Role of certain herbal substances and some feed additive mixtures as mold growth and mycotoxins inhibitors on infected corn grains

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Abstract:

Preliminary tests showed that the tested isolates of *Aspergillus flavus* and *Fusarium verticilloides* were capable to produce aflatoxins and fumonisins. Moreover, active ingredients of the tested herbal plant extracts were found to include monocyclic terpenes (limonene) and dicyclic terpenes (α-pinene). Besides, other active components were also detected, e.g. anethole in anise oil, thymol and carvacrol in thyme oil, eugenol and thymol in clove oil and dicyclic alcohol in peppermint. Feed additive mixtures (FAM), used for poultry (PAM) and cattle (CAM) were tested for their antagonistic and detoxification effect against the tested isolates. Individual herbal oils were more effective in reducing fungal growth than their mixtures. More inhibitory effect against *F.verticilloides* was obtained by clove oil, followed by thyme oil, caraway oil and peppermint. Oil mixtures without Sodium chloride were more effective in reducing fungal growth, followed by poultry additive mixtures. Feed additives and oil mixtures were more effective in reducing growth than suppressing mycotoxin production by *F.verticilloides*, whereas the contrary was observed in *A.flavus*. Oil mixtures with or without Na Cl were less effective in reducing fumonisin production than FAM, whereas the reducing effect was the same in aflatoxins production by *A.flavus*. Clove oil showed the highest reduction in fungal growth of both *F.verti*cilloides and *Aspergillus flavus*, compared with the other individual oils. Clove oil was more efficient in reducing fumonisins, whereas Na Cl was more effective in reducing aflatoxins.

Additional index words: Herbal substances, feed additives mixture, fungal growth inhibitors, Mycotoxins inhibition, corn grains.

Abbreviations: FAM = Feed additive mixtures; CAM = Cattle additive mixtures; PAM = Poultry additive mixtures; OM = Oil mixture; OM1 = Oil mixture and OM2 = Oil mixture2.

Introduction:

Corn (*Zea Mays* L.) is one of the most important grain crops all over the world. In Egypt, the cultivated area of maize grain production reached about 1.9 million Feddans in season 2005, which produced approximately 6.3 million, tons of corn grain (AOAD, 2006).

Corn grains are subjected to infection with many fungi. Transportation and storage of corn grains under certain conditions might induce undesirable changes that facilitate the spread of storage fungi such as *Fusarium verticilloides* and *Aspergillus flavus* with the possibility of producing mycotoxins (Jaime-Garcia and Cotty, 2006; Youssef *et al.*, 2003; Bluma and Etchechevery, 2008 and Bluma *et al.*, 2008).

Some of these fungi not only decrease the yield of crop and the quality of produced grains but also excrete mycotoxins; e.g. fumonisins, produced by *Fusarium verticilloides* (Youssef *et al.*, 2003; Afolabi*et al.*, 2007; Schjoth and Tronsmo, 2008; Murillo-Williams and Munkvold, 2008 and Elsamra *et al.*, 2012a and b) and aflatoxins produced by *Aspergillus flavus* (Youssef *et al.*, 2003; Frisvad*et al.*, 2007; Accinelli*et al.*, 2007; Probst and Cotty, 2007 ;Rahimi *et al.*, 2008; Zitomer *et al.*, 2009; and Diedhiou *et al.*, 2011 and Elsamra *et al.*, 2012a and b). These mycotoxins are known to cause diseases in plants, animals and humans who eat contaminated food (Takao *et al.*, 2001; Singh *et al.*, 2006; Jonathan H Williams et al., 2004 and Boonen, Jente *et al.*, 2012).

Youssef *et al.* (2003) demonstrated that *Fusarium sacchari*, *Fusarium verticilloides* and *Aspergillus flavus* are the most prevalent fungi associated with American maize imported from U.S.A during the period 1999-2000. Contamination of maize occurs mostly both in field and store (Bankole and Mabekoje, 2004). It was found that the extent of field contamination by fungi determined largely by the rate of deterioration of stored corn grains and subsequent production of mycotoxins (Bennet and Klich, 2005, Singh *et al.*, 2006 Vanara *et al.*, 2008 and Zitomer *et al.*, 2009).

It was found that sodium chloride (Thanaboripat *et al.*, 1992 and Chitaree *et al.*, 1993) and many essential oils and herbal plant extracts have antibiotic effects against both *A. flavus* and *F. verticilloides*, and significantly reduced fuminosins and aflatoxins production (Soliman and Badea, 2002; Ertas*et al.*, 2005; Kokoška *et al.*, 2005; Mohsenzadeh, 2007; Dambolena*et al.*, 2008, Alopsy, 2010 and Elsamra *et al.*, 2012a and 2012b).

Therefore, this study aimed to investigate the effect of feed additive mixtures and some medicinal plant oils mixtures on reducing growth and mycotoxins production by both *Fusariumverticilloides* and *Aspergillusflavus*.

Materials and methods:

1. Determination of mycotoxins production potentials of the tested isolates

Aspergillus flavus and *Fusarium verticilloides* isolated from samples 200 grains each of damaged corn grains were taken from four lots of grains imported from U.S.A. from October to December 2011. Isolates are purified, identified then identification was kindly verified in plant pathology section, Faculty of Agriculture, Alexandria University. Aflatoxins and fumonisins production capability in their cultures are verified. The Aflatoxins production capability of the tested isolate of *A. flavus* was determined using a qualitative method (Agar plug), recommended by Frisvad and Thrane (1995). Seven days

old culture of *A. flavus* was grown on YES medium and an agar plug was cut with the help of a flamed cork borer (inner Diameter approx. 0.4 cm) or a scalpel from the center of the colony.

