

ROLE OF ESSENTIAL OIL FOR CONTROL OF AVIAN ASPERGILLOSIS IN EXPERIMENTALLY INFECTED CHICKENS

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ABSTRACT

This study was designed to investigate the efficacy of essential oils as an alternative prophylaxis and treatment for avian aspergillosis. The in vitro susceptibility of *Aspergillus fumigatus*, *A. flavus*, and *A. niger* strains to antifungal drugs (Nystatin, Amphotericin B (AMP), Ketoconazole (KTC), Itraconazole (ITR) and Fluconazole (FLU), and to essential oils (Garlic, thyme, cinnamon, tea tree, clove and chamomile oils) was determined using the macro diffusion methods. All fungal isolates demonstrated variable degrees of drug resistances. All fungi showed 100% resistance to fluconazole, while ketoconazole is highly effective than itraconazole, amphotericin B and nystatin in all isolated fungi. Essential oils showed antifungal effect especially cinnamon oil followed by clove and garlic among tested EOs in vitro. Cinnamon oil showed the highest inhibition zone diameter to all fungi, so it was chosen for experimental trial based on the obtained antifungal activity results, Cinnamon oil was highly effective against *A. fumigatus* infection with a dose of 1 ml/kg ration and could be used in the field of poultry instead of other antifungal drugs.

Key word: Essential oils, Cinnamon oil, Aspergillosis

INTRODUCTION

Aspergillosis is the most common opportunistic mycotic infection of respiratory tract in birds causing high morbidity and mortality thus inducing significant economic losses especially in poultry (Tell, 2005).

Aspergillosis can be acute or chronic form. Acute aspergillosis generally occurs in young birds and resulting in high morbidity and mortality. The chronic form is sporadic and it causes lesser mortality and generally affects older birds, especially a compromised immune system due to poor husbandry condition. Inhalation of *A. fumigatus* asexual spores (conidia) can cause a spectrum of clinical manifestations depending on the immunological status of the host (Ben-Ami *et al.*, 2010; McCormick *et al.*, 2010).

Although aspergillosis is predominantly a disease of the respiratory tract, all organs can be infected, leading to a variety of manifestations ranging from acute to chronic infections. It is trusted that impaired immunity and the inhalation of a considerable amount of spores are important causative factors (Beernaert *et al.*, 2010).

Aspergillosis in poultry is caused by a fungal species under the genus *Aspergillus*. Organisms cultured from affected organ in decreasing frequency are: *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. glaucus* and *A. terreus*. From these spp, *A. fumigatus* is a common cause of the disease (salem and ali, 2014).

Treating fungal infections is a challenging task due to the limited number of effective antifungal drugs, the emergence of drug resistance and elevated renal and liver toxicities. Current treatments include the synthetic azoles (e.g. fluconazole and flucytosine) or the natural polyene amphotericin B (AmB). However usage is becoming limited by resistance development to the azoles, and acute toxicity of AmB. Also, the burden of antifungal resistance is becoming a major concern, and has thus intensified the search for new, safer, and more efficacious agents to combat serious fungal infections (Shreaz *et al.*, 2016).

Essential oils can represent one of the most promising natural products for fungal inhibition, many kinds of EOs obtained from different plants or herbs exhibited intense antifungal properties (Nazzaro *et al.*, 2017).

EOs' antifungal activity may be due to the inhibition of ergosterol biosynthesis and the disruption of membrane integrity and cell wall, which prompted the morphological deformations, collapse and

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deterioration of the conidia and/or hyphae (Pianalto and Alspaugh, 2016).

The fungitoxic properties of essential oil extracted from Cinnamon (*Cinnamomum zeylanicum* Breyn) bark have been investigated against five species of *Aspergillus* spp *A. flavus*, *A. fumigatus* Fresenius, *A. nidulans*, *A. niger* and *A. terreus* (Srivastava, 2015).

Cinnamon oil strongly inhibited the radial mycelial growth of *Aspergillus* species and associated with morphological changes as decreased condition, leakage of cytoplasm, loss of pigmentation and disrupted cell structure indicating fungal wall degeneration (Carmo *et al.*, 2008).

To our knowledge, no published data exist for examining Cinnamon oil treatment efficacy for avian aspergillosis in experimentally infected chickens. Therefore, the objectives of this study were (i) evaluation of the in vitro antifungal activity of EOs and antifungal drugs against *A. fumigatus*, *flavus*, *niger* (ii) assessment of cinnamon prophylaxis and treatment efficacy in an experimental avian aspergillosis model.

