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CHEMICAL TREATMENT WITH UREA FOR AFLATOXIN B1 AND ITS BIO EFFECT ON THE EGG EMBRYOS

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ABSTRACT: *Results showed the sovereignty of genus Aspergillus spp. By 68%, followed by genus Penicillium spp. And Rhizopus spp. 10% and Mucor spp. 2%, and Aspergillus flavus record highest existence percentages 39%. And given the isolation of A. flavus Link ex Fires given higher production of aflatoxin B1(AFB1) 3.5 µg / ml. And the concentration 5% urea is the best in the inactivation of the growth of A. flavus and its production of AFB1. And fogging cotton meal with urea 5% showed better results in reducing the existence of fungi percentage from 61% to 0.0 and the percentage fungus A.flavus from 39% to 0.0 in comparison with control 100%. Urea 5% showed as well as the protection of cotton meal for 8 weeks of fungal infection or contamination with AFB1 in comparison with control. AFB1 led to the killing of eggs embryos to 100% in comparison with control 0.0. And the cotton meal extract contaminated with AFB1 and treated with urea 5% led to reduce the proportion of feticide to 85% compared to the treatment of AFB1 100%.*

KEYWORDS: Cotton Meal, Afb1, Urea and Chicken Eggs

INTRODUCTION

Cotton increased attention in recent years in Iraq, as it was cultivated area in 2006 89153 dunam (2500 m² in Iraq), and total production 37 515 tonnes, This means that the productivity per dunam has reached 421 kg / dunam (statistics of the Ministry of Planning and Development - Central Statistical Organization and Information Technology - Agricultural Statistics Department), It is relatively low compared to areas planted cotton in the world because of infection in different agricultural pests that affect the quality and quantity of production [3,7]. Egypt and Syria is one of the leading cotton producing countries, where their production amounted to 83 580 and 835 000 tons of cotton in 1999, respectively, And Yemen suffers from low production of cotton, with annual production rate of 24 973 tonnes for 1999 as well [15]. Cotton is grown mainly for its fibers within the textile industry, and the seeds of great importance as a source of food oil for humans and animal feed. Where used since 1975 in England for the manufacture of edible oils and used the meal as feed for livestock, cotton meal and used in animal feed because they contain protein content 25-45%, and considered unsuitable for poultry feed because of their detrimental impact on the color of the yolk and the strength of the egg membrane to the presence of Gossypol and fatty acid cycloprobnoad [24], And the importance of diets in the success of the poultry projects, so the increased interest in cotton cake and conducted many scientific studies on improvement of it, and protection of natural contaminants, especially after the discovery of aflatoxins and other toxic compounds [29], And the seriousness of these toxins on humans and animals because of their impact carcinogenic [28], And with low levels of contamination [20]. Therefore the international organizations resorted to determine the permitted in contamination levels in food and feed [14]. Researchers interested in the reduction of mycotoxins in food whether to prevent fungal

infection in the store and field or remove or destroy the mycotoxins in The adoption of different methods for the disposal of mycotoxins or reduce their harmful effects such as physical, biological and chemical methods [21]. Some studies have pointed to the efficiency in the treatment of urea or destroy the aflatoxin B1 (AFB1) in feedstuffs and protection from injury in fungi producing toxin [5,16]. From the above this study aimed to:

-Testing the efficiency of urea to destroy AFB1

- Determining the percentage of effective urea to destroy AFB1 in contaminated cotton meal.

- Bio evaluation to cotton meal extract contaminated with AFB1 on eggs embryo.

MATERIALS AND RESEARCH METHODS

Isolate the fungi accompanying the cotton meal sample

We got a 50 kg of cotton meal from factory of vegetable oils / Baiji / Company of vegetable oils / Ministry of Industry and Minerals. After mixing and homogenizing, quantity divided into three equal sections in weight (three replicates). And took the 50 grams of each replicate, then took 10 grams of each replicates in a beaker 250 ml with 90 ml sterile distilled water Shake the flask for 15 minutes in an electric vibrator to get the first dilution 10^{-1} for the preparation of identified dilutions from 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively, By adding 1 ml of the first dilution to 9 ml sterile distilled water and the three replications. Then added 0.5 ml of each dilution on the medium component of the potato extract agar and sucrose subsidized in Achromycin (30 ppm) in Petri dishes (9 cm diameter), And it used 10 dishes / replicate and dishes incubated for 7 days at a temperature of 25 ± 2C˚, and adopted the dilution, which gave the highest number of fungi. the number of developing fungi were calculated according to the following equation:

%Fungus = The average number of colonies of Fungus × dilution inverse

Then diagnosed depending on the specialized taxonomic keys [10,23].