The plug was removed with a flamed needle and the agar side directly touched to a thin layer chromatography (TLC plate), for extra cellular toxins, and the mycelial side of the plug was wetted with a drop of chloroform and methanol mixture (1: 9V\V), then the mycelial side was made to touch the TLC plate for a few seconds. The diameter of the application spot should not exceed more than 0.6 cm. The standard and the spot were put on pre-coated TLC silica gel plate (20 x 20 cm) according to Frisvad and Thrane(1995).

The spot was left to dry, then, the TLC plates were developed in a suitable solvent. The eluent TEF (toluene\ethyl acetate \ 90% formic acid 0mixture (5: 4: 1v \v \v) was used for *Aspergilli*aflatoxins (Frisvad and Thrane, 1995). Dry developed TLC plates were viewed in day light and under short wave 254 nm. UV light plate was sprayed with H_2SO_4 40 % (H_2SO_4 in water as color reagent). Plates were viewed before and after heat treatment.

The inoculated flasks were kept for 30 days at 25°C (Marin *et al.* 1999). Then, the fungal mat was removed and the filtrate was taken with the purpose of toxins determination. The filtrate was kept frozen at -4°C until determination (Marin *et al.*, 1999). The fungal mat was oven dried at 105°C for 16 hours; the mycelium weight was recorded before and after dryness. The mycelium dry weight was determined.

Fumonisins producing ability of the tested *F. verticilloides*, grown on PSA medium for seven days, was detected using a qualitative method (Agar plug), recommended by Frisvad and Thrane, 1995and Wilson *et al.*, 2004. The method was the same as previously mentioned.

2. **Determination of active components in the tested Herbal oils**

Thymol and carvacrol were determined in thyme oil, L-menthol in peppermint oil, Carvone and Lemonene in caraway oil, eugenol and thymol in clove oil and methyl chavicol and anethole in anise oil. Detection process was carried out using GC method recommended by Michail *et al.* (1994), Edris *et al.* (2003) and Aursa *et al.* (2006) in Regional Center for Food and Feed in Cairo. Furthermore, the main components ratios, as mentioned in (feed additive mixture analysis certificate according to micro-plus Co, Germany, was registered and the active components in each tested concentration was calculated according to the feed additive mixture analysis certificate.

3. Effects of feed additive mixtures (FAM), individual herbal oils and their mixture as fungal inhibitors

The effects of FAM, individual herbal oils and their mixture as natural fungal inhibitors were tested by using the macrobroth dilution method, recommended by Nguefack *et al.* (2004), where 25 µl of a suspension of each tested fungus containing 1 x 10^6 spores/ml were added to 250 ml Erlenmeyer flask, containing 25 ml of broth (YES or PS) according to tested fungus (*A.flavus* and/or *F. verticilloides*) with various concentrations of oils or mixtures (0.1, 0.5, 1% and 2% with or without sodium chloride addition to mimic the same composition of the two tested feed additives (CAM / PAM used for feeding cattle and poultry). *A. flavus* cultures were incubated without shaking at 30°C for 8 days and at 25°C for 12 days for *F. verticilloides* cultures.

4. Determination of Fungal dry weight

At the end of the incubation period, the fungal mat was kept and oven dried at 105°C for 16 hours according to Aboul Naga and Abou Okada (1979), the mycelium weight was recorded before and after dryness. The mycelium dry weight was determined.

5. Effect of the tested additive feed mixtures (FAM), herbal oils and their mixtures on mycotoxins formation

The effects of FAM, herbal oils and oils mixtures on aflatoxins and fumonisins biosynthesis were evaluated by using corn grain (*Zea mays*) as substrate. Each tested treatment concentrations was inserted on 100g purified sterilized maize 1 day before inoculation with a disks 4 mm in diameter were taken from the margin of the developed fungus on Yes or PSA medium at the eighth days of the desired fungus *A.flavus* and/or *F. verticilloides* (Casa *et al.*, 1998). All parameters were incubated for one month at the suitable temperature as mentioned above.

6. Detection of fumonisins and aflatoxins

The effect of the FAM, herbal oils and their mixtures with or without sodium chloride on fumonisin production was determined after 30 days of incubation at 25°C. For each concentration, three conical flasks 250 ml were taken and stored at -18°C until extraction was performed. The content of each sampled flasks was separately ground using a blender. The method used for fumonisins extraction and sample clean-up was carried out using Frisvad and Thrane method (1995) and fumonisins estimation was carried out using HPLC process according to Samapundo*et al.* (2006) in central lab at Faculty of Pharmacy, Alexandria University. After fumonisins extraction, the pH of the sample extracts was adjusted where necessary to values between 5.8 and 6.5 by o-phosphoric acid. After sample cleanup process was achieved, fumonisins

was then eluted from column by 1% glacial acetic acid in methanol. The mobile phase consisted of methanol and 0.1M sodium dihydrogen phosphate was mixed in the ratio 3:1, respectively. The pH of the solution was adjusted to 3.35 with o-phosphoric acid. The derivatising reagent was prepared by adding 5ml of 0.1 M sodium tetraborate and 50µl of 2-mercaptoethanol to 40mg of o-pthaldialdehyde (OPA) dissolved in 1ml of HPLC grade methanol. HPLC apparatus equipped with a fluorescence detector was used to detect fumonisins. The same sampling protocol as described for fumonisins was used for aflatoxins determination. HPLC apparatus equipped with a fluorescence detector was also used to detect aflatoxins.

