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INHIBITORY EFFECTS OF SOME NATURAL OILS ON THE TOTAL AFLATOXIN PRODUCTION BY *Aspergillus flavus* IN FEED

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ABSTRACT

This research was carried out to study the effect of some natural oils at different concentrations and storing temperature on the production of aflatoxins in the different feed stuff by *Aspergillus flavus* . Five different oils were tested ; lemon (*Melissa officinalis*) , olive (*Olea europaea L.*) , black seed (*Nigella sativa*) , clove (*Syzygium aromaticum*) and peppermint (*Mentha piperta*) at three concentrations (0.05 % , 0.1 % and 0.15 %) were tested on sabouraud – yeast extract medium . It was found that there were no significant effect on weight either fresh or dry of the mycelia of *A . flavus* but there were significant increase in production of aflatoxin at 0.1 % and 0.15 % concentrations of olive and clove oils compared with other oils . Three different feeds (Starting , Growing and finishing) were provided with clove and olive oils at 0.1 % and 0.15 % and inoculated with concentration of $1*10^{-6}$ pores / ml of *A . flavus* and incubated at 25 °C , 30 °C and 35 °C for ten days . Results obtained showed that the effect of temperature was highly significant on reducing the total aflatoxin when stored at 35 °C and with 0.1 and 0.15% for olive and clove oils. . There were significant interaction among feeds , oils and their concentrations .

Keywords: Total aflatoxin – Lemon – Olive – Black seed – Peppermint – Clove - Feed - Temperature .

INTRODUCTION

Jenineke . *et . al* . (1989) and Pitt (1990) reported that mycotoxins are a group of structurally divers secondary fungal metabolites that occur world wide contaminated to grain . Among the various mycotoxins identified especially those affecting poultry , some occur significantly in naturally contaminated foods and feeds . Aflatoxicosis poisoning results from ingestion of aflatoxin in contaminated food or feed . The aflatoxins are group of structurally related toxic compounds produced by certain strains of the fungi *Aspergillus flavus* and *A . parasiticus* . Under favorable conditions of temperature and humidity . These fungi grow on certain foods and feeds , resulting in the production of aflatoxin . Gould (1996) reported that use of chemicals to enhance the safety of many foods is of great interest to the food industry . Chemical preservatives vary in their ability to kill microorganisms and how affects physical and chemical characteristics of foods . However , the presence of chemical residues in foods and labeling for preservatives on food packages are major concerns to consumers these days . Therefore , the need for naturally derived compounds and other natural products with antimicrobial and antifungal properties has been explored .

Herbs have been used for large range of purposes including medicine , nutrition , flavoring , cosmetics , charms , smoking and industrial uses (Smith and Winder , 1996) .

Investigations on the mode of action and potential uses of plant volatile oils have regained momentum . They appears revival to be used for traditional approaches to protect livestock and food from disease , pests and spoilage in industrial countries . This seems true in regard to plant volatile oils and their antimicrobial role which can be seen from the comprehensive range of organisms against which volatile oils have been tested . These have included food spoiling organisms . (Zaika , *et .al* . 1983 ; Connor & Beuchat, 1984 ; Janssen , *et . al* ., 1988 ; Outtara , *et . al.* 1997 and food poisoning organisms (Beuchat , 1976 ; Tharib , *et . al* ., 1983 ; Deans & Ritchie , 1987; Lis – Balchin and Deans , 1997) and mycotoxigenic filamentous fungi (Knobloch , *et . al* . 1989) . Hamza (2002) found that use of certain feed additives is very important in controlling many dangerous hazards present in animal and poultry feed such as mould inhibitors which are added to control mould growth promoters . The improper use of these additives may render the feed harmful for health of both animal and human . If mould inhibitors are added in sub therapeutic doses it will create multidrug resistant bacteria strains and if added in high doses it will make harmful residues in animal tissues . Jugal , *et . al* . (2002) suggested that commonly occurring mycotoxigenic fungi can be controlled with clove oil . (Mabrouk and El - Shayeh , 1980 at 0.1% level , Hitokoto , *et . al* . , 1980 at o.4 mg / ml , Liewelly , *et . al* . ,1981 and , El – Naghy , *et. al* . , 1992) at 1% concentration with 1% Tween 80 , Deans , *et . al* . (1995) ; El- maraghy (1995) ; Montes – Belmont and carvajal (1998) , at 1μ /ml $^{-1}$ broth .

Peppermint oil contains 50 – 78 % free methanol (5 – 20 % combined in various esters) , L – limonene methan . cineol and phellandrene (Claus, 1962). These constituents may be responsible of the inhibition of different fungi species (Campbell , 1967) .

