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# INHIBITION OF AFLATOXINS PRODUCED IN HUMAN AND ANIMAL FOOD BY THE FUNGI *Aspergillus flavus* AND *A. parasiticus* USING NEEM EXTRACT AND TOLCOLOFOS-METHYL

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## ABSTRACT

The usage of natural products and fungicides are the most promising methods which available to control fungal growth and toxins production in food. Our data indicated that the peanut samples that infected with 3.85 x $10^6$/ml Spore suspension have a quickly development after 4 days which increased in the positive control from 1, 2, 0 and 0 for B1, B2, G1 and G2 to 199, 148, 175 and 170 ng/g respectively. The levels of aflatoxins were developed to reach 219, 158, 205 and 185 ng\g after 8 days, respectively. Maximum levels were 224, 188, 235 and 160 ng/g after 12 days. It can also be noticed that a good minimization in toxin production was occurred by the direct usage of 5, 10 ppm Neem leaf extract. The data showed a gradual reduction of Tolcolofos-methyl (Rizolex) residue in the treated samples from 450 ppb at initial time to 10 ppb after 30 days. No detectable amount was observed after 45 days. That clearly indicates the outbreak in fungal growth after the first 45 days. That means when the Rizolex residue was completely disappeared after 45 days, fungal growth and aflatoxins B1 and B2 production started again with an abnormal high level because of the death of fungal antagonistic microorganisms. It can be concluded that when a storage period for more than 45 days, Maize needed a second treatment with 3g/kg Rizolex to guarantee a complete prevention of fungal growth and aflatoxins production.

## INTRODUCTION

Aflatoxins are a group of secondary metabolites produced by the molds of *Aspergillus flavus* (link ex fries). Little was known about its highly toxic and highly carcinogenic effects.

Aflatoxins had been found to be naturally occurring at a biological significant level in a wide range of food and feed stuff collected from various parts of the world, particularly from Africa such as barley, cassava, corn, cotton seeds, cow peas, millet, peanut, rice, sesame, sorghum, soybean and wheat.

The acute and chronic toxicity of aflatoxin exposure had been well studied (Heathcote and Hibbert, 1978). It was found that aflatoxin B1 was the most toxic for animals followed by aflatoxin G1, B2 and G2.

Detoxification of aflatoxins means all the techniques used to treat the contaminated products in order to remove, destroy or inactivate the contaminating toxins or inhibit the toxins production.

Removing aflatoxins by using a solvent system of 54% acetone, 44% hexan and 2% water (by weight) or a binary solvent system of 90% acetone and 10% water was noted by Gardner *et al*., (1968). By the same way, Rayner *et al.*, (1969) found that the treatment of cotton seeds and peanut meals with 90% and 95% aqueous ethanol at 75 $^{o}$C removed 94-96% of the aflatoxins in cotton seeds meal and 96-98% of aflatoxins in peanut meal.

A completely preventation of fungal growth and aflatoxins production had been achieved by El-Gazzar and Marth (1988) by adding 0.3 or 0.5% of hydrogen peroxide in glucose-yeast-salts medium inoculated with the fungus *A. parasiticus* NRRL 2999 and incubated at 14 or 28 $^{o}$C for 90 days.

The aqueous leaf extract of the subtrobical Neem tree seems to be a potential biological control inhibitor for the aflatoxins production of the fungus *A. flavus*. Zeringue and Bhatnagar (1990) reported that 98% inhibition in aflatoxin production was occurred when neem leaf extract was added to medium culture of *A. flavus.* Fungal growth was only 16% inhibited.

Garlic extract and sodium biocarbonate were reported by Singh and Chand (1993) as inhibitors for fungal growth and aflatoxin production. They found that 1.0 ml of Garlic extract per 100 ml medium inhibited the fungal growth with 19.7%, aflatoxin B1 production by 54.4% and total aflatoxin production by 30%. Sodium biocarbonate inhibited the fungal growth by 30.6%, B1 by 55.1% and total aflatoxin by 11%. Aflatoxins B2 and G2 increased in both Garlic extract and Sodium bicarbonate treatments.

