ANTIFUNGAL EFFECT OF SOME ESSENTIAL OILS AGAINST DIFFERENT FUNGI ISOLATED FROM BROILER FEED MIXTURES

YOUSEF, NAHED S.[1], HALA M. GAMAL EL–DEEN[1] AND REHAN A. ABDEL- MONSEF[2]

[1] *Faculty of Home Economics, Al-Azhar University, Tanta.*
[2] *Centeral Laboratory for Agricultural Climate, Agric. Res. Center, Ministry of Agric. and Land Recl., Egypt.*

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Abstract

Heat sterilized essential oils (EO), peppermint (*Mentha piperita*), lemon (*Melissa officinalis*), clove (*Syzgium aromaticum*), olive (*Olea europaea*), garlic (*Allium sativum*) and black seed (*Nigella sativa*) at 1, 2 and 3 % of the fungal medium were tested for their antifungal activity towards six isolates recovered from broiler feed mixtures. The fungal isolates were identified microscopically as *Mucor* sp., *Fusarium* sp., *Aspergillus ochraceus, Aspergillus terrus, Aspergillus niger,* and *Aspergillus fumigatus.* The sterilized clove oil exerted the most potent antifungal activity among the other tested essential oils. Complete fungal inhibition of sterilized clove oils was detected at 2% while strong inhibition was found at 1 %. The antifungal activity of heat sterilized and unsterilized clove oils was evaluated at concentrations of 0.1, 0.3, 0.5, 0.7 and 1.0 % of the fungal medium used. It was found that 0.7 % of unsterilized clove oil showed the strongest fungal inhibition.

Key words: Antifungal, Spoilage fungi, Essential oils, Mycotoxins.

INTRODUCTION

Mold spoilage is a great problem facing feed quality and storage. Fungal growth minimizes nutritional value and may result in the production of mycotoxins. Some species of *Aspergillus* and *Fusarium* are the most important mycotoxin-producing fungi (Betina, 1989). Among the different mycotoxins produced, Aflatoxins and Ochratoxins are produced by *Aspergillus ochraceus.* Fumagillin is produced by *Asp. fumigatus*, Citreoviridin is produced by *Asp. Terreus.* Fumonisins, Zearalenone and Trichothecenes are produced by *Fusarium* spp. (Young *et al.*, 1984). Morover, Dicoumerol is produced by *Mucor* spp. (Wohlers, 2002).

The stability of some foods against microbial attack is due to the fact that they contain naturally occurring substances with antimicrobial activity. Some spices are known to contain essential oils that possess antimicrobial activity, such as menthol in peppermint, citral in lemon, eugenol in cloves, polyphenol antioxidant in olive, diallyl sulphides (allicin) in garlic, and thymoquinone in black cumin (Menounos *et al.*, 1986; Galli and Visioli, 1999 and Tsao and Yin, 2001).

Investigations into the antimicrobial activities, mode of action and potential uses of plant volatile oils have regained momentum. There appears to be a revival in the use of traditional approaches for protecting livestock and food from diseases, pests and spoilage in industrial countries. This is especially true with regard to plant volatile oils and their antimicrobial evaluation, as can be seen from the comprehensive range of organisms against which volatile oils have been tested. These have included food spoiling organisms and food poisoning organisms, as well as spoiling and mycotoxigenic filamentous fungi (Lis-Balchin and Deans, 1997).

Therefore, the aim of the present study was to evaluate the antifungal activity of different types of essential oils against a number of spoilage fungi isolated from some feed components.

MATERIALS AND METHODS

Broiler chicken diets: starter, grower and finisher diets of broiler chickens were prepared and taken as a substrate for isolation of all spoilage fungi present. The ingredients of prepared broiler chicken diets are shown in Table 1 as recommended by NRC (1994).

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

Ingredients	Starter	Grower	Finisher
Yellow corn %	63.24	69.93	75.67
Soybean meal 44%	15.15	8.67	4.47
Corn gluten meal 62%	18.30	16.66	15.42
Dicalcium phosphate %	1.80	1.40	1.70
Limestone %	1.14	1.47	1.17
Salt %	0.30	0.30	0.30
Vitamins and mineral*	0.30	0.30	0.30
DL- Methionin %	0.02	0.50	0.10
L-Lycine %	0.40	0.47	0.51
Total	100	100	100
Calculated analysis:			
Energy K cal ME/kg	3124.1	3196	3234
Protein %	22.900	20.500	18.095
Crude fat %	3.020	3.290	3.153
Fiber %	2.92	2.39	2.57
Calcium %	0.90	0.93	0.88
Available phosphorus %	0.46	0.37	0.42
L. Lysine %	0.80	0.87	1.19
Methionine + cystine %	0.95	0.87	1.31

* Supplied per Kg of diets: vitamin A, 12000 IU, vitamin D_3, 2200 IU, vitamin E, 10 mg, vitamin K_3, 2 mg, vitamin B_1, 1 mg, vitamin B_2, 4 mg, vitamin B_6, 1.5 mg, vitamin B_{12}, 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, iodin, 1 mg, selenium, 0.1 mg, cobalt, 0.1 mg.