MATERIALS AND METHODS

Experimental inoculum

18 isolated strains (6 *A.fumigatus*, 6 *A.niger*, 6 *A.flavus*) were used for antifungal assay, all 18 strains of avian species with clinical aspergillosis (lungs and air sacs).

Spore suspensions of *A. fumigatus*, *A.flavus*, *A.niger* were prepared in sterile 0.85% NaCl with Tween 20 from fresh colonies grown on Sabouraud dextrose agar at 35 °C for 5 days, Cell concentration was adjusted to final concentrations of 0.4×10^4 to 5×10^4 . These suspensions were used directly for the inoculation (Espinel-Ingroff *et al.*, 2007).

Screening for antifungal activity of antifungal drugs and EOs in vitro

Disc diffusion test

M 44-A (CLSI/NCCLS 2004); (Espinel-Ingroff *et al.*, 2007)

A sterile swab was dipped in the suspension and the excess fluid drained by rotating the swab against the inside of the container, Potato dextrose plates was inoculated from the same suspension, with a fresh swab with a rotary movement. The plates were incubated at 35°C for 18–24 h, read, and then incubated for a further 18–24 h to determine the

optimal incubation time. Zone sizes were measured in millimeters with calibers.

The 18 isolated strains (6 *A.fumigatus*, 6 *A.niger*, 6 *A.flavus*) were tested against 5 anti-fungal discs of commonly used chemotherapeutic agents from Oxoid® Hampshire, UK: Nystatin (NY) 100 I.U, Amphotericin B (AMP) 20 µg, Ketoconazole (KTC) 10 µg, Itraconazole (ITR) 10 µg and Fluconazole (FLU) 25 µg. The following tentative zone diameter categories were assigned: susceptible, ≥ 17 mm (azoles and nystatin) and ≥ 15 mm (amphotericin B (AP)); intermediate, 14–16 mm (azoles and nystatin) and 13–14 mm (AP); resistant, ≤ 13 mm (azoles and nystatin) and ≤ 12 mm (AP).

Agar well diffusion test Balouiri *et al.* (2016) and Tartor and Hassan (2017)

Garlic, thyme, cinnamon, tea tree, clove and chamomile oils (ELCaptain Company and rose monde company) were screened for antifungal activity using agar well diffusion method.

The agar plate surface is inoculated by spreading 1ml of the microbial inoculum (5×10^4) over the entire agar surface, then, a hole with a diameter of 6 to 8mm is punched aseptically with a sterile cork borer or a tip, and a volume of 50 µl of each essential oils dissolved in DMSO is introduced into the well, then the agar plates are incubated for 48 hour at 37 °C. Zone sizes were measured in millimeters with calibers. Its considered positive when ≥ 10 mm.

Evaluation of cinnamon oil efficacy against *A.fumigatus* experimentally

A total of 65-One day old chicks were used, 5 killed at the beginning of the experiment (pre-inoculation control) lung sections were cultured on SDA plates incubated at 37 °C and checked for *A.fumigatus*, the remaining chicks were randomly assigned into 4 equal separated groups.

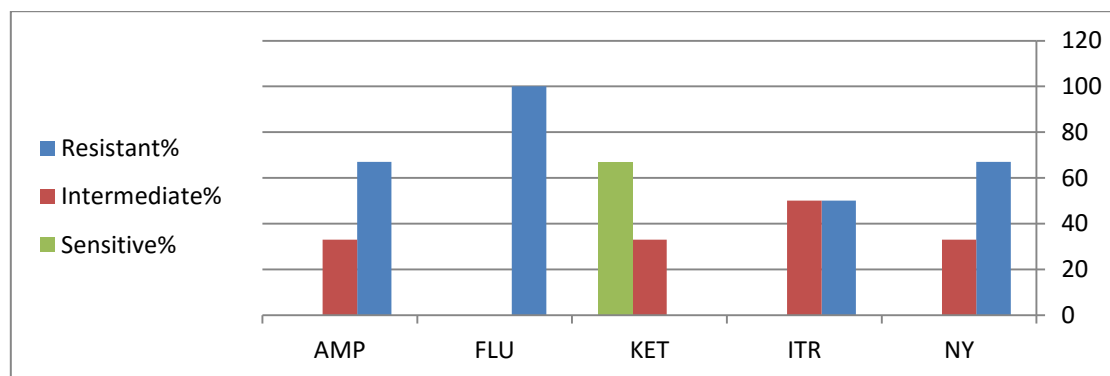
Chicks in groups 1, 2 (at 3-day-old) were inoculated intratracheally with 1ml *A.fumigatus* spore (2.7×10^6) detected by spectrophotometer (Hereba *et al.*, 2016), chickens in group 2 were treated with (1 ml / kg diet)cinnamon oil (the most effective in vitro sensitivity) and treatment started at the day of infection and continued for 15 days. Group 3, chicks were taken cinnamon oil (1ml / kg diet) for 15 day. Group 4, served as uninfected untreated control (negative control). All clinical signs, postmortem lesions and mortalities were recorded. At the end of the experiment, the remaining chicks were sacrificed and subjected to post mortem and mycological examination.