This research was designed to evaluate the efficacy of using some oils at different levels and different storing temperature to protect the feeds .

MATERIALS AND METHODS

Fungi used

Eight strains of *Aspergillus flavus* were isolated from feedstuff. The cultures were purified and maintained on sabouraud – yeast extract agar plates (20 gm glucose , 10 gm peptone , 10 gm yeast extract , 15 gm agar and one L . distilled water) . For seven days at 26 - 28 ° C. After this period the conidia were harvested gently with an inoculums loop . The spores were diluted with the isotonic NaCl physiological solution , vortexes to break up any clumps of spores and then count in homocytometer .

Detection of aflatoxin

Producing fungi , isolated from feedstuff fungal cultures were grown on sabouraud yeast extract agar plates and the ability of isolated fungi to produce aflatoxin were compared under UV . light (366 nm) . One strain of *A. flavus* which gave highest intense fluorescence was isolated . This strain ,

was exposed to different types of oils for control aflatoxins production of . Various concentrations of lemon (*Melissa officinalis*) , olive (*Olea europaea L.*) , black seed (*Nigella sativa*) , clove (*Syzygium aromaticum*) and peppermint (*Mentha piperta*) oils were tested . Three concentrations of each oil (0.05% , 0.1% and 0.15%) were used . Fungal cultures were grown in 250 ml conical flask containing 50 ml of sabouraud yeast extract medium and oils at different concentrations (three replica for each concentration) which inoculated with spore suspension at 10^{-6} spores / ml . The inoculated flasks were incubated under stationary culture conditions at 26 - 28°Cfor seven days. At the end of incubation period , the fungal mat was collected by filtration and washed twice with distilled water , then weighed to estimate the biomass as fresh weight and dried at 80°C in an oven until constant weight .

Production of aflatoxins on feedstuff was carried out in flasks (according to Fabbri ,*et al.*, 1980). Each flask containing 20 gm feedstuff (T1, T 2 and T3) and different concentrations of oils was inoculated with 10^{-6} spore concentration of *A. flavus* isolate and after being moistured with distilled water to 18.5 % moisture content . Incubation lasted for one week at 28 °C , then each flask was tested for total aflatoxin . Enzyme immunoassay was applied for the quantitative analysis of aflatoxins .

Test procedure : It was carried out according to Ridasceen® Aflatoxin Total Enzyme immunoassay for the quantitative analysis of aflatoxin (R – Biopharm AG , Darmstadt , Germany).

The basal experimental diets as shown in Table (1) were formulated to meet the nutrients requirements of broiler chickens based on the recommendation of NRC (1994) .

Table (1) : Composition of starter , grower and finisher diets for broiler.

Ingredients	Starter (T1)	Grower (T2)	Finisher (T3)
Corn	63.24	69.93	75.67
Soybean	15.15	8.67	4.74
Corn gluten	18.3	16.66	15.42
Dicalcuim	1.8	1.4	1.7
Lime stone	1.14	1.47	1.17
Salt	0.3	0.3	0.3
Vitamins*	0.3	0.3	0.3
Methionin	0.02	0.5	0.1
Lysin	0.4	0.47	0.51
Total	100	100	100
Calculated analysis			
CP	22.9	20.5	18.095
ME	3124.1	3196	3234
Ca	0.9	0.93	0.88
P	0.46	0.37	0.42
C. fiber	2.92	2.39	2.57
C. fat	3.02	3.29	3.153
L. lysine	0.8	0.87	1.19
Meth. + cys.	0.95	0.87	1.31
Meth.	0.55	0.54	0.97

***Supplied per kg of diets : vit . A , 12000 IU ; vit . D3 ,2200 IU ; vit . E , 10 mg ; vit . k3 , 2 mg , vit . B1 , 1 mg ; vit . B2 , 4 mg ; vit . B6 ,1.5 mg ; vit . B12 , 10 µg ; Nicotinic acid , 20 mg ; Folic acid 1 mg ; Pantothenic acid , 10 mg ; Biotin , 50 µg ; Cholin chloride , 500 mg ; Copper , 10 mg ; Iron , 30 mg ; Manganese , 55 mg ; Zink , 50 mg ; Iodine , 1 mg ; Selenium , 0.1 mg ; Cobalt , 0.1 mg .**

Statistical analysis

The data were statically analyzed according to completely random design . Analysis of variance (F value) and Duncan's Multiple Range were used for the analysis of the results according to SAS (1985) .