Essential oils: peppermint, lemon, clove, olive, garlic and black cumin were collected from local market.

Isolation of spoilage fungi: one-gram samples of broiler feed were mixed with 9 ml sterile distilled water and a series of dilutions were made. Aliquots of one ml of these dilutions were pipetted in sterile Petri-dishes filled with PDA medium. Petri-dishes were incubated at 28°C for 3-4 days. Developed fungi were counted and pure fungal isolates were obtained after repeated subculturing.

Identification of fungi : slide culture was prepared with PDA as described by Haley and Calloway (1978). Cultures were incubated at 25°C. Microscopic examination of the slide cultures was performed using bright field and phase microscopy (40 and 100 X objectives).

Antifungal activity : the anti-fungal activity of the essential oils was tested against the isolated fungal cultures at concentrations of 1, 2 and 3 %. Essential oils at these tested concentrations were added to PDA medium, then the mixture was autoclaved at 121°C for 15 minutes. PDA medium mixed with essential oils was poured in sterilized Petri-dishes and oil free-PDA was used as a control. In order to evaluate the effect of heat on the antifungal activity, a second experiment with clove oil at concentrations of 0.1, 0.3, 0.5, 0.7 and 1.0 % was performed. Clove oil was added to PDA either before or after sterilization of the medium. The center of each Petri-dish was inoculated with 5 mm disc cut from periphery of a 5-day old fungal culture grown on PDA. Plates were incubated at 28°C till control plates showed full growth (9 cm), then colony diameter was measured. Data collected was the mean of four plates (Chaudhri and Sen, 1982).

Statistical analysis: statistical analysis was computerized by statistical program SAS (1988). Duncan's multiple range test was used to detect significant differences between treatments.

RESULTS AND DISCUSSION

The total fungal counts of the different feed samples were 3.2×10^4, 5.4×10^4 and 5.5×10^5 CFU/g for starter, grower and finisher feed diets, respectively. It was noticed that increasing the yellow corn in chicken broiler diets (as shown in Table 1) stimulated fungal propagation. Based on morphology features, the molds isolated from feed diets were identified as *Aspergillus*, *Mucor* and *Fusarium* species. The *Aspergillus* spp. were further identified to be *Asp. ochraceus*, *Asp. terrus*, *Asp. niger* and *Asp. fumigatus*. These species are common contaminants of food products (Sherf and

Macnab, 1986). *Aspergillus niger* and *Asp. flavus* were reported to be the most common fungi causing spoilage of the bakery products (Guynot *et al.,* 2003). Moreover, it has been reported that *Aspergillus, Penicillium* and *Fusarium* are among the most important mycotoxin-producing fungal genera (Betina, 1989) and most recently *Mucor* spp. has been reported to be a mycotoxin-producer (Wohlers, 2002). The possibility of using some essential oils to suppress this fungal contamination worth to be considered.

Data presented in Tables 2-7 show the antifungal effect of the sterilized tested essential oils. They showed different antifungal activity. Peppermint oil exhibited positive antifungal activity against *Asp. ochraceus* and *Asp. niger* at the three tested concentrations of the essential oils. However, at the 3%, the growth of all tested fungi was inhibited (Table 2). Lemon oil showed positive antifungal activity against all the tested *Aspergillus* spp. in addition to *Fusarium* spp., while *Mucor* sp. was only affected at 3 % of the tested oil (Table 3). Sterilized clove oil exerted complete inhibition of *Mucor* sp. and *Asp. fumigatus* at 1 % concentration and at 2 % concentration all tested fungi were entirely inhibited (Table 4). Sterilized olive, garlic and black cumin oils have moderate antifungal effects on the tested *Aspergillus* spp. These oils, on the other hand, showed very weak antifungal effect on both *Mucor* sp. and *Fusarium* sp. (Tables 5, 6 and 7).

