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ANTIFUNGAL EFFECT OF CARVACROL ON FUNGAL PATHOGENS ISOLATED FROM BROILER CHICKENS

RADWAN, I.A.[1]; ABED, A.H.[1] and ABDALLAH, A.S.[2]
[1] Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.
[2] Department of Microbiology, Animal Health Research Institute, Beni-Suef, Branch, Egypt.

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ABSTRACT

Fungal diseases of poultry have become problematic as bacterial and viral diseases. This study was designed to investigate the prevalence of fungal agents in broiler chickens and to detect the activity of carvacrol using agar dilution method against these isolates. Mycological examination was conducted on 227 broiler chickens` samples in El-Fayoum Governorate in the period from February 2015 to May 2016. Forty nine fungal isolates were recovered with a prevalence of 21.6% including 29 moulds (12.8%) and 20 yeasts (8.8%). Mould isolates were recognized as 5 *A. fumigatus* (2.2%), 6 *A. flavus* (2.6%), 10 *A. niger* (4.4%), 1 *A. nidulans* (0.4%), 1 *Cladosporium* (0.4%) and 4 *Penicillium* species (1.8%). All yeast isolates were recognized as *Candida* species. *C. albicans* was the most prevalent; 11 isolates (4.8%), followed by *C. krusei*; 4 isolates (1.8%), *C. tropicalis*; 3 isolates (1.3%), and *C. glabrata*; 2 isolates (0.9%). The antifungal activities of carvacrol against the recovered fungi revealed that carvacrol completely inhibited the growth of all tested fungal isolates at concentrations of 0.1, 0.25, 0.5, and 1% while a concentration of 0.01% has no effect on the fungal growth.

Key words: Broiler chicken, *Aspergillus* species: *C. albicans*, Carvacrol.

INTRODUCTION

Fungal pathogens pose serious problems worldwide for both human and animal health, especially in the subtropical and tropical regions. Fungi, bacteria and their toxins are natural contaminants of environment particularly foods even when the best condition of culture, harvest, storage and handling were used. Microorganisms, such as bacteria, moulds, yeasts and viruses, in the living environment are often pathogenic and cause severe infections in both human beings and animals (Reddy, 2007).

Fungal infections are frequently associated with morbidity and mortality in birds (Radwan *et al.,* 2016). Among the fungi, *Penicillium* and *Aspergillus* species are dominantly present (Plewa-Tutaj and Lonc, 2014). Few fungal species are common pathogens in avian species especially *Aspergillus spp.*; the cause of aspergillosis (Rippon, 1982). Manifestations of aspergillosis depend on the organs affected and whether infection is localized or disseminated. Aspergillosis appears to be more significant in confinement situations where stress factors may be involved or where moldy litter or grain is present (Radwan *et al.,* 2016). Contaminated poultry litter is often the source of Aspergillus conidia (Dyar *et al.,* 1984). A possible effect of aspergillosis is the possible transmission of fungal mycotoxin residues to meat and eggs from infected chickens, which is potentially hazardous to public health (Anath and Faryd, 2000).

Aspergillus fumigatus is considered as a major pathogen in birds (Radwan *et al.,* 2016). Other species like *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* may also be isolated from avian cases of aspergillosis (sometimes in mixed infections) but much less frequently than *A. fumigatus* (Kunkle *et al.,* 2003 and Martin *et al.,* 2007). Active fungal proliferation and sporulation of *A. fumigatus* on organic material produce large amounts of airborne small-sized conidia that are easily dispersed in air, then potentially inhaled and deposited deep in the respiratory tract of broilers and develops as a bronchopneumonia (Milos *et al.,* 2011).

Moreover, *Candida* species are widely spread throughout the poultry producing areas of the world (Radwan *et al.,* 2016). In the past, *C. albicans* was assumed to be the only pathogenic yeast of the genus Candida. However, it is now known that of the more than 100 species of candida, seven are of medical

Corresponding author: Dr. ABDALLAH, A.S.
E-mail address: asosa.sayed@gmail.com
Present address: Department of Microbiology, Animal Health Research Institute, Beni-Suef, Branch, Egypt.

significance (Hopfer, 1985).