RESULTS AND DISCUSSION

Detection of aflatoxin produced of *Aspergillus flavus* isolated from various feedstuff

This experiment was carried out to study the ability of eight isolates of *A . flavus* isolated from feed stuff to produce aflatoxins on saburaud – yeast extract agar plate . The results in Table (2) showed that two isolates of *A . flavus* showed some fluorescence (+) , four isolates showed slight fluorescence (++) , one isolate showed moderately intense fluorescence (+++) and one isolate showed highly fluorescence (++++) . This results is in agreement with Abdel – Fattah (1987) who reported that thirty three fungal isolates from fungal contaminated food and feed samples were able to produce aflatoxin and the most active producer were *A . flavus* isolates .

Table (2): Color and intensity of fluorescence produced by eight isolates of (*Aspergillus flavus*) isolated from different feed stuff as viewed under UV light (366 nm) .

Number of strain	Fluorescence	
	Color	Intensity
1	Green	1 + Some fluorescence
2	Green	2 + Slight fluorescence
3	Blue	2 + Slight fluorescence
4	Bluish green	3 + Moderately fluorescence
5	Green	2 + Slight fluorescence
6	Blue	2 + Slight fluorescence
7	Bluish green	4 + Highly fluorescence
8	green	1 + Some fluorescence

Effect of oils on mycelia

Data in Table (3) show the effects of different oils at different concentrations on weight of mycelia and intensity of florescence .

Lemon oil :It is clear from these data that there were no significant differences in mycelia growth and aflatoxin fluorescence under different levels of lemon oil . The present results agree in part with that of Tantoui – Elaraki and Beraoud (1994) who showed that lemon was unable to inhibit totally the mycelial growth and aflatoxin synthesis even at 1% concentration .

Olive oil : The results show that olive oil levels ; 0.1 % and 0.15 % had the higher effects on mycelia weight and the lowest fluorescence , but this differences were not significant . Phenols , widely distributed in vegetables , are found in high concentrations in the typical components of the Mediterranean diets . The complex phenols found in olive oil include the glycoside oleuropein , and its hydrolysis product hydroxytyrosol (3 , 4 –

dihhydroxyphenyl ethanol) . The amounts of these olive oil " minor components or nonnutrients " vary , depending on number of factors including production and storage (Brenes , *et . al* .1999) . However , concentrations of 2.3 – 9 mg / l of oleuropein and 1.4 – 5.6 mg / l of hydroxytyrosol had been reported (Montedoro, *et . al* . 1992) . Olive oils , have received less attention and little information exists regarding the biological activities of olive oil on aflatoxin.

Black seed oil : The data show that there were no clear effects on mycelia weight and fluorescence of aflatoxin by using different black seed oil levels (0.05 % , 0.1% and 0.15 %) . The present results agree with Abdel – Malek , *et . al* . (1994) who showed that black cumin was of moderate inhibitory effects on yeast tested . In the other hand , Mouhajir , *et . al* . (1999) made antimicrobial tests of the crude seed extract (*Nigella sativa*) against ten microbial species , including two fungi , showed activity against *Bacillus subtillis* , *Klebsiella pneumoniae* , *Mycobacterium phlei* and *Mathicillin sensitive* and resistant *Staphylococcus aureus* . De , *et . al* . (1999) indicated that black cumin have potent antimicrobial activities against the tested organisms *Bacillus subtilis* , *Escherichia coli* and *Saccharomyce cerevisiae* . This seems due to the difference between species .

Clove oil : Data in Table (3) clear that the highest effect on fluorescence obtained at 0.1 % and 0.15 % . Fluorescence tend to decrease at 0.05 % . Means show that there were no significant effect on mycelia weight either at dry or fresh base . Our results disagree with many studies concerning the effect of clove oil on mycelia of *A . flavus* . The resultus disagree with Cherry (1999); Mau , *et . al* . (2001) ; Mabrouk and El – shayeb (1980) ; at 0.1 % ; Hitokoto , *et . al* . (1980) , at 0.4 mg / ml ; El – Naghy , *et . al* . (1992) ; Abedel – Mallek , *et . al* . (1994) , on yeast species , Deans , *et . al.* (1995) Calderone , *et . al* . (1994) . On Paeibacillus lavae in vitro testes at concentration of 1000 ppm , Montes – Belmont and Carvajal (1998) ; Jugal , et . al . (2002) found that clove completely inhibited the fungal growth . Bara and Vanetti (1995) suggested that although the amount of eugenol in the essential oil fraction of cloves is particularly high the high tannin content of this spices provides additional antimicrobial effects .