Table 2. Effect of sterilized peppermint oil on mycelial growth (cm) of isolated fungi

Isolated fungi	Oil concentration (%)				Overall
	0	1	2	3	
Mucor sp.	9.00 ± 0.086	9.00 ± 0.086	8.25 ± 0.086	2.73 ± 0.086	7. 244 ± 0.043
Fusarium sp.	9.00 ± 0.086	3.90 ± 0.086	2.60 ± 0.086	1.00 ± 0.086	4.125 ± 0.043
Aspergillus ochraceus	9.00 ± 0.086	4.48 ± 0.086	3.93 ± 0.086	3.50 ± 0.086	5.225 ± 0.043
Aspergillus terrus	9.00 ± 0.086	6.53 ± 0.086	4.43 ± 0.086	3.08 ± 0.086	5.756 ± 0.043
Aspergillus niger	9.00 ± 0.086	3.83 ± 0.086	3.40 ± 0.086	3.33 ± 0.086	4.888 ± 0.043
Aspergillus fumigatus	9.00 ± 0.086	6.00 ± 0.086	3.48 ± 0.086	2.93 ± 0.086	5.35 ± 0.043

Table 3. Evaluation of antifungal activity of sterilized lemon oil against mycelial growth (cm) of isolated fungi

Isolated fungi	Oil concentration (%)				Overall
	0	1	2	3	
Mucor sp.	9.00 ± 0.086	9.00 ± 0.086	8.25 ± 0.086	2.73 ± 0.086	7.244 ± 0.043
Fusarium sp.	9.00 ± 0.086	5.90 ± 0.086	4.35 ± 0.086	2.25 ± 0.086	5.375 ± 0.043
Aspergillus ochraceus	9.00 ± 0.086	3.90 ± 0.086	2.55 ± 0.086	0.50 ± 0.086	3.988 ± 0.043
Aspergillus terrus	9.00 ± 0.086	5.70 ± 0.086	2.35 ± 0.086	0.50 ± 0.086	4.388 ± 0.043
Aspergillus niger	9.00 ± 0.086	3.73 ± 0.086	3.15 ± 0.086	0.50 ± 0.086	4.093 ± 0.043
Aspergillus fumigatus	9.00 ± 0.086	5.00 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	3.75 ± 0.043

Table 4. Mycelial growth (cm) of isolated fungi affected by sterilized clove oil

Isolated fungi	Oil concentrations (%)				Overall
	0	1	2	3	
Mucor sp.	9.00 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	2.625 ± 0.043
Fusarium sp.	9.00 ± 0.086	0.73 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	2.681 ± 0.043
Aspergillus ochraceus	9.00 ± 0.086	2.25 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	3.063 ± 0.043
Aspergillus terrus	9.00 ± 0.086	0.70 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	2.625 ± 0.043
Aspergillus niger	9.00 ± 0.086	0.80 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	2.700 ± 0.043
Aspergillus fumigatus	9.00 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	0.50 ± 0.086	2.625 ± 0.043

Table 5. Evaluation of antifungal activity of sterilized olive oil on mycelial growth (cm) of isolated fungi

Isolated fungi	Oil concentration (%)				Overall
	0	1	2	3	
Mucor sp.	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.000 ± 0.043
Fusarium sp.	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.000 ± 0.043
Aspergillus ochraceus	9.00 ± 0.086	5.55 ± 0.086	5.53 ± 0.086	5.50 ± 0.086	6.394 ± 0.043
Aspergillus terrus	9.00 ± 0.086	6.40 ± 0.086	6.38 ± 0.086	6.43 ± 0.086	7.050 ± 0.043
Aspergillus niger	9.00 ± 0.086	4.85 ± 0.086	4.80 ± 0.086	4.55 ± 0.086	5.800 ± 0.043
Aspergillus fumigatus	9.00 ± 0.086	8.00 ± 0.086	8.00 ± 0.086	7.85 ± 0.086	8.213 ± 0.043

Table 6. Evaluation of antifungal activity of sterilized garlic oil against mycelial growth (cm) of isolated fungi.