Purification isolates of fungus *Aspergillus flavus*

Scrubbed the isolates of *A. flavus* fungus on potato extract agar and sucrose in Petri dishes (9 cm diameter) and diagnosed with the same way as above, the dishes were incubated at a temperature of 25 ± 2 C˚ for seven days.

The portability test of *A. flavus* isolates fungus to produce AFB1 at the medium of yeast extract and sucrose

I took a random isolates of *A. flavus* fungus growing on Czapek Dox Agar medium and planted on the Yeast Extract Sucrose (YES) medium, In flasks size 100 ml and by 50 ml / flask and 2 replicates for each isolation. Flasks were sterilized in autoclave at a temperature of 121 C˚ And pressure of 1.5 kg / cm² for 20 minutes and then inoculated in fungal isolates (7 mm diameter) and a week old. Flasks incubated at a temperature of 25 ± 2 Cº for two weeks [12]. Extraction process conducted by the way Jones [18] and modified by Al-Jarrah [6] and extracts preserved in the frozen (–20 Cº) until the examination.

Detection of AFB1 by using Thin Layer Chromatography (TLC).

Were used Thin Layer Chromatography (TLC) of silica gel G60 in the TLC Scanner II apparatus (GAMAG Swiss-made) To detect AFB1 in the plantation extract of *A. flavus* fungus by using the standard AFB1 and a concentration of 5 ppm supplier from Fluka AG, chem, Fabrik CII. 9470 Buchs-switzerland by using the separation System (Chloroform: Methanol, 97:3), pulled 10 µl of extract of each isolation using micro tubes placed in spots on the sheets silica gel and a distance of 1.5 cm between the spot and the other to the right of the plate, Putting the standard of AFB1 to left and leaving until the drought, and then placed in pre-separation solution. After the arrival of the solution to a distance of 2 cm below the upper end of the plate, plates output was examined under ultraviolet, Rf deportations was matching coefficient and color brilliance and severity with the standard AFB1 [11].

Confirmatory tests for the existence of AFB1

The chemical method

Followed the method of Romer [25] by using a mixture of sulfuric acid with 20% ethyl alcohol(Ethanol) To make sure the existence of AFB1 depending on brilliance color change from the blue to yellow, which indicates the existence of the same material.

Bidirectional test

Followed the method of Cocker et al. [11] to ensure the existence of AFB1 by using separation system chloroform: Gasoline: water (46: 35: 19) After separation system chloroform: methanol (97: 3) to make sure no fragmentation the spot from the standard AFB1.Was selected best isolates fungus *A. flavus* production of AFB1.

Determine the effective concentration of urea on the growth of *A. flavus* fungus in medium

I tested the effectiveness of the eleven concentration of urea 0.5, 1, 2,3, 4, 5,6, 7,8,9 and 10% on the growth of *A. flavus link ex Fires* fungus on medium of potato extract agar and sucrose and subsidized in Achromycin in concentration 30 ppm in Petri dishes medium inoculated in the isolation of fungus (7 mm diameter) produced AFB1 toxin and a week-old, And by 10 replicates / concentration, The dishes were incubated at a temperature of 25 ± 2 Cº for 14 days, Then recorded the results depending on the fungus growth of various concentrations of urea compared to the control treatment.

Effect of urea on the vitality *A. flavus* fungus and inactivation of the production of AFB1 in the cotton meal sample

I attended the glass flasks (500 ml) and put in each 100 grams of cotton meal, After wetting cake to 65%, Flasks sterilized twice in autoclave at a temperature of 121 Cº and pressure of 1.5 kg / cm² for 20 minutes Within 48 hours. Flasks inoculated in the isolation of fungus-producing AFB1 toxin, And treated with urea concentrations of 0.5, 1, 2,3, 4, 5,6, 7,8,9 and 10%, And incubated for 14 days at a temperature of 25 ± 2 Cº, with Shaking the Flasks manually for 4 days after inoculation To ensure the homogeneity of inoculation, Then planted Flasks components on Petri dishes containing potato extract agar and sucrose subsidized in Achromycin And by 10 dish / concentration. The dishes were incubated for 7 days at a temperature of 25 ± 2 Cº. Then recorded the results depending on the existent or non-existence

of fungus in urea concentrations compared to control treatment. Then took 50 grams of each concentration for the purpose of analysis and extraction with Setz and Moher [26] method.