Unfortunately, few anti-fungus medicines are available for treating fungus infections, not to mention that most of them have serious side effects (Wang *et al.*, 2012). The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies (Rapp, 2004).

The uses of plant-derived products as disease control agents have been studied since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Lee *et al.*, 2007). Therefore, there has been increasing interest to replace synthetic preservatives with natural, effective and nontoxic compounds. Those are; in the first place, extracts and essential oils of spices and herbs (Smid and Gorris, 1999). The essential oils from many plants are known to possess antibacterial and antifungal activity (Pinto *et al.*, 2006) but the spectrum of activity and mechanisms of action remain unknown for most of them. Previous studies have reported antifungal activity for clove oil and eugenol against yeasts and filamentous fungi, such as several food-borne fungal species (López *et al.*, 2007). It has also seen that some of the essential oils such as oregano, thyme, rosemary and clove essential oils and some of their main constituents such as eugenol, carvacrol and thymol show efficient antifungal activity against Aspergillusniger among which thymol and carvacrol proved to have better anti-Aspergillus effect than eugenol (Latifa *et al.*, 2012). Major components of oregano extract, which include the terpenoid phenols carvacrol, thymol, and eugenol, have potent antifungal activity of their own (Alma *et al.*, 2003).

The purpose of this study was to investigate the prevalence fungal pathogens in broiler chickens and to detect the activity of carvacrol against these isolates and possibility for their application in the veterinary medicine.

MATERIAL AND METHODS

1. Samples

A total of 227 samples were collected from broiler from different areas in El-Fayoum Governorate during the period from February 2015 to May 2016. The manifestations of studied chickens showed dyspnea, gasping, accelerated breathing, depression, emaciation, ruffled feathers, profuse watery diarrhea, blindness, torticollis, lack of equilibrium, and stunting growth. Postmortem lesions of diseased and freshly dead chickens showed congestion of the lungs, airsacculitis, mucous enteritis with sloughing of the intestinal mucosa and some of them showed greenish gray lesions and caseated nodules of 1-2 mm thickness distributed in lungs, livers, proventriculus, gizzard, intestine and abdomen.

The chickens' samples were collected mainly from crop (n=20), air sacs (n=80), pericardium (n=80), proventriculus (n=27) and liver (n=20). All samples were transferred directly to the laboratory of Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University for mycological examinations.

2. Fungal isolation

All samples were taken immediately and transferred directly into pre-enrichment broth Malt extract broth, (Oxoid) and incubated at 37^{o}C and 25^{o}C for 24-48 h, then cultured on Sabouraud dextrose agar medium (Oxoid) and incubated at 37^{o}C for 24-48 h.

3. Identification of fungal isolates

The recovered fungi were identified morphologically according to Rippon (1988). Mycelial fungi were identified by examination of mycelial morphology, the reverse colour as well as examination of colonial smears using lactophenol cotton blue stain. Yeast like fungi were identified by colonial morphology and examination of Gram`s stained culture.

4. Biochemical identification of yeast isolates by using API kit

The appropriate API kit (API 20 C AUX, Oxoid) was used according to the manufacturer's instruction.

5. Agar dilution method for detection of antifungal activity of carvacrol

According to the method of Jeff-Agboola *et al.* (2012) the antifungal activity of carvacrol against 22 randomly selected fungal isolates was done. The tested isolates included 10 *C. albicans*, 3 *A. flavus*, 3 *A. fumigatus*, 2 *A. niger* and 2 *A. nidulans* as well as 2 *Penicillium* species. Briefly, the tested fungi were grown on SDA at 35C for 48 h, then cells were suspended in physiological saline (0.9% NaCl), and the suspension was adjusted to 1×10^{6} CFU. SDA was prepared and autoclaved at 121˚C for 15 minutes and kept at 55˚C. Carvacrol (Segmaaldrech co.) was sterilized by filtration (pore size, 0.45 µm), and were mixed with SDA according to the tested concentrations (0.01, 0.1, 0.25, 0.5, and 1%). The oil-agar medium (10 ml) was then poured into sterile petri dishes and was solidified. Equal amounts of the fungal suspensions were inoculated and speared onto the agar plates. The plates were then incubated at 37 ^{o}C and 25 ^{o}C for 24h and then examined daily for 8 days.