RESULTS

Table 1: Sensitivity of *A. fumigatus* strains to antifungal drugs.

Antifungal drugs	Interpretation of zone diameter (mm) (Espinel-Ingroff <i>et al.</i> , 2009)			Result (n=6)		
	Resistant	Intermediate	Sensitive	Resistant%	Intermediate%	Sensitive%
Nystatin	≤ 13	14–16	≥ 17	67	33	0
Itraconazole	≤ 13	14–16	≥ 17	50	50	0
Ketoconazole	≤ 13	14–16	≥ 17	0	33	67
Fluconazole	≤ 13	14–16	≥ 17	100	0	0
Amphotericin B	≤ 12	13–14	≥ 15	67	33	0

A. Fumigatus isolates showed 100 % resistant to Fluconazole, 67% resistant to both Nystatin and Amphotericin B while 50 % resistant to Itraconazole and only sensitive to Ketoconazole with 67%

**Figure (1):** Degree of sensitivity of *Aspergillus fumigatus* to antifungal drugs – disk diffusion method**Table 2:** Sensitivity of *A. flavus* strains to antifungal drugs.

Antifungal drugs	Interpretation of zone diameter (mm) (Espinel-Ingroff <i>et al.</i> , 2009)			Result		
	Resistant	Intermediate	Sensitive	Resistant%	Intermediate%	Sensitive%
Nystatin	≤ 13	14–16	≥ 17	100	0	0
Itraconazole	≤ 13	14–16	≥ 17	100	0	0
Ketoconazole	≤ 13	14–16	≥ 17	0	0	100
Fluconazole	≤ 13	14–16	≥ 17	100	0	0
Amphotericin B	≤ 12	13–14	≥ 15	100	0	0

A. flavus isolates showed 100 % resistant to Fluconazole, Itraconazole, Nystatin and Amphotericin B while 100% susceptible to Ketoconazole.

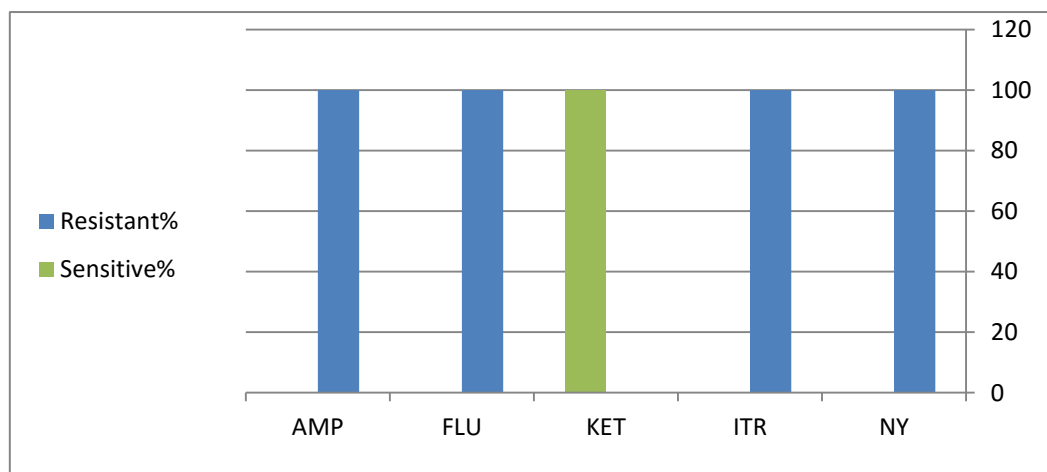
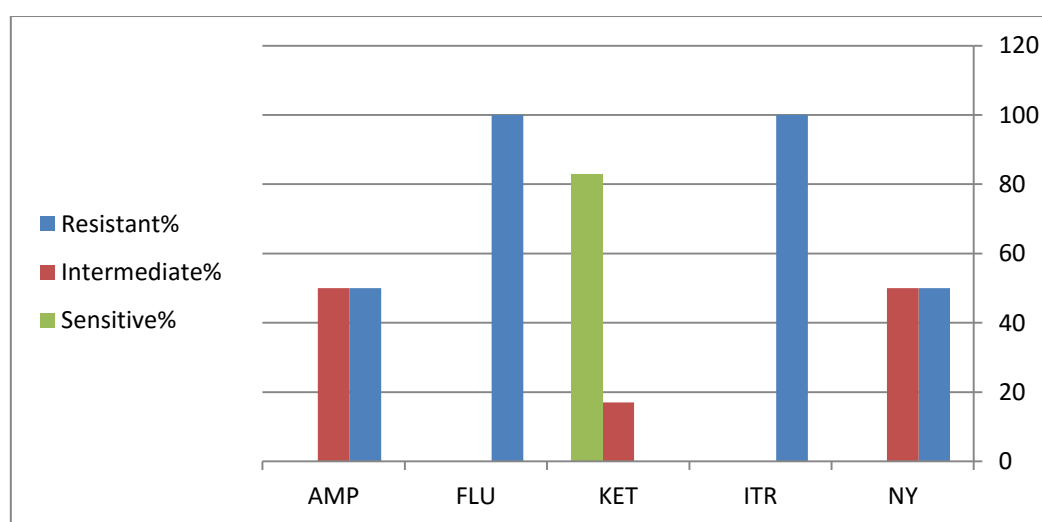
**Figure (2):** Degree of sensitivity of *Aspergillus flavus* to antifungal drugs – disk diffusion method.