Effect of urea treatment on fungi associated with the cotton meal sample in dusting method

I attended the flasks (1000 ml), put in each 250 grams of cotton meal after estimating the humidity and by three flasks per meal, Then add urea powder in the flasks at concentrations of 0.5,1,2,3,4,5,6,7,8,9 and 10% to study the effect of urea in the fungus associated with the meal. Flasks incubated in the laboratory temperature for 14 days, I attended the dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} for each concentration, taking 0.5 mL of each dilution and dissemination on medium of potato extract agar and sucrose in Petri dishes, operation were repeated 5 times / dilution, The dishes were incubated at a temperature of 25 ± 2 Cº for 7 days, and adopted the dilution which gave the highest number of fungi, Results recorded depending on the growth of fungi in comparison with control.

Evaluation the effectiveness of urea in AFB1 product in the cotton meal sample

I attended the flasks (500 ml), put in each 100 grams of cotton meal and three flasks / concentration of urea concentrations tested. After wetting meal to the level of humidity 65%, and sterilized twice in autoclave at a temperature of 121 Cº and pressure of 1.5 kg / cm² within 48 hours. Flasks inoculated in the isolation of *A. flavus* fungus producing AFB1 toxin, Incubated at a temperature of 25 ± 2 Cº for a period of 3 weeks. Then conducted the analysis and extraction operation according to method of Setz and Moher [26] after confirming meal containing on AFB1, The remaining meal (50 g) in each flask treated in urea concentrations tested. And two weeks after treatment in urea, conducted the analysis and extraction operation[26] as well as, AFB1 was detected on the TLC, and was a quantitative estimate of AFB1 in cotton meal using Electronic Scanner .

The effect of urea treatment duration in the treatment of cotton meal sample contaminated in AFB1

I attended 30 kg of cotton meal, was distributed in three bags of polyethylene (10 kg / bag) after wetting meal to the level of humidity 65% and sterilization in 2% formalin for 48 hours, and ventilating for 72 hours to dispose of formalin. The bags contents inoculated in the *A. flavus* isolation developing on the medium potato extract agar and sucrose in a two-week-old and by the density of pollen 64×10^4 spore / ml. Bags incubated in the laboratory temperature for two weeks. After the detection of AFB1 on the TLC, and conducting confirmatory tests to prove that meal containing AFB1. Meal treated with effective urea concentration of 5% except for the control treatment, and left in the laboratory temperature, and after 2, 4, 6 and 8 weeks of treated with urea, took the 50 grams of meal for the purpose of analysis and extraction at the same previous method, extracts preserved in the frozen (– 20 Cº), was detected AFB1on TLC and a quantitative estimate of AFB1using Electronic scanner.

Bio-evaluating the effectiveness of urea in Destruction AFB1 in a contaminated cotton meal sample on egg embryos Processing of Eggs

It was obtained eggs From the Al-Wefaq Company / Baghdad / Iraq Biological evaluation was conducted for cotton meal extract contaminated in AFB1 after extraction by method of Setz and Moher [26] and treated with urea 5% on egg embryos, Followed the method of Al-Heeti [4] to determine the basic qualities of the eggs Needed for the test, as follows:

- Fertilization rate in eggs is higher than 75%.

- Determine air space for each egg and cleared by ethanol 70%.

- Puncture the egg shell in the middle of the air space using Tooth driller.

- Caught the egg to an end between the index finger and thumb and recycling semi-circle to two consecutive and then injected by 30 µl of each treatment.

- Close the holes with adhesive transparent tape after injection and put the egg vertically for an hour before entered into the incubator.

- Adjust the temperature of the incubator at 99.75 ˚F (37.8 ˚C) and humidity of 60% the day before the introduction of eggs, 196 fertilized eggs were used in the experiment at a rate of 28 eggs / treatment Table 1, Eggs laying in the incubator in the modern hatchery Al-Ameri / Baghdad / Iraq. And it began examination of eggs every day since the fourth day until eighteenth day to hold back dead embryos of treatments. And record the number of hatched chicks each treatment after 21 days of incubation [13] For calculating the percentage of killing in treatment, as follows:

$$\%Killing = \frac{A\_B}{D-B} \times 100$$

as the: A = number of dead embryos as a result of treatment

B = number of dead embryos in the control treatment. D = number of eggs in each treatment.