RESULTS

1. Prevalence of fungal pathogens recovered from broiler chickens.

Out of 227 samples collected from different lesions of

broiler chickens, 49 fungal isolates were recovered; with a prevalence rate of 21.6%, including 29 (12.8%) mould isolates and 20 (8.8%) yeast isolates. Out of 20 crop, 7 isolates (35%) were from of which 2 (10%) moulds and 5 (25%) yeasts while from 80 air sacs, 17 isolates (21.3%) were recovered of which 12 (15%) moulds and 5 (6.3%) yeast. Also, 10 isolates (12.5%) were recovered from pericardium (n=80); 7 (8.8%) moulds and 3 (3.8%) yeast. From 20 livers, 5 isolates (25%) were recovered; 3 (15%) moulds and 2 (10%) yeast; while 10 isolates (37%) were recovered from proventriculus (n=27); 5 (18.5%) moulds and 5 (18.5%) yeast (Table 1).

Table 1: Prevalence of fungal pathogens recovered from broiler chickens.

Source	No. of samples	Recovered Fungi					
		Mycelial Fungi		Yeasts		Total	
		No.	%	No.	%	No.	%
Crop	20	2	10	5	25	7	35
Air sac	80	12	15	5	6.25	17	21.25
Pericardium	80	7	8.75	3	3.75	10	12.5
Liver	20	3	15	2	10	5	25
Proventriculus	27	5	18.5	5	18.5	10	37
Total	227	29	12.8	20	8.8	49	21.6

%: Percentage was calculated according to the corresponding No. of samples.

2. Identification of the recovered fungi.

2.1. Identification of mould isolates.

Mould isolates recovered from broiler chickens (n=29) were recognized as 5 A. fumigatus (2.2%), 6 *A. flavus* (2.6%), 10 *A. niger* (4.4%), 1 *A. nidulans* (0.4%), 1 *Cladosporium* (0.4%) and 4 *Penicillium* species (1.8%) (Table, 2).

Table 2: Prevalence of mould isolates recovered from broiler chickens.

Total No. sample	*A. fumigatus*		*A. flavus*		*A. niger*		*A. nidulans*		*Cladosporium* spp.		*Penicillium* Spp.		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
227	5	2.2	6	2.6	10	4.4	1	0.4	1	0.4	4	1.8	29	12.8

%: Percentage calculated according to Total No. of samples.

2.2. Identification of yeast isolates.

Out of 227 samples from chickens' samples, 20 yeast isolates (8.8%) were recovered and all isolates were recognized as *Candida* species. *C. albicans* was the most prevalent with a total of 11 isolates (4.8%). Then, 4 isolates of *C. krusei* (1.8%), 3 isolates of *C. tropicalis* (1.3%) and 2 isolates of *C. glabrata* (0.9%) (Table, 3).

Table 3: Prevalence of yeast isolates recovered from broiler chickens and their environment.

Source	Total No. sample	*C. albicans*		*C. tropicalis*		*C. krusei*		*C. glabrata*		Total	
		No.	%	No.	%	No.	%	No.	%	No.	%
Broiler chicken	227	11	4.8	3	1.3	4	1.8	2	0.9	20	8.8

%: Percentage was calculated according to the corresponding Total No. of samples.

3. Antifungal activity of carvacrolon the growth of different fungal isolates.

Carvacrol at concentrations of 0.1, 0.25, 0.5, and 1% completely inhibited the growth of all the tested fungal isolates using agar dilution method. On the other hand, a concentration of 0.01% has no effect on the fungal growth (Table 4).

Table 4: Antifungal effect of carvacrol on the growth of different fungal isolates.