Results and discussion:

Detection of aflatoxins and fumonisins production by *A .flavus* and *F.verticilloides* was performed using the plug agar technique recommended by Frisvad and Thrane, (1995) and Wilson *et al.* (2004). According to plug agar technique, spraying with specific colour reagent spray for each mycotoxin resulted in fluorescence violet and blue colors, characteristic of fumonisins andaflatoxins observed at their retention times. These findings confirmed the presence of fumonisins and aflatoxins in the tested cultures of *F. verticilloides* and *A.flavus* isolates.

1. Active components in the tested Herbal oils and the Feed additive mixtures (FAM):

The main active components in the tested treatments, presented in Table 1 showed that the main group in all the tested oils is composed of monocyclic terpenes such as limonenene and dicyclic terpenes such as α-pinene which occurred. Furthermore, phenolic compounds such as anethole in anise oil, thymol and carvacrol in thyme oil and eugenol and thymol in clove oil are the main components in the tested oils. In addition, dicyclic alcohol was higher in peppermint oil. These findings are relatively in agreement with Sulieman *etal.* (2007), Dambolena *et al.* (2008) and Kozlowski and Walentowska(2008).

Main active components in FAM as mentioned in analysis certificate from the origin country (Germany) for poultry and dairy cattle nutritional process were presented in Table (2), whereas the main components of the same FAM used as mycotoxins detoxifying agents were calculated according to the tested concentrations in Table (3). Active components in Tables (2 and 3) indicated that ratios of all the calculated active components applied for detoxification process were higher than those applied as nutritive agent, except for sodium chloride.

2. Effectiveness of feed additive mixtures (FAM), individual oils and their mixtures (OM) on inhibiting *F.verticilloides* and *A.flavus* growth:

The inhibitory effect of the tested FAM, individual oils and their mixtures on fungal growth of *F.verticilloides* and *A.flavus* was presented in Tables (4 and 5).

2.1. *F. verticilloides:*

Individual herbal oils in general are more effective in reducing fungal growth than their mixtures. Clove oil was the best at conc.0.5% (MIC 0.1%), followed by thyme oil with the same MIC, caraway oil at MIC 0.1%. then peppermint oil at MIC 0.1% and anise oil. These findings are closely in agreement with those of Kalemba and Kunicka (2003); Martinez-de Oliveira (2004); Leopold *et al.* (2006) and Reddy *et al.* (2010), who reported that among 22 studied essential oils, clove, thyme, mint, origanum and cinnamon were found to possess the strongest antagonistic effect. Furthermore, in case of FAM and OMs, PAM and its local alternative, OM_1 were more effective than CAM and OM_2 in inhibiting *F.verticilloides* growth, most probably, due to the essential oil components of each mixture and the synergistic effects among them. These findings are in harmony with those of Reddy *et al.* (2009), who reported that the synergistic interaction between different essential oils mixtures or between mixtures of oils depends on their individual components.

2.2. *A.flavus*:

In case of individual herbal oils, clove oil realized the best growth inhibition, followed by anise oil at MICs 2% and 0.5%, respectively. These results were in agreement with those of Kalemba and Kunicka (2003), Reddy *et al.* (2009), Gujar and Talwankar (2012) and Deabes *et al.* (2012), who reported that the synergistic interaction between the mixtures of oils and their individual components are too low to be of any practical importance.

The antifungal activity of the tested individual oils was ranked in the following order:

- ***F.verticilloides* fungal growth inhibition experiment:**
  Clove < Caraway <Thyme <Anise < Peppermint < Sodium chloride.
- ***A.flavus* fungal growth inhibition experiment:**
  Clove < Anise < Thyme < Caraway ≤ Peppermint < Sodium chloride.

These findings are highly coincided with those of Erdogan and Daniel (2007), Alopsy (2010) and Reddy *et al.* (2010).

According to results presented in Tables 4 and5, OM1 without addition sodium chloride at conc. 0.5% (MIC 0.1%) was more effective in reducing

fungal growth followed by PAM at 1% (MIC 0.1%). Furthermore, addition of sodium chloride at concentration 0.5% was more inhibitory. These findings were in agreement with those reported by Diyaolu and Adebajo (1994), Castells *et al.* (2009) and Youssef (2009), who found that addition of sodium chloride only at concentrations more than 1.7%, were more effective in fungal growth suppression. On the contrary, OM1 with sodium chloride was less effective than PAM at low concentrations (0.1% and 0.5%) due to, probably, the effect of sodium chloride on the reduction of synergistic effects among oil mixtures . These results were relatively in agreement with those of Kalemba and Kunicka (2003), reported that the mechanism of essential oil actions depends on their hydrophilic or lipophilic characters.