Table 1. Treatments used in biological testing on egg embryos

Seq.	Treatments	AFB1(µg/ml)	% Urea
T1	Cotton meal extract contaminated with AFB1	3.5	0.0
T2	Cotton meal extract contaminated with AFB1 and urea	3.5	5.0
T3	Urea	0.0	5.0
T4	Methanol	0.0	0.0
T5	The holes	0.0	0.0
T6	Cotton meal extract	0.0	0.0
T7	Control (fertilized eggs only)	0.0	0.0

RESULTS AND DISCUSSION

Fungi associated with Cotton meal sample

The results showed the presence of a number of fungi associated with cotton meal Table 2, Aspergillus genus sovereignty with the highest percentages 68%, followed by genus Penicillium 10%, Rhizopus 10% and Mucor 2%, And was the highest existence percentage of the fungus *A. flavus* 39%, and these findings are consistent with that of Hussein [16] sovereignty fungus *A. flavus* in maize in the stores, And with that of Al-Jubouri [5] and Al-wershan [9] of sovereignty Aspergillus genus in the diet, And with Al-Heeti and Majeed [5] of sovereignty *A. flavus* fungus in blocks of forage. The reason for this is attributed to Aspergillus species genus susceptibility on the growth in medium in low moisture contents, whether these moisture during harvest or when Storage [17]

Table 2. Fungus accompanying the cotton meal sample and its existence percentages

Meal Type	Fungi	%Fungus
Cotton meal sample	*Aspergillus* spp.	39
	A. flavus	39
	Penicillium spp.	10
	Rhizopus spp.	10
	Mucor spp.	2

Portability of *A. flavus* isolates to produce AFB1 on the yeast extract and sucrose medium

The results of the analysis on TLC in the plantations of *A. flavus* Fungus extracts showed Portability of several isolates of the fungus to produce AFB1 on the yeast extract and sucrose medium it was sure that there AFB1 in chemical and Bidirectional examination on TLC [11]. Isolates were varying in the production of AFB1 and was selected isolation *A. flavus Link ex Fires* which gave the highest production for AFB1(3.5 µg/g) to be used in subsequent studies. And attributable the reason for this disparity isolates in the production of AFB1to its genetic ability [19].

Determine the effective concentration of urea in the inactivation of the growth of *A. flavus* fungus in medium

Results showed that the different effects of urea in the growth of the isolation of *A. flavus* fungus on potato extract agar and sucrose, and increasing the influence of urea directly proportional in the concentration increased. and urea concentrations 1, 2 and 3% have shown effectiveness in affecting on the growth of fungus and without bacteremia, and concentrations of 4.5, 6, 7, 8, 9 and 10% have shown wholly inhibition of the fungus growth when compared with the control treatment, These findings are consistent with the findings of the Al-Heeti and Majeed [5] The effectiveness of urea in the inhibition of fungal growth in the feedstuff and with the findings of Hussein [16] The effectiveness of urea in maize protection from fungal infection in stores, and these results may be due to the toxic effect of urea or their degradation products toxic for fungi as ammonia [2].

Effect of urea in vital fungus *A. flavus* and production of AFB1 in Cotton meal sample

The results showed a significant disparity in the effect of urea in vitality of *A. flavus* fungus depending on the nature of the medium and the chemical composition and physical qualities of the meal and the concentration of urea where the growth of *A. flavus* fungus isolation slightly and weak in the concentration of urea in 1, 2 and 3%, the concentration 5% urea gave wholly inhibition for fungus growth And the production of AFB1 (3.5 µg/g) in Cotton meal sample In comparison with control table 3, these results correspond with the findings of Al-Hitee and Majeed [5] the effectiveness of urea in the inhibition of fungal growth and the production of AFB1 in feedstuff blocks, and with the results of Zaman and Owen [30], which pointed to the effectiveness of urea in preventing the emergence of Or the presence of fungi in the feedstuff of high moisture content. and it has attributable the reason for the inhibition of *A. flavus* fungus growth on meal and these results may be due to the toxic effect of urea or their toxic degradation products for fungi as ammonia[2].

Table 3. The effect of urea in vital fungus *A. flavus* and the production of AFB1 in Cotton meal sample

Meal Type	***A. flavus***	**% Urea**											
Cotton meal sample		**0.0**	**0.5**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**	**6.0**	**7.0**	**8.0**	**9.0**	**10.0**
	Fungus growth	*	*	*	*	*	*	**	**	**	**	**	**
	AFB1	+	+	+	+	+	+	-	-	-	-	-	-

*= Fungus growth **=No growth +=AFB1 existence -=No AFB1

Dusting cotton meal sample in urea and its effect on associated fungi

Results in the laboratory showed the isolation of fungi associated with cotton meal which dusting in different concentrations of urea and in the moisture content of 9.5% Table 4 that the concentration of urea 5% may cause a complete inactivation of the growth of fungi associated with meal sample, And low percentage of existence fungi of 61% in the control treatment (0.0 urea) to 0.0 in concentration 5% urea, and low percentage of existence fungus *A. flavus* of 39% in the control treatment to 0.0 in 5% urea.