Isolated fungi	Oil concentration (%)				Overall
	0	1	2	3	
Mucor sp.	9.00 ± 0.086	9.00 ± 0.086	9.0 ± 0.086	9.00 ± 0.086	9.000 ± 0.043
Fusarium sp.	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.000 ± 0.043
Aspergillus ochraceus	9.00 ± 0.086	5.450 ± 0.086	5.40 ± 0.086	4.98 ± 0.086	6.206 ± 0.043
Aspergillus terrus	9.00 ± 0.086	6.10 ± 0.086	6.00 ± 0.086	5.98 ± 0.086	6.769 ± 0.043
Aspergillus niger	9.00 ± 0.086	4.83 ± 0.086	4.80 ± 0.086	4.75 ± 0.086	5.844 ± 0.043
Aspergillus fumigatus	9.00 ± 0.086	7.70 ± 0.086	7.70 ± 0.086	7.70 ± 0.086	8.025 ± 0.043

Table 7. Effect of sterilized black cumin oil on mycelial growth (cm) of isolated fungi

Isolated fungi	Oil concentration (%)				Overall
	0	1	2	3	
Mucor sp.	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.00 ± 0.086	9.000 ± 0.043
Fusarium sp.	9.00 ± 0.086	8.58 ± 0.086	8.43 ± 0.086	8.20 ± 0.086	8.55 ± 0.043
Aspergillus ochraceus	9.00 ± 0.086	5.20 ± 0.086	5.00 ± 0.086	5.00 ± 0.086	6.056 ± 0.043
Aspergillus terrus	9.00 ± 0.086	5.88 ± 0.086	5.80 ± 0.086	5.75 ± 0.086	6.606 ± 0.043
Aspergillus niger	9.00 ± 0.086	6.00 ± 0.086	3.48 ± 0.086	2.93 ± 0.086	5.219 ± 0.043
Aspergillus fumigatus	9.00 ± 0.086	7.85 ± 0.086	7.20 ± 0.086	7.20 ± 0.086	7.813 ± 0.043

Table 8 shows the statistical analysis of the last six tables. It is clear from the table that the various essential oils at their tested concentrations exerted significantly different antifungal effects on the diverse fungal cultures.

Mau *et al.* (2001) in their studies proved that after heating the antimicrobial activity of the mixed extracts produced from cinnamon, corni fructose and Chinese chive remained stable or was even slightly enhanced. The authors mentioned that this enhanced effect might be due to the low concentration of the mixed extract as a result of evaporation.

Table 8. Means colony diameter (cm) of isolated fungi as affected by different essential oils and different concentrations.

Fungi	Means*	Oils	Means*	Conc. (%)	Means*
Mucor sp.	7.352^{A}	peppermint	5.431^{D}	0	9.0^{A}
Fusarium sp.	6.727^{B}	Lemon	4.806^{E}	1	5.501^{B}
Asp. ochraeus	4.972^{E}	Clove	2.728^{F}	2	4.834^{C}
Asp. terrus	5.452^{D}	Olive	7.576^{A}	3	4.147^{D}
Asp. niger	4.757^{F}	Garlic	7.474^{B}		
Asp. fumigatus	5.963^{C}	Black cumin	6.207^{C}		

* Data in the column followed by different letters are significantly different ($P < 0.01$).

Table 9. Evaluation of antifungal activity of sterilized and unsterilized clove oil against mycelial growth (cm) of isolated fungi

Isolated fungi	Sterilized clove oil (%)					Overall[1]	Unsterilized clove oil (%)					Overall[1]
	0.1	0.3	0.5	0.7	1.0		0.1	0.3	0.5	0.7	1.0	
Mucor sp.	9.00 ± 0.046	9.00 ± 0.046	8.53 ± 0.046	1.00 ± 0.046	0.50 ± 0.046	5.605^{a}	9.00 ± 0.046	1.05 ± 0.046	0.60 ± 0.046	0.60 ± 0.046	0.50 ± 0.046	2.350^{c}
Fusarium sp.	8.23 ± 0.046	6.40 ± 0.046	2.05 ± 0.046	1.30 ± 0.046	0.73 ± 0.046	3.740^{d}	5.00 ± 0.046	1.45 ± 0.046	0.65 ± 0.046	0. 50 ± 0.046	0.50 ± 0.046	1.620^{d}
Asp. ochraeus	8.65 ± 0.046	6.43 ± 0.046	6.00 ± 0.046	2.33 ± 0.046	2.25 ± 0.046	5.130^{b}	7.25 ± 0.046	4.50 ± 0.046	3.60 ± 0.046	0.50 ± 0.046	0.50 ± 0.046	3.285^{a}
Asp. terrus	9.00 ± 0.046	6.60 ± 0.046	5.23 ± 0.046	1.20 ± 0.046	0.70 ± 0.046	4.545^{c}	4.70 ± 0.046	1.65 ± 0.046	0.70 ± 0.046	0.50 ± 0.046	0.50 ± 0.046	1.610^{d}
Asp. niger	9.00 ± 0.046	8.55 ± 0.046	8.03 ± 0.046	1.35 ± 0.046	0.80 ± 0.046	5.545^{a}	6.65 ± 0.046	3.93 ± 0.046	1.18 ± 0.046	0.50 ± 0.046	0.50 ± 0.046	2.550^{b}
Asp. fumigatus	9.00 ± 0.046	9.00 ± 0.046	8.40 ± 0.046	1.05 ± 0.046	0.50 ± 0.046	5.590^{a}	9.00 ± 0.046	1.08 ± 0.046	0.58 ± 0.046	0.50 ± 0.046	0.50 ± 0.046	2.330^{c}
Overall[2]	8.813^{A}	7.660^{B}	6.370^{C}	1.371^{D}	0.913^{E}		6.933^{A}	2.275^{B}	1.230^{C}	0.520^{D}	0.500^{D}	