The usage of fungicides and insecticides to reduce aflatoxin production of the fungus *A. flavus* was investigated by El-Kady *et al*., (1993). Aflatoxin production was inhibited by 27, 82, 100 and 100% when Vitavax-captan was added at 10, 25, 50 and 100 ppm, respectively. Sumisclex inhibited also the aflatoxin production but Actellic no effect. The most affective pesticide tested on aflatoxin production was Rizolex-T on both maize grain and sunflower seeds.

The most overall purpose of this work is to through some lights on the possibility of using natural compounds such as Neem extract and the fungicides Tolclofos-methyl Rizolex) to inhibit fungal growth and aflatoxins production in human and animal food resources.

## MATERIALS AND METHODS

### A. Monitoring of Aflatoxins in Foodstuff:

A total of one hundred and ninety two samples were subjected to qualitative and quantitative analysis of Aflatoxins (B1, B2, G1, G2 and M1). Forty-eight samples of each of peanut, maize, biscuit, and Milk were collected from four different local markets (Shobra, Heliopolis, Maadi and Dokki) from 1$^{st}$ October 1993 to 1$^{st}$ March 1994. The samples were subjected every 15 days to the Aflatoxins analysis. Each sample consists of 1kg. Samples were reduced in size using mill, food cutter, and grinder. A fine powdered mixing sample was needed. Each sample was divided into two portions; one for analysis purpose and the other kept under freezing at -20 $^{o}$C.

Extraction of a aflatoxins from all samples, fraction of the crude extract, purification of all fractions and identification of different types of aflatoxins were carried out according to official methods of analysis (Anonymous, 1984).

**B. Control of aflatoxins production:**

**B.1. Treatment of peanut samples with neem extract (Azadirachtin):**

Twenty-one peanut samples were moistured to 86% R.H. level and artificially infected with 1 ml suspention contained 3.85 x $10^6$ spore/ml of *A. flavus*. The infected samples were incubated at 28 $^oC$ for 12 days as recommended by Cotty (1991). Each sample was approximately one kg. Six samples were treated with 5 and 10 ppm neem extract, six samples for positive control and three samples for negative control. Negative control samples were analyzed before treatment or storage in initial time to determine the amount of toxins in row peanut. Qualitative and quantitative assays for the developed aflatoxins had been carried out every four days according to AOAC (1990).

**B.2. Treatment of maize with Rizolex (Tolcolofos-methyl):**

Thirty-two samples of Maize were analyzed. Twenty-one samples were analyzed for determination of four types of aflatoxins B1, B2, G1 and G2. One of them was in initial time and twenty (ten from treated samples and ten from control) samples during the experimental period, one from the treated samples and one from control every two weeks. Eleven samples were analyzed for determination of Tolcolofos-methyl. One was in initial time and ten during the experimental time every two weeks.

**C. Determination of Tolcolofos-methyl (Rizolex) Residues in Maize:**

Samples of 50g maize were extracted with 150ml ethyl acetate using warring blender for 3 min. Extracts were decanted through a funnel covered with cotton and sodium sulfate. Extracts were concentrated to 2ml using rotary evaporator. A gas chromatographic analysis using phillips 4500 equipment with flame photometric detector of organophosphorus residues was carried out. A chromatographic column Pyrex (7 ft length x 4 mm i.d.) packed with 4% SE -30/6% OV -210 on Gas. Chrom 80 -100 mesh was used. Carrier gas, N2 30 ml/min, H2 30 ml/min, Air 60 ml/min. coulmn temperature was 210 $^oC$, detector cell temperature was 220 $^oC$. Injector temp. was 225 $^oC$. Retention time (Rt) was 3.3 min.

## RESULTS AND DISCUSSION

**1- Monitoring:**

Aflatoxins are considered the most hazardous contaminates to human health. Different countries and international organizations, Egyptian standardization organization (ESO) and the Egyptian Ministry of Agriculture have set maximum residue limits (MRL'S) of aflatoxins in different items of food. These levels have been also internationally set by (Anonymous, 1995).

As data given in table (1), aflatoxin $G_1$ and $G_2$ are the most contaminates biscuit. Aflatoxin B2 was not detected in any of the analyzed Biscuit samples. The highest mean value of the four analyzed toxins was that of G1 as it reached 7 ppb. Aflatoxins $B_1$ and $B_2$ were clearly dominating in Maize. The highest level of $B_1$, B2, $G_1$ and $G_2$ was detected in peanut proving that peanut was the most contaminated commodity. The great difference in minimum and maximum values of aflatoxins in peanut samples might leads to the conclusion that peanut crop is exposed to different storing conditions of which some were favorable for fungal infection. The study of Cotty (1991) on the effect of storage conditions on aflatoxins production in cotton seads indicates clearly our conclusion. Data in the same table reported that milk samples were contaminated with only aflatoxin M1.