Table.4 Dusting cotton meal sample in urea and its effect on associated fungi

Meal Type	**Fungi**	**%Fungus**	
		0.0 Urea	**5%Urea**
Cotton meal sample	*Aspergillus* spp.	61	0.0
	Penicillium spp.		
	Rhizopus spp.		
	Mucor spp.		
	A. flavus	39	0.0

These findings are consistent with the findings of Al-Heeti and Majeed [5] The effectiveness of urea in the inhibition of fungi growth in the feedstuff. And the reason is attributed to the toxicity of urea or their degradation products for fungi.

The effect of treatment duration of the urea in the treatment of cotton meal sample contaminated in AFB1

The results of analysis on TLC Table 5 and bi-directional testing and chemical examination, the absence of AFB1 in cotton meal contaminated in *A. flavus* fungus producing AFB1, and at 65% moisture content After 2, 4, 6 and 8 weeks of treatment with urea 5% compared to the control treatment that showed the existence of AFB1 at a concentration of 3.5 µg/g.

Table. 5 The effect of treatment duration of the urea in the treatment of cotton meal sample contaminated in AFB1

Meal type	Treatments		AFB1 / week			
	AFB1(µg/g)	% Urea	2	4	6	8
Cotton meal	3.5	0.0	+	+	+	+
	3.5	5.0	-	-	-	-

+= Existence AFB1 – = No existence AFB1

And these results are consistent with the findings of the Zuber et al., [31] the effectiveness of urea in destruction of toxins AFB1 for 12 weeks in maize and with the results of Shantha [27] who managed to wholly destruction of AFB1 in peanuts By using urea, And with the results of Hussein [16] who pointed out the effectiveness of urea in reducing the contamination at AFB1 when the treatment is in the urea 4%, and these results are convergent the results of Al-Heeti and Majeed [5] who pointed to the effectiveness of urea in the protection of blocks of feedstuff contamination in AFB1 for four months. And the reason is attributed to the toxicity of urea or their destruction products as ammonia for destruction AFB1.

The effectiveness of urea in destroy AFB1 in a Cotton Meal sample And their vital impact on chicken embryos.

The results in Table 6 have shown negative impact of AFB1 in the concentration of 3.5 µg/mL and that led to the killing of egg embryos, 100% when compared to the control treatment 0.0. while the treatment of AFB1 in urea 5% have shown high improvement in reducing the impact of AFB1, and reducing the proportion of killing to 17%, that urea caused a decline in the proportion of killing of egg embryos increased by 83% when comparing with AFB1 treatment 100%. And these results are consistent with the findings of the El-Behadli [13] and Al-Heeti [4] Who indicated that the concentration of AFB1 in 10 µg/ml Leads to the the killing of egg embryos,

Table.6 The effectiveness of urea in destroy AFB1 in a Cotton Meal sample And their vital impact on chicken embryos

Treatments	AFB1(µg/g)	% Urea	% Dead embryos
Control	0.0	0.0	0.0
AFB1	3.5	0.0	100
AFB1+Urea	3.5	5	17
Urea	0.0	5	16
Methanol	0.0	0.0	13
Perforating	0.0	0.0	16
Cotton meal extract	0.0	0.0	8

100%, The reason for the low percentage of killings in chicken embryos when processing AFB1 in urea is due to to the efficiency of urea as material destroy for AFB1 in foods and feedstuff according to Piva and et al. [22] and Al-Heeti and Majeed [5] studies. as well as with studies that indicate the efficiency of ammonia as one of the products of the decomposition of urea to destroy AFB1 In food and feedstuff [1].

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

1. Contamination of cotton meal in the number of fungi and most existence *A. flavus* Product for AFB1.

2. AFB1 also led to the killing of all egg embryos.

3. The treatment of contaminated cotton meal in AFB1 at urea5% Leads to Minimize the negative impact of AFB1 at the eggs embryos.

Recommendations

1- Interest in conducting surveys To determine the extent of contamination of feedstuff Or raw materials used in Especially manufacturing waste Including contaminated meals at mycotoxins.

2- Use of urea in reducing the toxic effect of AFB1 Or destroyed at poultry feed and animals.

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