3. Effectiveness of feed additive mixtures (FAM), individual oils and their mixtures (OM) on suppressing the production of fumonisins by *F.verticilloides* and aflatoxins by *A.flavus*:

The effect of the tested FAM, individual oils and their mixtures on suppression the production of fumonisins by *F.verticilloides* and aflatoxins by *A.flavus* was presented in Tables 6 and 7.

3.1. Fumonisins:

The antifungal activity of the tested individual oils in suppressing fumonisins production was ranked in the following order: Clove = Sodium chloride < Thyme < Peppermint < Caraway < Anise. Clove oil completely inhibited fumonisins produced by *F.verticilloides.* This result was in harmony with those reported by Velluti *et al.* (2003), Velluti *et al.* (2004) and Leopold *et al.* (2006).

Table(1): Active components in the tested herbel oils determined by G.C.

Active components µg/ml	Clove oil	Dominance %	Thyme oil	Dominance %	Anise oil	Dominance %	Caraway oil	Dominance %	peppermint oil	Dominance %
α-Pinene	1.2097	0.0079	50.57	6.5062	6.1029	45.8248	0.9508	0.0958	1.9426	0.00923
Anethole					6.92	51.964	0.5000	0.0504		
Cinole					0.06	0.451				
Terpinol	0.1201	0.0008			0.235	1.7645				
Limonene	1.0477	0.0068	0.5	0.0643			5.8315	0.5877	0.7991	0.003796
Phellandrene									1.5968	0.00759
Menthol									20426*	97.0396
Benzyl Benzoate	0.4889	0.00318	0.20	0.0257					0.0706	0.00034
Thymol	1091.99*	7.1006	116.35*	14.9694						
Eugenol	14283.98*	92.881								
Carvacrol			609.6*	78.4300						
Camphene			0.034	0.00437						
Carvone							984.8937*	99.26	618.3031*	2.9374
Ox. comp.	15378.8	100.00					0.0601	0.0065		
Traces				——			——			——
Total	15378.84	100.00	777.254	99.99997	13.3179	100.00	992.2362	100.00	21049.1305	99.998

N.B. * = main component in the tested herbal oil

Ox. comp. = Oxygenic components.

Table (2): Composition of feed additive mixtures used as animal nutrients in this study according to Micro-plus Lab. Germany:

Feed mixture composition per 100g				Dose added for nutrition		Active component ratio in the diet	
For Poultry	Component ratio %	For Dairy cattle	Component ratio%	For Poultry	For Dairy cattle	Poultry Diet (per150g/10^6g)	Dairy cattle Diet(per2-3g)/animal
L-Menthol (peppermint)	8.00	L-Menthol (peppermint)	2.40	150g/1000Kg	2-3g /animal *daily	12×10^{-6}	0.048-0.072
Eugenol (clove)	2.00	Thymol (Thyme)	1.25	----	----	3×10^{-6}	0.025-0.0375
Anethole (Anise)	3.40	L-Carvon (Caraway)	0.98	----	----	5.1×10^{-6}	0.0196-0.0294
Sodium chloride	86.6	Sodium chloride	95.37	----	----	129.9×10^{-6}	1.9074-2.8611
Total	100%	Total	100%				

N.B. FAM= feed additive mixtures.

N.B=* Animal weight varied between 350 kg to 580Kg. These mixtures are added at the rate of 150 g per kg diet in the case of poultry, while in the case of dairy cattle the other structure was added with average of 2 grams to 3 grams per daily diet according to animal body weight, which ranges between 350 kg to 580 kg.

Table (3): Calculation of the main active components ratios in each feed additive mixtures (FAM).

FAM	Treatment	Main active components (mg/Kg)			
		L.menthole	Eugenol	Anethole	Sodium chloride
Poultry mixture (P.M)	0.1%	0.008	0.002	0.0034	0.086
	0.5%	0.04	0.01	0.017	0.43
	1.0%	0.08	0.02	0.034	0.86
	2.0%	0.16	0.04	0.068	1.72
FAM	**Treatment**	**Main active components (mg/Kg)**			
		L.menthole	Thymol	Carvon	Sodium chloride
Dairy cattle mixture (CA.M)	0.1%	0.0024	0.00125	0.00098	0.09537
	0.5%	0.012	0.00625	0.0049	0.47685
	1.0%	0.024	0.0125	0.0098	0.9537
	2.0%	0.045	0.025	0.0196	1.9074

Table (4): Effectiveness feed additive mixtures (FAM), of individual oils and their mixtures (OM) on inhibiting *F. verticilloides* growth.