Table 3: Sensitivity Susceptibilities of *A. niger* strains to antifungal drugs.

Antifungal drugs	Interpretation of zone diameter (mm) (Espinel-Ingroff <i>et al.</i> ,2009)			Result		
	Resistant	Intermediate	Sensitive	Resistant%	Intermediate%	Sensitive%
Nystatin	≤ 13	14–16	≥ 17	50	50	0
Itraconazole	≤ 13	14–16	≥ 17	100	0	0
Ketoconazole	≤ 13	14–16	≥ 17	0	17	83
Fluconazole	≤ 13	14–16	≥ 17	100	0	0
Amphotericin B	≤ 12	13–14	≥ 15	50	50	0

A.niger strains isolates showed 100 %resistant to Fluconazole and Itraconazole while 50 % resistance to Nystatin and Amphotericin B while only susceptible to ketoconazole with 83%.

**Figure (3):** Degree of sensitivity of *Aspergillus niger* to antifungal drugs – disk diffusion method.

Results of sensitivity of fungi isolates to different essential oils (n=6):

Sensitivity of *A. fumigatus* strains to essential oils

Cinnamon oil, tea tree oil and clove oil displayed antifungal activity for *A.fumigatus* isolates, with the highest efficacy by Cinnamon oil. Cinnamon oil showed the highest inhibition zone diameter (27 ± 2 mm) followed by tea tree oil (13 ± 2 mm), and clove (12.5 ± 1 mm). Garlic oil has moderate activity (10 ± 1 mm), thyme oil and chamomile oil did not exhibit any antifungal sensitivity for *A.fumigatus* isolates. Cinnamon oil was chosen for experimental trial in vivo based on the obtained antifungal activity results.

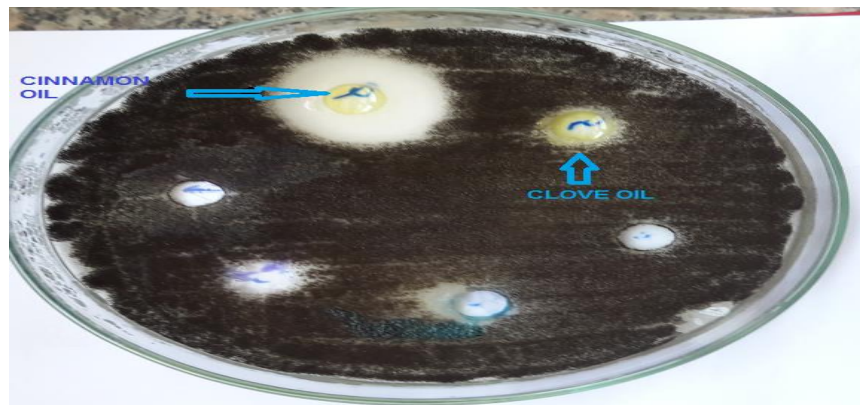
Sensitivity of *A. flavus* strains to essential oils

Cinnamon oil, garlic oil and clove oil displayed antifungal activity for *A.flavus* isolates, with the

highest efficacy by Cinnamon oil, which showed the highest inhibition zone diameter (27.8 ± 3 mm) followed by garlic oil(12.3 ± 2 mm), and clove oil (10 ± 2 mm), chamomile oil, thyme oil and tea tree oil did not exhibit any antifungal sensitivity for *A.flavus* isolates.

