (Hatem et al., 1982). However, the inhibition zones are the net work of bacterial species, strain, density of bacterial suspension (count/ml) and both quality and quantity of aflatoxins used. So, this finding indicates the necessity of standardizing

the strain and the density of bacterial suspension in order to get reporducable and comparable results.

Candida albicans isolate was resistant to the highest concentration of crude aflatoxins tested (40 ug/ml). This result is parallel with that of Burmeister and Hesseltine (1966) who found taht 38 species of yeasts from 6 different genera were not inhibited by 30 ug/ml of aflatoxins.

The biological assay for detection of aflatoxins in field commodities showed that 6 out of 50 samples were positive using both the biological and TLC assays, while the remaining samples were negative in both tests. Data of positive samples showed approximately linear relationship between the chemical and biological methods **(Table 3)**. However, it should be noted that the biological test is only screening test whose result should be confirmed by TLC, firstly to exclude the inhibition of bacteria due to other chemicals or any biological active materials and secondly to determine the type and exact amounts of aflatoxins.

SUMMARY

Production of aflatoxins was conducted using an identified toxin-producing strain of *Aspergillus flavus* and both natural (rice and corn) and semisynthetic (YES) media. Qualitative data showed that the strain of *A. flavus* was capable to produce four types of aflatoxins, namely B_1, B_2, G_1 and G_2 on rice, corn and YES media. Quantitative data showed that the concentrations of aflatoxins B_1 and G_1 produced were much higher, being 52, 40.3 & 34.6 and 64.7, 45.0 & 28.9 ug for 50 gm of rice, corn and YES media, respectively, while, the yielded amounts of aflatoxins B_2 and G_2 were 11.5, 17.9 & 17.5 and 28.9, 40.3 & 39.5 ug for 50 gm of rice, corn and YES media, respectively.

A bioassay was conducted using 5 standard bacterial strains namely, *Bacillus megaterium, Bacillus subtilis, Streptococcus faecalis, Staphylococcus epidermidis* and *Paracoccus denitrificans* as well as a field strain of *Candida albicans*. All strains except *P. denitrificans* showed varied degrees of inhibition when applying crude aflatoxins at 5-40 ug/ml, while, the minimum concentration of crude aflatoxins needed to inhibit *P. denitrificans* was not less than 10 ug/ml. Moreover, *Candida albicans* was not inhibited at any concentration of aflatoxins applied in this work.

Both undiluted and diluted (1/10, 1/100 & 1/1000) broth cultures showed direct relationship between the length of inhibition zones and the concentrations of crude aflatoxins. Mean length of (7.0 - 20.5), (5-14), (4.5-13.0), (3-12) and (1.5-11.0) mm were observed when various concentrations of aflatoxins were applied using *B. megaterium, S. epidermidis, S. faecalis, B. subtilis* and *P. denitrificans*, respectively.

Field trials were applied to testify the validty of such data. A 1/100 dilution was prepared from each strain of 4 different species to estimate aflatoxins in samples of contaminated corn. Both chemical and biological assays were carried out at the same time. Data revealed that the most sensitive organism which was inhibited by as low as 7.5 ug aflatoxins/ml was *B. megaterium* giving an inhibition zone of 10.5 mm, followed by *S. epidermidis* with an inhibition zone of 7.5 mm, while, the other two organisms were relatively less sensitive to crude aflatoxins. Similarly, such biological assay was applied to detect aflatoxins in some samples of wheat, corn, peanut, rice and poultry rations. Out of 14 samples of wheat 4 samples were positive (28.6%). Also, 2 samples of 10 corn samples were positive. The same results were obtained using TLC techniques of detection.

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