[1] Data in the column followed by different small letters are significantly different (P< 0.01).

[2] Data in the row followed by different capital letters are significantly different (P< 0.01).

Data presented in Table 9 show the antifungal effect of sterilized and unsterilized clove oil at concentrations of 0.1, 0.3, 0.5, 0.7 and 1.0 %. Sterilized clove oil up to 0.5 % did not exert antifungal effect on most of the tested fungi with a weak positive effect on *Fusarium* spp. Meanwhile, 0.7 and 1.0 % sterilized clove oil produced stronger antifungal activities which reached almost complete inhibition on the majority of tested fungi at 1.0 %. It was of great interest to notice that all of the tested concentrations of the unsterilized clove oil supported positive antifungal effect compared with the sterilized one on all the tested fungal cultures. Significant differences among the used concentrations of both sterilized and unsterilized clove oil against the isolated fungi were recorded. The antifungal activity of clove oil demonstrated in this study had been well documented (Walsh and Pease, 2002).

In conclusion, the strong antifungal activity of the heat sterilized and unsterilized clove oils might open the way in the production of organic food and feed where safety natural products might replace hazardous chemical preservatives. Sterilized clove oil might be added to canned and cooked foods. While unsterilized clove oil is recommended to be added to fresh and pickled foods in addition to different feed mixtures for broilers.

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مقدرة بعض الزيوت العطرية كمضادات للفطريات المعزولة من بعض مخاليط علف الدواجن

ناهد سامى يوسف[1] ، هالة محمد جمال الدين[1] ، ريهان عادل عبد المنصف[2]

[1] *كلية الاقتصاد المنزلى – جامعة الأزهر – طنطا .*

[2] *المعمل المركزى للمناخ الزراعى – مركز البحوث الزراعية – وزارة الزراعة واستصلاح الأراضى – مصر.*

تم عزل الفطريات *Mucor* sp., *Fusarium* sp., *Aspergillus ochraceus, Aspergillus terrus, Aspergillus niger,* و *Aspergillus fumigatus* من مخلوط علف دجاج التسمين. وتم أيضاً اختبار زيت النعناع والليمون والقرنفل والزيتون والثوم وحبة البركة كمضاد فطرى على هذه الفطريات بتركيزات ١ و ٢ و ٣ % من بيئة مستخلص البطاطس والدكستروز والتى تم تسخينها لدرجة حرارة التعقيم.

وأوضحت النتائج أن زيت القرنفل المسخن لدرجة حرارة التعقيم له تأثير فعال ضد الفطريات أقوى من الزيوت الأخرى. فعند استخدام زيت القرنفل بتركيز ١% أعطى تثبيط قوى ضد الفطريات أما عند استخدامه بتركيز ٢% فقد حدث تثبيط كامل لكل الفطريات المستخدمة.

ولقد تم تقييم التأثير المثبط لزيت القرنفل المعقم وغير المعقم بتركيزات ٠,١ و ٠,٣ و ٠,٥ و ٠,٧ و ١,٠ % وأوضحت النتائج أن زيت القرنفل غير المعقم عند تركيز ٠,٧ % ثبط الفطريات بدرجة كبيرة. وهذه النتائج قد تتيح الفرصة لاستخدام الزيوت العطرية كزيت القرنفل كمادة طبيعية آمنة بدلاً من استخدام المواد الكيميائية عند تصنيع الأغذية المعاملة بالحرارة وكذا عند تصنيع أعلاف الدواجن.