Sensitivity of *A. niger* strains to essential oils

Cinnamon oil, clove oil and garlic oil displayed antifungal activity for *A.niger* isolates, with the highest efficacy by Cinnamon oil, which showed the highest inhibition zone diameter (26.8 ± 1 mm) followed by clove oil (14.5 ± 1.5 mm), and garlic oil (11.8 ± 2 mm), chamomile oil has weak activity (9.7 ± 2 mm), thyme oil and tea tree oil did not exhibit any antifungal sensitivity.



Figure(4): Agar well diffusion test showing susceptibility of *A.niger* to cinnamon oil and clove oil.

Results of Experimental design:

Clinical signs: (Group 1): Two days after the inoculation, general and respiratory signs of aspergillosis were observed. Clinical signs included ruffled feathers, gasping, dyspnea, dullness, greenish watery diarrhea, loss of weight, cloudy eye and anorexia. Deaths were recorded starting from the third day of the inoculation. All chicks in infected untreated group (group 1) died within 15 days post the inoculation. While in infected and treated group (group 2), 10 chicks (66.7%) survived and no deaths or clinical signs were occurred after day 8 post inoculation.

(Groups 3, 4): Neither death nor clinical signs of aspergillosis were observed in both treated and control groups.

Post mortem: The birds in all groups were necropsied and the most common gross findings were noticed in Group (1). There were whitish

cheesy materials of fungal growth appeared dispersed in the air sacs and lung tissue. Marked congestion of the lungs and thickening of the air sac membranes occur were observed. Miliary necrotic foci develop in lungs around the sites of fungal growth and result in the formation of nodules. These nodules were discrete, circumscribed, and white in color and randomly distributed throughout the lung tissue and air sac, myocardium, thoracic wall and abdominal serosa, some cases showed congested kidney. (Group 2): This lesion observed in only 5 dead birds from treated group and disappeared after 8 day post infection (day 10). (Group 3, 4): no post mortem lesions were observed.

In (Group 1): the inoculated *A.fumigatus* was re-isolated from the lungs, air sacs from all infected chicks, while in (Group 2): the inoculated *A.fumigatus* was re-isolated from the lungs, air sacs from the only five dead birds and re-isolation was unsuccessful in the survivor chicks.

Table 4: Mortality percent in the four groups.

Days (PI)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16-30	Survivors	Dead	Total	% survived	% mortality
GROUP 1 N=15 Infected untreated	-	-	1	1	2	2	-	2	-	-	2	2	1	1	1	-	0	15	15	0	100
GROUP 2 N=15 Infected treated	-	-	1	1	2	-	-	1	-	-	-	-	-	-	-	-	10	5	15	66.7	33.3
GROUP 3 N=15 Take cinnamon oil in ration	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	0	15	100	0
GROUP 4 N=15 (negative control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	0	15	100	0

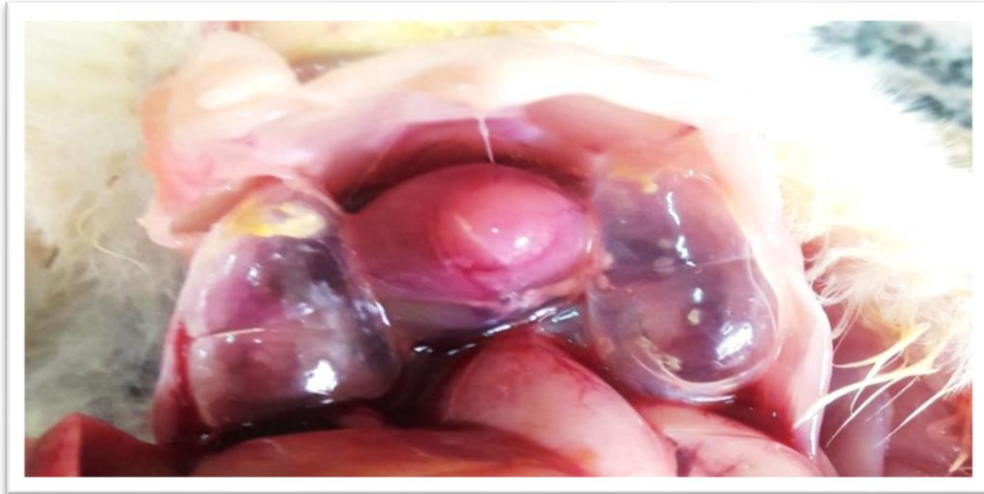


Figure (5): Air sacs are thickened and lung tissues contain a lot of Miliary necrotic foci around the sites of fungal growth.