**Table (1): Aflatoxin levels in ppb in different commodities collected from Egyptian local markets in the period from October 1993 to March 1994**

| Aflatoxin | Biscuit | | | Peanut | | | Maize | | | Milk | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | min | max | mean | min | max | mean | min | max | mean | min | max | mean |
| **B1** | 2 | 3 | 2 | 2 | 66 | 15.41 | 2 | 33 | 19.33 | ND | ND | ND |
| **B2** | ND | ND | ND | 2 | 82 | 13.12 | 3 | 11 | 7.45 | ND | ND | ND |
| **G1** | 3 | 11 | 7 | 3 | 30 | 10.27 | 3 | 3 | 3 | ND | ND | ND |
| **G2** | 5 | 6 | 4.7 | 5 | 30 | 12.57 | 3 | 11 | 5.50 | ND | ND | ND |
| **M1** | ND | ND | ND | ND | ND | ND | ND | ND | ND | 0.5 | 1.0 | 0.75 |

MRL'S of ESO and CCFAC, number of violated samples and % of violation are indicated in table (2). The number of MRL'S given by ESO sometimes is the same number which given by CCFAC in some foods such as peanut and milk. In Biscuit and maize there is a difference in MRL'S by ESO and CCFAC. The difference can be seen in Biscuit, which is 0, 0 in total aflatoxins, and $B_1$ according to ESO and 5, 5 according to CCFAC. Therefore, the No. of violation of Biscuit samples ranged from 10 - 5 samples for total aflatoxins and 5 - 0 samples for aflatoxin $B_1$. Thus the violation percentage ranged from 20.8 to 10.4% for total aflatoxin and 10.4 to 0 for aflatoxin B1.

**Table (2): Different of MRLs of aflatoxins given by different organizations and its effects on No. of violation and %violation**

| | Biscuit | | Peanut | | Maize | | Milk |
|---|---|---|---|---|---|---|---|
| | Total (ppb) $B_1+B_2+G_1+G_2$ | B1 (ppb) | Total (ppb) B1+B2+G1+G2 | B1 (ppb) | Total (ppb) $B_1+B_2+G_1+G_2$ | B1 (ppb) | M1 |
| **MRL'S Ppb** | 0-5 | 0-5 | 10 | 5 | 10-20 | 5-10 | 0.05 |
| **No. of violation** | 10-5 | 5-0 | 21 | 16 | 11-9 | 12-11 | 10 |
| **Violation %** | 20.8-10.4 | 10.4-0 | 43.75 | 33.30 | 22.9-18.75 | 25-22.9 | 20.8 |
| **MRL of ESO** | 0 | 0 | 10 | 5 | 20 | 10 | 0.05 |
| **MRL of CCFAC** | 5 | 5 | 10 | 5 | 10 | 5 | 0.05 |

MRL'S of ESO and CCFAC have the same number of total aflatoxins and aflatoxin $B_1$ in peanut and in $M_1$ in milk. Thus, there is only one number of violated samples and % of violation. Twenty-one sample were violated for total aflatoxin, but 16 samples were violated in aflatoxin $B_1$ in peanut as illustrated in table (2). It means that 43.75% violation in total aflatoxin and 33.30% in $B_1$. This is the highest level of contamination, which observed in peanut.

Maize is an animal feedstuf unusual humanly consumed. Therefore, the MRL'S by ESO and CCFAC show the highest numbers. It's 20 ppb in total aflatoxin and 10 ppb in aflatoxin B1 by ESO. International numbers by CCFAC are lower than ESO. They are 10 ppb in total aflatoxin and 5 in aflatoxin $B_1$.

According to the same observation MRL'S ranged from 10-20 in total aflatoxin B1. Also, the No of violated samples ranged from 11-9 in total aflatoxin and 12-11 in aflatoxin $B_1$. Violation % generally in maize come in the second stage after peanut. It ranged from 22.9-18.75% in total aflatoxin and from 25-22.9% in aflatoxin $B_1$. By the same way, the data proved a highly contamination in milk by aflatoxin $M_1$. Violation percentage was 20.8%.