FAM and OM				Individual oils			
Treatment	Conc. %	Average of Dry weight (g)	Growth inhibition ER%	Treatment	Conc. %	Average of Dry weight (g)	Growth inhibition ER%
F.v.control	0.0	2.27	0.0	F.v. control	0.0	2.27	0.0
Poultry Mixture (PM)	0.1	0.117	94.845	Thyme oil	0.1	1.120	50.66
	0.5	0.110	95.154		0.5**	0.400	82.370
	1**	0.04	98.238		1	0.574	74.18
	2	0.583	74.317		2	0.600	72.18
Oil mixture 1 (OM_1) without Sod. Chloride	0.1	0.0454	98.00	Peppermint oil	0.1	1.200	46.94
	0.5	0.0079	99.652		0.5**	1.000	55.597
	1**	1.365	39.868		1	1.203	46.75
	2	1.839	18.987		2	1.534	32.56
Oil mixture 1 (OM_1) with Sod. Chloride	0.1	1.890	16.740	Clove oil	0.1	0.159	92.995
	0.5	1.841	18.899		0.5**	0.0002	99.991
	1**	0.164	92.775		1	0.00	100.0
	2	0.138	93.921		2	0.00	100.0
Dairy Cattle mixture (CAM)	0.1	2.10	7.489	Caraway oil	0.1**	0.103	95.455
	0.5	1.007	55.639		0.5	1.107	95.300
	1**	1.48	37.007		1	1.204	46.790
	2	2.204	2.907		2	1.500	33.640
Oil mixture 2 (OM_2) without Sod. Chloride	0.1	0.868	61.762	Anise oil	0.1	1.617	28.930
	0.5	1.225	46.035		0.5	1.400	38.307
	1**	1.392	38.678		1**	1.000	55.845
	2	1.587	30.088		2	8.54	-2.782
Oil mixture 2 (OM_2) with Sod. Chloride	0.1	1.779	21.630				
	0.5	1.477	34.934				
	1**	2.180	3.965				
	2	5.084	-12.396				
PM + CAM	0.1	0.259	88.595	Sodium chloride	0.1	3.476	-53.128
	0.5	1.078	52.511		0.5	2.979	-31.233
	1**	0.633	72.115		1	2.199	03.128
	2	0.650	71.366		2**	1.419	37.489
OM_1 + OM_2	0.1	0.911	59.886				
	0.5	0.252	88.904				
	1**	0.788	65.302				
	2	3.490	-53.677				
L.S.D.$_{0.05}$ for conc.	0.1	0.06395		L.S.D.$_{0.05}$ for conc	0.1	0.1581	
	0.5	0.06555			0.5	0.1164	
	1**	0.1304			1**	0.26499	
	2	0.2916			2	0.44223	

(**=MIC (minimum inhibitory)concentration).

Table (5): Effectiveness of feed additive mixtures (FAM), individual oils and their mixture (OM) on inhibiting *A.flavus* growth.

FAM and O.M				Individual oils			
Treatment	Conc. %	Average of Dry weight (g)	Growth inhibition ER%	Treatment	Conc. %	Average of Dry weight (g)	Growth inhibition ER%
A.fcontrol	0.0	0.54	0.0	A.f.control	0.0	0.54	0.0
Poultry Mixture (PM)	0.1	1.10	-103.70	Thyme oil	0.1**	0.45	16.67
	0.5	0.76	-40.74		0.5	0.46	-14.81
	1	0.74	-37.04		1	0.73	-35.18
	2**	0.40	25.925		2	0.57	-5.556
Oil mixture 1 (OM_1) without sod. chloride	0.1**	0.52	3.704	Peppermint oil	0.1**	0.47	12.960
	0.5	0.61	-12.963		0.5	0.63	-16.67
	1	0.66	-22.223		1	1.14	-111.12
	2	1.65	-205.556		2	0.90	-66.67
Oil mixture 1 (OM_1) with sod. chloride	0.1	0.60	-87.037	Clove oil	0.1	0.61	-12.963
	0.5	1.01	-85.185		0.5	0.46	14.815
	1	1.00	-11.11		1	0.45	16.67
	2**	0.43	20.370		2**	0.21	61.12
Dairy Cattle (CAM)	0.1	0.72	-33.334	Caraway oil	0.1**	0.47	12.963
	0.5	0.44	18.52		0.5	0.51	5.556
	1	0.40	25.92		1	0.54	0.000
	2**	0.33	38.88		2	0.54	0.000
Oil mixture 2 (OM_2) without sod. chloride	0.1**	0.11	1.852	Anise oil	0.1	0.38	29.629
	0.5	0.53	-68.518		0.5**	0.29	46.296
	1	0.91	-68.421		1	0.38	29.629
	2	1.71	-217		2	0.50	7.410
Oil mixture 2 (OM_2) with sod. chloride	0.1	1.42	-162.963				
	0.5	0.93	-72.223				
	1	0.62	-14.815				
	2**	0.51	5.556				
OM_1 + OM_2	0.1**	0.11	79.630	Sodium chloride	0.1	0.841	-55.741
	0.5	0.53	1.852		0.5	0.716	-32.592
	1	0.91	-68.519		1	0.545	-0.926
	2	1.71	-216.667		2**	0.488	9.6296
PM + CAM	0.1**	0.52	3.704				
	0.5	0.61	-12.963				
	1	0.66	-22.223				
	2	1.65	-205.556				
L.S.D.$_{0.05}$ for conc.	0.1	0.10522		L.S.D.$_{0.05}$ for conc	0.1	0.04672	
	0.5	0.0966			0.5	0.05131	
	1	0.1978			1	0.05001	
	2	0.3884			2	0.04202	

(**=MIC (minimum inhibitory concentration).

Tables (6): Efficacy ratios(ER %) of the tested mixtures and their individual oils on fumonisins (Fums) inhibition.