Peppermint oil : The mycelia weight , fresh and dry , and intensity of fluorescence as a result of addition of peppermint oil are presented in Table (3). There were no significant differences among mycelia weights and intensity of fluorescence at different levels of peppermint , 0.05 % , 0.1 % and 0.15 % . These results agree with the findings of Ibrahim and Ougumoded (1991) who showed that peppermint exhibited no significant preservative action against *P . aeruginosa* . On the other hand, Mabrouk and El – Shayeb (1980) showed that no aflatoxin was produced when peppermint used at level 10 % . El – Naghy , *et . al* . (1992) showed the high fungustic effect of peppermint oil . They also observed that this oil when added at 1% concentration with 1% Tween 80 inhibited growth of the tested fungi seriously. Montes – Belmont and Crvajal (1998) studied the effect of *Menta piperta* (Peppermint) on maize kernel .

Table (3) : The effect of different oils at different concentrations on mycelia weights and intensity of florescence .

Type of oil	Concentrations %	Weight of mycelia		Fluorescence (intensity)
		Fresh wt . gm	Dry wt . gm	
Control		5.094 ± 1.43	0.32 ± 0.15	+ + + +
Lemon	0.05	6.178 ± 1.65	0.265 ± 0.14	+ + + +
	0.1	5.384 ± 1.4	0.163 ± 0.14	+ + + +
	0.15	7.107 ± 1.76	0.33 ± 0.16	+ + + +
Olive	0.05	7.126 ± 0.05	0.235 ± 0.03	+ + + +
	0.1	4.872 ± 1.48	0.235 ± 0.15	+ +
	0.15	4.792 ± 1.41	0.15 ± 0.01	+ +
Black seed	0.05	7.161 ± 0.07	0.316 ± 0.02	+ + + +
	0.1	8.366 ± 1.48	0.405 ± 0.06	+ + + +
	0.15	7.123 ± 4.3	0.335 ± 0.14	+ + + +
Clove	0.05	8.139 ± 1.68	0.35 ± 0.21	+ + +
	0.1	4.631 ± 0.79	0.288 ± 0.12	+ +
	0.15	6.023 ± 0.96	0.395 ± 0.05	+ +
Peppermint	0.05	6.237 ± 5.7	0.29 ± 0.07	+ + + +
	0.1	4.88 ± 2.08	0.42 ± 0.21	+ + + +
	0.15	5.908 ± 1.58	0.52 ± 0.02	+ + + +

Total aflatoxin

Table (4a, 4b and 4c) show the effect of different oils, oil concentrations on total aflatoxin produced *by A . flavus* on different types of feed (ng / gm) and when stored at different temperatures .The means of different feed showed significant decreasing in total aflatoxin with clove oil and olive oil .Adding olive and clove oils at different concentrations decreased total aflatoxin . Differences were significant at 25° C, 30° C and 35° C . In contrast , Suttajit (1990) indicated that several practices have been recommended to keep the condition unfavorable for any fungal growth . These include store commodities at low temperature when ever possible . Mislivec (1990) in his experiment , virtually all of the mycotoxin producing species detected in Thailand grow poorly , when stored at 10 ° C or less . However , as lowering temperature facilities are virtually nonexistent this temperature control is not the answer .

Data also showed significant differences in total aflatoxin under different temperature, 35°C (610.69 ng/gm) comparing with other temperature 25°C (3108.3ng/gm) and 30 °C (3240.33ng/gm). Total aflatoxin decreased significantly when feed treated with clove oil (2235.07ng/g) followed by olive oil (2404.47 ng/g).

Briant (2003) reported that moldy feed may have reduced digestibility and energy content, yet need to be adjusted down by 5 % . Molds growth and propagating derive energy from feeds protein , fat and carbohydrate . Dietary fat in particular is reduced in mold infected feeds which suggested that book values for energy better to be multiplied by 95% in the presence of substantial amounts of mold (95 % of energy value) . From Table (4a) it is clear that total aflatoxin decreased significantly with starting (2282.91ng/gm)and finishing feeds (2283.48ng/gm) comparing with growing feed (2392.93 ng/gm) . Fungal growth in feed stuffs is associated with the

utilization of nutrients from the host and consequent alteration in the nutritional content of the feed stuff . The germ of the grain is the main site for *Aspergillus sp* development , which leads to a greater potential for the synthesis of AFB1 (Brekke , et . al . 1975) . Since the germ is the richest fat contributor in cereal , a major consequence from mould development is the reduction in its energy content. Broiler are usually fed – high energy feeds , which require supplemental fat . Any reduction in the energy content of grains leads to extra oil supplementation , which increases feed cost .Moulds may be affected by different levels of nutrients .

Table (4a) The effect of different oils and different types of feed on total aflatoxin produced by *Aspergillus flavus* (ng / g) .