This monitoring study through an extensive light on the problems of food contamination in Egypt. It is a really prognostic study proving that the fungal growth and the production of fungal aflatoxins in human and animal food must be removed

The usage of the naturally products and fungicides are the most promising methods which available to control fungal growth and toxins production in food. Data in table (3) show that the peanut samples which infected with 3.85 x $10^6$/ml spore suspension have a quickly development after 4 days which increased in the positive control from 1, 2, 0 and 0 for $B_1$, $B_2$, G1 and G2 to 199, 148, 175 and 170 ng/g respectively. The level of aflatoxins were developed to reach 219, 158, 205 and 185 ng\g respectively after 8 days. Maximum levels were after 12 days. They were 224, 188, 235 and 160 ng/g. It can be noticed in the same table that a good minimization in toxin production was occurred by the direct usage of 5, 10 ppm neem leaf extract.

**Table(3) : Effect of the treatment of Neem extract on the aflatoxins level (ng) on the Peanut samples infected with spores of *A. flavus***

| Period of sampling | Positive control | | | | Treatment with Neem | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 5 ppm | | | | 10 ppm | | | |
| | B1 | B2 | G1 | G2 | B1 | B2 | G1 | G2 | B1 | B2 | G1 | G2 |
| 4 days | 199 | 148 | 175 | 170 | 94 | 28 | 55 | 60 | 29 | 8 | 25 | 20 |
| 8 days | 219 | 158 | 205 | 185 | 164 | 118 | 105 | 80 | 54 | 43 | 70 | 65 |
| 12 days | 224 | 188 | 235 | 160 | 179 | 163 | 140 | 120 | 109 | 88 | 100 | 100 |

The data in table (4) presented the percentage of toxins reduction after 4, 8 and 12 days post treatment with 5 and 10 ppm of neem extract. Five ppm neem exract caused percentage reductions of 53, 81, 69 and 65% for the production of B1, B2, G1 and G2 respectively after 4 days. Percentage of reduction decreased after 8 days to reach 25, 25, 49 and 57 % respectively.

**Table (4) : Reduction % of aflatoxins production as a result of the treatment with Neem extract**

| Period (day) | % Reduction | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 ppm | | | | 10 ppm | | | |
| | B1 | B2 | G1 | G2 | B1 | B2 | G1 | G2 |
| 4 days | 53 | 81 | 69 | 65 | 85 | 95 | 86 | 88 |
| 8 days | 25 | 25 | 49 | 57 | 75 | 73 | 66 | 65 |
| 12 days | 20 | 13 | 40 | 25 | 51 | 53 | 57 | 34 |

Decreasing in reduction was noticed after 12 days. It was only 20, 13, 40 and 25%. By increasing the concentration of neem extract to 10 ppm, a maximum reduction was occurred after 4 days to 85,95 86 and 88 %respectively and decreased to 75, 73 66 and 65 % 51% respectively after 8 days. The level of reduction decreased more to reach to 51, 53, 57 and 34 % after 12 days.

It means that neem extract was effective only in the first four days. The effect was declined and reached its minimal at 12 days post treatment. This could be due to the degradation in active ingredients of the neem extract, which were responsible for the inhibition of the fungal growth and its toxins production.

The treatment of maize with 3g/kg Rizolex was enough to prevent the production of all kinds of aflatoxins for about 45 days. Data in table (5) reported that:

**1st The untreated Tolcolofos-methyl ( Rizolex ) samples:**

1- All the samples at initial time were aflatoxins free.
2- Aflatoxins B1 and B2 were detected after 15 days in the untreated samples with a concentration of 34 and 17 ppb respectively.
3- The amount of B1 and B2 were gradually increased in the untreated samples to reach their maximum level after 5 months. They were 87 and 54 ppb respectively.
4- Through the experimental period in the untreated samples, G1 and G2 aflatoxins were not detected.

**2nd The treated Tolcolofos-methyl ( Rizolex ) samples:**

1- Any types of aflatoxins were not detected at 45 days.
2- At 60 days B1 was observed at concentration of 34 ppb. B2, G1 and G2 were also not detected.
3- Unexpected results were observed and detected when the concentration of B1 and B2 were gradually increased to become higher than their in the untreated samples.