FAM and OM				Individual oils			
Treatment	Conc. %	Average FUMS. (ppm)	FUMS. inhibition ER%	Treatment	Conc. %	Average FUMS. (ppm)	FUMS. inhibition ER%
Control + Maize	0.0	49.432	0.0	Control + Maize	0.0	2.27	0.0
Poultry Mixture (PM)	0.1	37.366	24.409	Thyme oil	0.1	33.138	33.138
	0.5	36.714	25.728		0.5**	35.230	35.230
	1**	36.713	25.730		1	33.624	33.624
	2	36.850	25.453		2	30.381	30.381
Oil mixture 1 (OM_1) without Sod. Chloride	0.1**	48.882	01.113	Peppermint oil	0.1	22.216	22.216
	0.5	48.962	0.9508		0.5**	24.421	24.421
	1	50.847	-2.8625		1	25.271	25.271
	2	51.467	-4.1168		2	25.494	25.494
Oil mixture 1 (OM_1) with Sod. Chloride	0.1	49.007	0.8598	Clove oil	0.1	92.505	92.505
	0.5**	48.962	0.9508		0.5**	100.0	100.0
	1	50.802	-2.7715		1	100.0	100.0
	2	51.852	-4.8956		2	100.0	100.0
Dairy Cattle mixture (CAM)	0.1	38.220	22.682	Caraway oil	0.1**	23.023	23.023
	0.5	37.881	23.367		0.5	24.134	24.134
	1	37.775	23.582		1	25.006	25.006
	2**	37.156	24.834		2	24.440	24.440
Oil mixture 2 (OM_2) without Sod. Chloride	0.1	46.902	05.118	Anise oil	0.1	04.845	04.845
	0.5**	49.097	06.777		0.5	04.268	04.268
	1	59.632	-20.634		1**	02.498	02.498
	2	76.987	-55.743		2	01.590	01.590
Oil mixture 2 (OM_2) with Sod. Chloride	0.1**	47.102	4.7135	Sodium chloride	0.1	-17.743	-17.743
	0.5	49.636	-0.4127		0.5	46.8199	46.8199
	1	49.722	-0.5867		1	80.0655	80.0655
	2	74.222	-50.149		2**	100.00	100.00
L.S.D.$_{0.05}$ for conc. %	0.1	3.67767		L.S.D.$_{0.05}$ for conc %	0.1	0.2231	
	0.5	0.59391			0.5	0.0989	
	1**	0.80114			1**	0.1386	
	2	0.56755			2	0.64025	

(**=MIC (minimum inhibitory concentration).

Table (7): Efficacy ratios(ER %) of the tested mixtures and their individual oils on aflatoxins (Afla) inhibition.

FAM and OM				Individual oils			
Treatment	Conc. %	Average Aflatoxin (ppb)	Afla inhibition ER%	Treatment	Conc. %	Average Aflatoxin (ppb)	Afla inhibition ER%
Control + Maize	0.0	49. 203	-	Control + Maize	0.0	49.203	0.0
Poultry Mixture (PM)	0.1	41.042	16.586	Thyme oil	0.1**	18.419	62.565
	0.5	38.855	21.031		0.5	18.433	62.537
	1	31.214	36.391		1	19.159	61.061
	2**	18.449	62.504		2	20.575	58.183
Oil mixture 1 (OM_1) without Sod. Chloride	0.1**	18.276	62.856	Peppermint oil	0.1	39.719	19.275
	0.5	18.506	62.388		0.5	39.474	19.773
	1	27.689	43.725		1**	38.809	21.125
	2	53.094	-07.908		2	38.914	20.911
Oil mixture 1 (OM_1) with 0Sod. Chloride	0.1**	18.969	61.447	Clove oil	0.1	50.549	-02.736
	0.5	20.609	58.114		0.5	20.292	58.759
	1	37.390	24.009		1	19.623	60.118
	2	43.419	11.755		2**	18.406	62.592
Dairy Cattle mixture (CAM)	0.1	42.886	12.839	Caraway oil	0.1	44.404	09.753
	0.5	19.766	59.828		0.5	44.066	10.440
	1	19.638	60.088		1**	38.879	20.982
	2**	18.839	61.712		2	40.773	17.133
Oil mixture 2 (OM_2) without Sod. Chloride	0.1**	18.599	62.199	Anise oil	0.1	39.813	19.084
	0.5	33.869	31.165		0.5	22.509	54.253
	1	39.159	20.413		1	19.979	59.395
	2	41.734	15.180		2**	19.263	60.850
Oil mixture 2 (OM_2) with Sod. Chloride	0.1**	20.854	57.616	Sodium chloride	0;1	52.823	-7.3573
	0.5	38.774	21.196		0.5	27.891	43.314
	1	39.509	19.702		1	16.406	66.656
	2	45.149	08.239		2**	5.3239	89.180
$L.S.D._{0.05}$ for conc. %	0.1	1.910728		$L.S.D._{0.05}$ for conc%	0.1	2.2361	
	0.5	1.914198			0.5	2.04291	
	1	1.751211			1	1.81236	
	2	1.792422			2	1.81206	

(**=MIC (minimum inhibitory concentration).

FOAM were more effective in reducing fumonisins than OMs, which may due to the occurrence of some forms of contradiction forces among OMs essential oils. Furthermore, FAM can be sometime acting as individual herbal oil due to its content of active components which occurred approximately in equilibrium form, whereas this form of equilibrium was broken in case of certain OMs because of the occurrence of small ingredients or traces, which may hinder or support the work of essential components according to their structures and types. These explanations agreed with those of Reddy *et al.* (2010). On the other hand, synergistic effects among oils of OM_2 were very strong, compared antagonistic effects occurred among oils of OM1 which may explain their behavior in reducing mycotoxins. These findings were in great simulator with those of Mert and Ekmekçi (1987), Akgü l*et al.*(1988) and Youssef (2009), who found that there were disadvantageous antagonistic effects weakening the essential oil action as compared with their constituents (Table 1, 2 and 3).