Conc.	Growth Examination				
	***A. niger* (*n=2*)**	***A. flavus* (*n=3*)**	***A. fumigatus* (*n=3*)**	***A. nidulans* (*n=2*)**	***C. albicans* (*n=10*)**
0.01%	+	+	+	+	+
0.1%	-	-	-	-	-
0.25%	-	-	-	-	-
0.5%	-	-	-	-	-
1%	-	-	-	-	-

- : No growth, +: Positive growth.

DISCUSSION

Fungi are found on a wide variety of substances such as soil, plants, water and exudates of animals (Radwan *et al.,* 2014). Among the infectious diseases, the fungal diseases have their own importance and seem to be one of the great obstacles for the poultry farmers.

Species of the genus Aspergillus are important fungal infection, which affects the respiratory tract of the birds causing high morbidity, mortality and production losses (Richard *et al.,* 1991). Aspergillosis appears to be more significant in confinement situations where stress factors may be involved or where moldy litter or grain is present. Contaminated poultry litter is often the source of Aspergillus conidia (Dyar *et al.,* 1984). Moreover, mycological examination to investigate the mycotic flora of chicken population revealed isolation of fungal isolates such as *A. niger*, *A. fumigatus* and *A. flavus* indicating the ubiquitous nature of these fungi (El - Badry and Sokkar, 1988). Moreover, *Candida* species are widely spread throughout the poultry producing areas of the world (Radwan *et al.,* 2016). Poultry of all ages are susceptible to the effects of such organism.

Recently, the growing economic value of poultry has led to the increase of research of poultry diseases. The fungal diseases of poultry have become problematic as bacterial and viral diseases (Darwish, 1989). Therefore, the current study was designed to investigate the prevalence of fungal agents in broiler chickens and their environment.

In the present study, the prevalence of fungal isolation from broiler chickens was 21.6% including 12.8% mycelial fungi and 8.8% yeast isolates (Table 1). The present prevalence was lower than that of Radwan *et al.* (2014) who reported that the prevalence rate of fungal isolation from broiler chickens was 39% of which 3% were mycelial fungi and 36% were yeast and these results were opposite to those obtained in the present study. Also, Radwan *et al.* (2016) recorded a prevalence rate 53.1% (42% mycelial fungi and 11.1% yeast). These results were nearly similar to those of Pennycott *et al.* (2003) who isolated yeast from chicken samples in a percentage of (12.15 %).

Initial contamination of poultry farms may occur through use of a mouldy litter or introduction of one-day old birds that has retained conidia in hatchery facilities. Further contamination may involve inappropriate bedding management (Dyar *et al.,* 1984). A short-time exposure to heavily contaminated wood shavings induced an experimental pulmonary aspergillosis in chickens (Julian and Goryo, 1990). The negative correlation between relative humidity and the number of Aspergillus conidia in air may indicate that xerophilic Aspergillus conidia more readily discharge in dry conditions than in humid atmosphere. Interestingly, high counts of *A. fumigatus* conidia in air coincided with high levels of respirable dust particles of poultry houses suggesting a possible physical association or a similar response to indoor conditions (Debey *et al.,* 1995).

In the present study, the mycological identification of the recovered moulds from broiler chickens (n=29) revealed that the isolates were identified as *A. fumigatus* (2.2%), *A. flavus* (2.6%), *A. niger* (4.4%), *A. nidulans* (0.4%), *Cladosporium* (0.4%) and *Penicillium* species (1.8%) (Table, 2). These result run parallel to those obtained by (Radwan et al., 2016) who recorded 2.2% *A. fumigatus*, 8.4% for both *A. flavus* and *A. niger*, 1.3% *A. nidulans*, 0.4% *Cladosporium* spp. and 1.8% *Penicillium* spp. Abd El-Aziz (2015) reported that *A. fumigatus* represented 28.0%, *A. niger* 18.6%, *Cladosporium* spp. 11.9% and *Penicillium* Spp. 8.5%. Similarly, the presence of the aforementioned fungal species was described from poultry farm by (El-Zarka, 1988), who reported the presence of *A. niger*, *A. fumigatus*, *Penicillium* spp. and *Cladosporium* spp. in addition to *A. flavus*. Comparable results were also stated where fungal isolates represented about 41.7 % of poultry samples and *A. niger*, *A. fumigatus* represented the majority of these isolates with 12.5% and 10%, respectively (El-Badry and Sokkar, 1988). In another study conducted by Moustafa (1995), 35.2% of recovered isolates were *A. flavus*, 27.5% *A. niger*, 23.1% *A. fumigatus* and 14.3% *A. terreus*. However, neither *Cladosporium* nor *Penicillium* spp. were recovered.