These outbreak in fungal growth and aflatoxin production might be attributed the imbalance between *A. flavus* and its antagonistic microorganisms in maize, which are to be more sensitive to Rizolex more than *A. falvus.*

Table (5) shows also the gradually reduction of Rizolex residue in the treated samples from 450 ppb at initial time to 10 ppb after 30 days. No detectable amount was observed after 45 days. That indicates clearly the outbreak in fungal growth after the first 45 days.

**Table (5) :Effect of Rizolex treatment on aflatoxins levels in Maize samples and the Rizolex in maize at different times**

| Period of sampling | Aflatoxin in untreated maize as ppb | | | | Aflatoxin in treated maize as ppb | | | | Residues of Rizolex In maize at different time |
|---|---|---|---|---|---|---|---|---|---|
| | B1 | B2 | G1 | G2 | B1 | B2 | G1 | G2 | |
| **Initial time** | ND | ND | ND | ND | ND | ND | ND | ND | 450 |
| **After 15 days** | 34 | 17 | ND | ND | ND | ND | ND | ND | 120 |
| **After 30 days** | 52 | 23 | ND | ND | ND | ND | ND | ND | 10 |
| **After 45 days** | 59 | 27 | ND | ND | ND | ND | ND | ND | ND |
| **After 60 days** | 65 | 30 | ND | ND | 34 | ND | ND | ND | ND |
| **After 75 days** | 69 | 33 | ND | ND | 40 | 17 | ND | ND | ND |
| **After 90 days** | 72 | 34 | ND | ND | 134 | 84 | ND | ND | ND |
| **After 105 days** | 78 | 36 | ND | ND | 167 | 100 | ND | ND | ND |
| **After 120 days** | 84 | 50 | ND | ND | 200 | 134 | ND | ND | ND |
| **After 135 days** | 87 | 54 | ND | ND | 234 | 144 | ND | ND | ND |

That means when the Rizolex residue was completely disappeared after 45 days, fungal growth and aflatoxins B1 and B2 production started again with an abnormal high level because of the death of fungal antagonistic microorganisms. It can be concluded when a storage period more than 45 days, a second treatment with 3g/kg Rizolex is really needed to guarantee completely preventation of fungal growth and aflatoxins production.

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## تثبيط الأفلاتوكسين المنتجة بواسطة فطرى الأسبرجلس فلافس وباراستيكس مستخدماً مستخلص النيم والتولكوفوس ميثيل فى غذاء الإنسان والحيوان

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**** المعمل المركزى للمبيدات بالدقى.**

يهدف البحث إلى عمل تقصى للأفلاتوكسينات فى مجموعة الأغذية التى تم تحليلها وهى البسكويت والفول السودانى والذرة واللبن والتى تم جمعها من أسواق القاهرة الكبرى (شبرا - هليوبلس - المعادى - الدقى). ولقد أظهرت عينات الفول السودانى أعلى مستوى تلوث بالأفلاتوكسينات جميعها كما ثبت أن عينات البسكويت أكثر تلوثاً بالـ G2، G1 فى حين أنها أقل تلوثاً بالـ B2، B1 وعلى العكس ثبت أن الذرة ملوثة أكثر بالـ B2 ، B1 أكثر من الـ G2، G1. كما أظهرت عينات اللبن التى تم تحليلها تواجد الأفلاتوكسين M1 فقط. وكمحاولة للحد من نمو الفطر المنتج للأفلاتوكسينات والحد من إنتاج هذه التوكسينات إستخدم كل من مستخلص النيم ومبيد التولكوفوس ميثيل (الريزولكس). بالنسبة لمستخلص النيم فقد ثبت أن معاملة الفول السودانى بتركيز 10 جزء فىالمليون كان لها تأثير أقوى فى الحد من إنتاج الأفلاتوكسينات من المعاملة بتركيز 5 جزء فى المليون. أما مبيد الريزولكس فقد أظهرت النتائج أن المعاملة بالمبيد الفطرى قد أدت إلى الحد من نمو الفطر وبالتالى إنتاج الأفلاتوكسينات ولكن بعد مدة معينة زاد إنتاج الأفلاتوكسينات إلى فوق الحد العادى وذلك بعد إختفاء المبيد.