3.2. Aflatoxins:

The antifungal activity of the tested individual oils in reducing aflatoxins production was ranked in the following order: Sodium chloride < clove <thyme < anise < peppermint < caraway. Clove oil has showed a maximum antifungal activity against both of the tested fungi. These coincide with those of Mert and Ekmekçi. (1987), Sulieman *et al.* (2007), Dambolena *et al.* (2008) and Zyani *et al.* (2011). In addition, it was noticed that those essential oils of peppermint; caraway and anise had moderate effect against these fungi. The present data were highly coincided with those of Chitaree*et al.* (1993), Mohsenzadeh (2007), El Nekeety*etal* (2011) and El-Ha0bib (2012). Moreover, data of the antifungal effect of thyme oil were similar to those of El Nekeety*et al.* (2011) and Sumalan *et al.* (2013).

Results presented in Table 4,5 ,6 and 7 showed also both of feed additives (PAM and CAM) and oils mixtures (OM_1 and OM_2) were more effective in reducing fungal growth than mycotoxin production in case of *F.verticilloides*, whereas the contrary was observed in case of *A. flavus*. These were in line with those of Le Bars *et al.* (1994), who mentioned that mycotoxin production was inversely proportional to fungal growth.

OM_1 and OM_2 with or/and sodium chloride are less effective against fumonisin producion by *F.verticilloides* than FAM, whereas they exerted the same effectiveness against aflatoxin production by *A.flavus*. Similar conclusions were recorded by Kalemba and Kunicka (2003), who reported that oil mixture exhibited its dependence on fungal species and oil mixture structure. The combined use of oils mixture and sodium chloride exhibited a

synergistic antifungal effect in case of *A. flavus* only. Furthermore, strong synergism between essential oils and food additives components may lead to using smaller amounts of flavored food preservatives. On the other hand, our results may explain that the addition of substa0nces individually almost achieved the best results. This explanation was in line with that recommended by Reddy *et al.* (2009).

Conclusion:

The addition of feed additives mixtures (FAM) at the tested percentages can be used as detoxifying agent beside its initial role as nutritive agent. The attempt of using oil mixtures (OM_1 and OM_2) as alternatives to FAM was failed in inhibiting fumonisins production; however it can be considered a successful alternative to FAM in reducing aflatoxins production and *F.verticilloides* growth. The addition of medicinal oil to maize grains individually realized the best results in reducing fungal growth and mycotoxin production. Sodium chloride can be added to feed at 2% as a cheap detoxifying agent beside its nutritive role as source of sodium and chloride. Further nutritive studies must be applied to accomplish the nutritive value of the succeeded treatment ratios which reduce fungal growth and mycotoxin production.

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الملخص العربي

دور بعض المواد النباتية وبعض مخاليط اضافات الأعلاف كمثبطات لنمو فطريات العفن وما تنتجه من سموم فطرية فى حبوب الذرة المصابة

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١-المركز الاقليمي للاغذية والاعلاف مركز البحوث الزراعية-الاسكندرية

٢- المركز الاقليمي للاغذية والاعلاف مركز البحوث الزراعية-القاهرة

يعتبر محصول الذرة الصفراء من أهم محاصيل الحبوب في مصر والعالم ويمثل تلوث الاغذية والاعلاف خطرا بالغا يهدد صحة الانسان والحيوان ويهدد الاقتصاد بسبب زيادةنسبة الفقد نتيجة التلف الناتج عن الاصابة للفطرية وانتاج السموم الفطرية. وتستلزم عملية تقليل نسبة التلوث بالسموم الفطرية و المحافظة علي سلامة وجودة الحبوب اثناء التخزين استراتيجية خاصة تتطلب الامان الصحي للانسان والحيوان وسهولة تطبيق هذه الاستراتيجية علي نطاق تجارى بالاضافة الي رخص تكلفة المعاملات .

تمهيد:

لقد زادت في الآونة الأخيرة أهمية إستخدام موادا طبيعية آمنة صحيا مثل استخدام الزيوت النباتية كبدائل لمثبطات الأعفان و إضافة مخاليط الزيوت النباتية كبدائل للمضادات الحيوية و رفع القيمة التغذوية لعلائق الدواجن والماشية.

هدف البحث:

اختبار كفاءة بعض إضافات الأعلاف (FAM) المستوردة من الخارج (بغرض رفع القيمة التغذوية لعلائق الدواجن والماشية) كمثبطات نمو للفطرين اسبرجلس فلافس و فيوزاريوم فيرتسيلويدس والسموم الفطرية الافلاتوكسين والفيومنسين المنتجة فى حبوب الذرة الصفراء الواردة من الخارج. كما تهدف الدراسة الي عمل مخاليط زيوت نباتية محلية تحاكي المستوردة واستخدامها كمثبطات للنمو الفطري وانتاج التوكسينات ومقارنة مدي فاعليتها كمخاليط وكزيوت مضافة فرادي مع اضافات الاعلاف المستوردة وذلك في محاولة لتوفير النقد الاجنبى . وتشتمل الدراسة ايضا علي دراسة تأثير كلوريد الصوديوم المضاف فردياً او كمكون من مكونات المخاليط المستوردة او المحلية وذلك في محاولة للوصول الى طريقة آمنة صحيا و زهيدة التكاليف وسهلة التطبيق فى آن واحد .