Identification of the recovered yeast isolates from broiler chickens illustrated in table (3) revealed that all isolates (n=20, with a total prevalence of 8.8%) belonged to Candida species and arranged as C. albicans which was the most prevalent with a prevalence of 4.8% followed by *C. krusei*, *C. tropicalis* and finally, *C. glabrata* with prevalences of 1.8%, 1.3% and 0.9%, respectively. These results were lower than those of Pennycott *et al.* (2003) who isolated *C. albicans* from chicken's samples in a percentage of (12.15 %). On the other hand, these results run hand to hand with those of Radwan *et al.* (2014) who found that *C. albicans* was the most prevalent (19%) followed by *C. pseudotropicalis* (6 %) and *C. krusei* (5%). Also, Radwan *et al.* (2016) recorded the same where *C. albicans* was the most prevalent (2.7%) followed by *C. krusei* (2.2%). Moreover, Wyatt and Hamilton (1975) found that *C. albicans* comprised 95% of the isolates and the mean incidence of Candida in the crops was 32.3%. They studied the crops from four field outbreaks of crop mycosis. Three of the four cases of crop mycosis were characterized by multiple strains of *C. albicans* in the crop. They also found that less than 1% exhibited visible lesions attributable to Candida. *C. albicans* comprised 95% of the isolates. The population of Candida in the crops of birds found positive was of low magnitude in the majority of the chickens examined.

The uses of plant-derived products as disease control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Lee *et al.,* 2007). Therefore, there has been increasing interest to replace synthetic preservatives with natural, effective and nontoxic compounds. Those are; in the first place, extracts and essential oils of spices and herbs (Smid and Gorris, 1999).

One of the Major components of oregano extract is the terpenoid phenol carvacrol, which have a potent antifungal activity of their own (Alma *et al.,* 2003). Previous studies have reported antifungal activity for carvacrol (Remmal *et al.*, 1993; Ultee *et al.,* 2002; Alma *et al.*, 2003; Gutiérrez *et al.,* 2010, Zhang *et al.,* 2010 and Zabka and Pavela, 2013).

In the current study the antifungal activity of carvacrol on the growth of different fungal isolates were studied and the results were illustrated in table (4). Carvacrol at concentrations of 0.1, 0.25, 0.5, and 1% completely inhibited the growth of all the tested fungal isolates using agar dilution method. On the other hand, a concentration of 0.01% has no effect on the fungal growth. These results run hand to hand with those of Gutiérrez *et al.* (2010) who reported that carvacrol showed complete inhibition of *C. albicans* and *A. flavus*. Remmal *et al.* (1993) assessed the antifungal properties of eugenol and carvacrol against *C. albicans* in-vitro and found that carvacrol was more efficacious than eugenol. Also, Zabka and Pavela (2013) evaluated carvacrol as the most effective against the genera Fusarium, Aspergillus and Penicillium. Moreover, carvacrol was shown to be effective regardless of the maturity of the biofilm (Abdel-Massih *et al.,* 2010) as it was able to inhibit biofilms of several strains of Candida, including *C. albicans*, *C. glabrata*, and *C. parapsilosis*. Moreover, Latifa *et al.* (2012) found that eugenol, carvacrol and thymol show efficient antifungal activity against Aspergillusniger among which thymol and carvacrol proved to have better anti-Aspergillus effect than eugenol.