Type of oil / Type of feed	Olive	Clove	Overall
Starter	2242.88±15.09	2322.92±15.09	2282.91[b]
Grower	2411.77±15.1	2374.07±15.1	2392.93[a]
Finisher	2558.74±15.09	2008.22±15.9	2283.48[b]
Overall	2404.47[a]	2235.07[b]	

Means with the different letter are significantly different.

Table (4b) The effect of different oils concentration and different types of feed on total aflatoxin produced by *Aspergillus flavus* (ng/ g).

Concentration / Type of feed	Control	0.1%	0.15%
Starter	2850.1±18.49	2206.05±18.49	1792.5±18.49
Grower	2338.7±18.5	2560.17±18.5	2279.83±18.5
Finisher	2940.11±18.5	2782.8±18.5	1127.4±18.5
overall	2709.67[a]	2516.37[b]	1733.28[c]

Means with the different letter are significantly different.

Table (4c) The effect of temperature and different types of feed on total aflatoxin produced by *Aspergillus flavus* (ng / g) .

Temperature / Type of feed	25° C	30° C	35° C
Starter	3386.16±18.49	3263.5±18.49	199.0±18.5
Grower	3185.94±18.49	3626.38±18.5	366.4±18.5
Finisher	2752.7±18.5	2831.05±18.5	1266.6±18.5
Overall	3108.3[b]	3240.33[a]	610.69[c]

Means with the different letter are significantly different.

Table (5) shows the statistical analysis of variance for the variation in total aflatoxin produced by *Aspergillus flavus* on different types of feed , oils , concentration of oils and stored at different degree of temperature. The statistical analysis showed that the variation in total aflatoxin was highly significant for different types of feed , oils , concentration of oils , different degree of temperature and interaction between its .

Table (5): Analysis of variance (F value) for the effect of different types of feeds, oils, concentration of oils and temperature o the total aflatoxins produced by *Aspergillus flavus* .

Source	F value
Feed	35.21**
Oils	188.18**
Concentration of oils(Con)	2345.26**
Temperature (temp)	19257.9**
Feed & oils	246.54**
Feed & Con	749.25**
Feed & temp	849.55**
Temp & oil	370.65**
Temp & con	290.44**
Oil & con	220.00**
Feed & temp & oil	323.14**
Feed & temp & con	267.38**
Feed & oil & con	444.33**
Temp & oil & con	95.33**
Feed &temp & oil & con	95.87**

** = Highly significant at P ≤0.01

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التأثير المثبط لبعض الزيوت الطبيعية على الأفلاتوكسينات الكلية المنتجة في الأعلاف

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تم القيام بهذه التجربة في كلية الاقتصاد المنزلي – جامعة الأزهر بهدف حماية العلف من التلوث بالأفلاتوكسينات

هذا البحث أجري لدراسة تأثير بعض الزيوت الطبيعية بتركيزات مختلفة على إنتاج الأفلاتوكسينات في الأعلاف بواسطة فطر الأسبرجلس فلافس عند تخزينها عند درجات حرارة مختلفة .

وقد اختبرت خمس زيوت طبيعية هي زيت الليمون , الزيتون ،القرنفل ، حبة البركة والنعناع عند ثلاث تركيزات هي ٠,٠٥ ،٠,١ و ٠,١٥% على إنتاج الأفلاتوكسينات في بيئة سابورود – مستخلص الخميرة ووجد أنه يوجد تاثير معنوي لزيت الزيتون والقرنفل عند تركيزي ٠,١ و ٠,١٥% على إنتاج الأفلاتوكسينات بالمقارنة بالكنترول .

ثلاث أنواع من أعلاف الدواجن هي البادي , النامي و الناهي كل منها أضيف إليه زيت الزيتون والقرنفل عند تركيزي ٠,١ و ٠,١٥% وحقنت بجراثيم تركيزها ١* ٦١٠

من فطر اسبرجلس فلافس وحضنت عند درجة حرارة ٢٥و٣٠ و٣٥ م لمدة عشرة أيام

وأوضحت النتائج أنه يوجد تأثير معنوي على إنتاج الأفلاتوكسينات عند تخزينها عند درجات حرارة مختلفة كما يوجد تأثير معنوي على إنتاج الأفلاتوكسينات بين نوع العلف والزيت وتركيزهذه االزيوت .

تحت نفس الظروف لهذه التجربة وجد أن الأفلاتوكسينات الكلية قلت مع الأعلاف المختلفة عند درجة حرارة ٣٥ ?م مع استخدام زيت الزيتون أو القرنفل بنسبة ٠,١ % ،٠,١٥ % .