نتائج الدراسة:

أظهرت الاختبارات الأولية أن العزلات المختبرة من الاسبرجلس فلافس والفيوزاريوم فيرتسيلويدس كانت قادرة على إنتاج السموم الفطرية الافلاتوكسين و الفيومنسين. وعلاوة على ذلك، تم العثور على مركبات نشطة فى المستخلصات النباتية العشبية مثل تريين أحادي الحلقة كال-(الليمونين) وتريين ثنائي الحلقة (α-بينين). الى جانب ذلك، أيضا تم الكشف عن المكونات النشطة الأخرى، على سبيل المثال anisole في زيت اليانسون، والثيمول و الكارفاكرولI في زيت الزعتر، الأوجينول والثيمول في زيت القرنفل والنعناع في الكحول الحلقية الأليفاتية.

المخاليط المضافة للاعلاف بغرض تغذوى (FAM)، وتستخدم لتربية الدواجن (PAM) والماشية (CAM) تم اختبار فاعليتها لإزالة السموم وتأثيرها ضد العزلات المختبرة. وكانت الزيوت العشبية الفردية أكثر فعالية في الحد من نمو الفطريات عن مخاليطها. تم الحصول على التأثير الأكثر تثبيطا ضد *F.verticilloides* من زيت القرنفل، يليه زيت الزعتر وزيت الكراوية والنعناع. وكانت الخلطات من الزيوت المحلية بدون كلوريد الصوديوم مضاف أكثر فعالية في الحد من نمو الفطريات، تليها اضافات مخاليط مضافة تغذية الدواجن المضافة ومخاليط محلية البديلة OM_1 كانت أكثر فعالية في الحد من نمو الإنتاج من السموم بواسطة

F.verticilloides، في حين لوحظ العكس في *A.flavus*. مقارنة مع الزيوت الأخرى، زيت القرنفل كان أكثر كفاءة في خفض الفيومنسين، في حين كان كلوريد الصوديوم أكثر فعالية في الحد من الأفلاتوكسين.

فى حالةالنمو الفطرى للفطر فيوزاريوم فيرتسيلويدس كان ترتيب الزيوت النباتية المختبرة من حيث الكفاءة كالتالى: القرنفل > الكراوية>الزعتر>الينسون>النعناع > كلوريد الصوديوم بينما كانت كفاءة هذه الزيوت فى خفض الفيومنسين كالتالى :القرنفل = كلوريد الصوديوم >الزعتر>النعناع > الكراوية >الينسون. أما فى حالةالنمو الفطرى للفطر أسبرجلس فلافس كان ترتيب الزيوت النباتية المختبرة من حيث الكفاءة كالتالى: القرنفل > الينسون >الزعتر> الكراوية ≤ النعناع > كلوريد الصوديوم +النعناع و بلغت كفاءة هذه الزيوت فى خفض انتاج الافلاتوكسين كالتالى: كلوريد الصوديوم > القرنفل>الزعتر> الينسون>النعناع > الكراوية .

أظهر كلوريد الصوديوم فاعلية كبيرة تساوت مع فاعلية زيت القرنفل فى خفض انتاج الفيومنسين. أظهرت اضافات الاعلاف (PAM)والمستخدمة في علائق للدواجن فاعلية كبيرة فى تثبيط النمو الفطري للفطرين فيوزاريوم فيرتسيلويدس و اسبرجلس فلافس حيث بلغت ٩٨,٢٤% و٩٠، ٢٥% ، علي التوالى، فى حين بلغت نسبة خفضه لانتاج الفيومنسين والافلاتوكسين ٢٥.٣٧% و٦٢.٥٠%.على صعيد اخر كانت كفاءة اضافات الاعلاف (CAM) و المستخدمة في علائق ماشية اللبن اعلى في تثبيط النمو الفطري للفطر فيوزاريوم فيرتسيلويدس (٥٥%) عن الفطر اسبرجلس فلافس (٣٨.٨٨%) بينما بلغت نسبة خفضه لانتاج الفيومنسين (٢٤.٨٣%) و الافلاتوكسين (٦١.٧١٢%).

تأثرت فاعلية مخاليط الزيوت المحلية باضافة ملح كلوريد الصوديوم تأثرا ايجابيا فى حالة تثبيط النموالفطري للفطر اسبرجلس فلافس حيث ارتفعت نسبة الكفاءة من (٣.٧٠٤ %) الي (٢٠.٣٧%) في حالة المخلوط OM1 و المحاكى لل PAM بينما ارتفعت من (١.٨٥٢%) الي (٥.٥٣٦%) فى حالة المخلوط OM2 و المحاكى لل CAM ولكن هذا التاثير الايجابى كان اقل من تأثير كل من PM و CAM على حدة بينما كانت فاعلية و تأثير هذه المخاليط المحاكية تقريبا متساوية مع اضافات الاعلاف المستوردةFAM) فى حالة خفضهم لانتاج الافلاتوكسين مما يعنى امكانية نجاحهم كبدائل محلية للFAM فى خفض الافلاتوكسين.