Terpenoid phenols such as carvacrol which is present in oregano and some of the other plants essential oils act as a potent antifungal agents and acts on a wide range of pathogens. Major components of oregano extract, which includes the terpenoid phenols carvacrol, thymol, and eugenol, have been shown to possess potent antifungal activities of their own, however, carvacrol showed the strongest antifungal activity against *C. albicans* biofilms that are resistant to many antifungal drugs, with a MIC of 0.03% (Dalleau *et al.,* 2008). The study demonstrated that carvacrol is responsible for the disruption of both Ca2+ and H+ homeostasis in yeast and that these disruptions likely lead to loss of cell viability and loss of metabolic activity that occur within minutes of carvacrol treatment (Zhang *et al.,* 2010) and they reasoned that 15 min. following drug exposure would be the ideal time to capture the transcriptional effect of carvacrol.

Moreover, the antifungal effect of carvacrol may be interpreted by reduction of ergosterol content; the major sterol component in fungal cell membrane. Therefore, carvacrol was found to display a broad fungicidal activity through the disruption of cytoplasmic membrane integrity leading to leakage of vital intracellular compounds (Vale-Silva *et al.,* 2010). Thymol and carvacrol were suggested to affect cell membrane structure by generating asymmetries and membrane tensions. This is confirmed by the fact that terpenes alter cell permeability by entering between the fatty acyl chains making up the membrane lipid bilayers, disrupting lipid packing, and changing membrane fluidity. All of these phenomena lead to major surface alterations and deformities that also reduce the ability of fungi to adhere to mucosal cells, and decrease their virulence and infectiousness (Radwan *et al.,* 2014).

CONCLUSION

The fungal diseases of poultry have become problematic as bacterial and viral diseases. The prevalence of fungal pathogens isolation in examined broiler chickens was 21.6. Aspergillus species was the most prevalent moulds while *C. albicans* was the most prevalent yeast recovered from broiler chickens.

The antifungal activities of carvacrol against the recovered fungi revealed that carvacrol completely inhibited the growth of all tested fungal isolates at concentrations of 0.1, 0.25, 0.5, and 1% while a concentration of 0.01% has no effect on the fungal growth.

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تأثير الكارفاكرول كمضاد للفطريات على الفطريات الممرضة المعزولة من بدارى التسمين

إسماعيل عبد الحفيظ رضوان ، أحمد حسين عابد ، اثار سيد عبد الله

E-mail: asosa.sayed@gmail.com Assiut University web-site: www.aun.edu.eg

امراض الفطريات التي تصيب الدواجن تمثل مشكلة مثلها مثل الامراض البكتيرية والفيروسية. هذه الدراسة صممت لتحري نسب الاصابة بالفطريات في بدراي التسمين ولتحديد تاثير الكارفاكرول ضد هذه المعزولات باستخدام طريقة تخفيف الاجار. تم القيام باختبارات خاصة بالفطريات علي ٢٢٧ عينة من بداري التسمين في محافظة الفيوم في الفترة مابين فبراير ٢٠١٥ الي مايو ٢٠١٦. تم عزل ٤٩ معزولة فطرية بنسبة حدوث ٢١.٦% مشتملة علي ٢٩ فطر خيطي (١٢.٨%) و٢٠خمائر (٨.٨%). تم التعرف علي معزولات الفطريات الخيطية كالتالي ٥ اسبرجيللس فيوميجاتس (٢.٢%)، ٦ اسبرجيللس فلافس (٢.٦%)، ١٠ اسبرجيللس نايجر (٤.٤%) و٤ بنسلييوم (١.٨%). كل معزولات الخمائر تم التعرف عليها ككانديدا. تم عزل١١ معزولة كانديدا البيكانز كاكثر نسبه حدوث بنسبة (٤.٨%)، يتبعها كانديدا كروزيي ٤ معزولات (١.٨%)، كانديدا توربيكاليز ٣ معزولات (١.٣%) وكانديدا جلابراتا ٢ معزولة (٠.٩%). كشف تاثير الكارفاكرول علي الفطريات المعزولة ان الكارفاكرول منع تماما نمو الفطريات المعزولة عند تركيزات ٠,١، ٠,٢٥، ٠,٥ و ١% بينما تركيز ٠,٠١% لم يكن له اي تاثير علي نمو الفطريات.