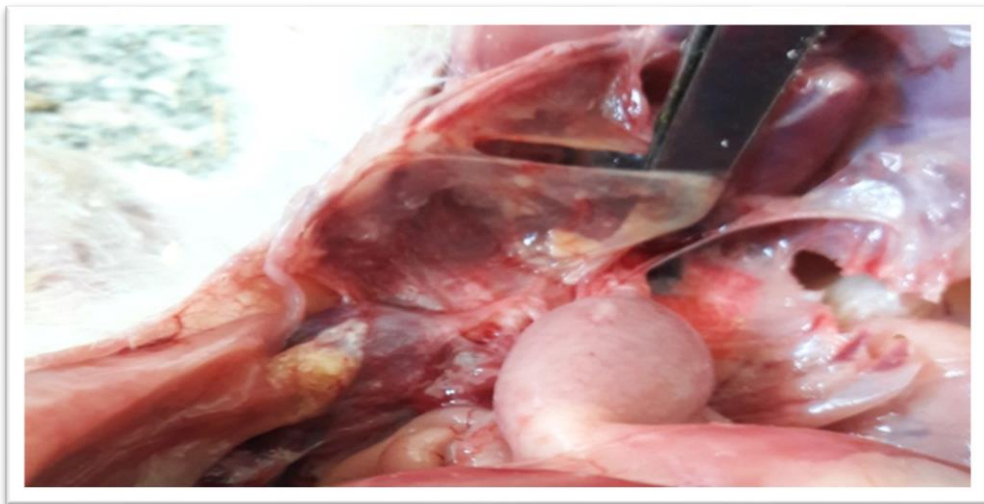


Figure (6): lung showing discrete, circumscribed, white in color and randomly distributed nodules throughout the lung tissues.

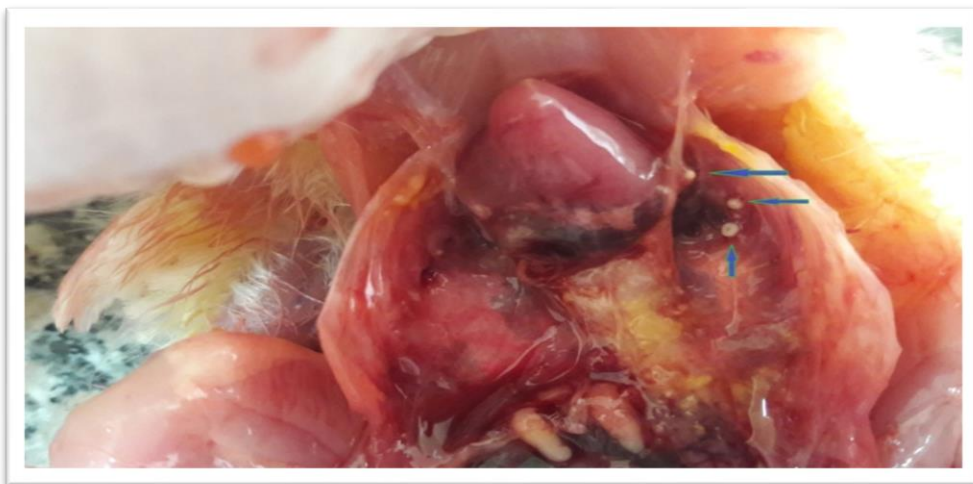


Figure (7): Air sacs are thickened and covered with small whitish cheesy materials of fungal growth.

DISCUSSION

In recent years, a fungal infection have emerged as a world-wide health problem and has become an important cause of respiratory infection in poultry owing to extensive use of broad spectrum antibiotics, corticosteroids and increasing population of terminally ill and debilitated patients which trigger an interest to examine the source and reservoir of such fungi. Among the infectious diseases fungal diseases have their own importance and seem to be one of the great obstacles for the poultry farmers in the form of high morbidity, mortality and production losses Asfaw and Dawit (2017).

In vitro sensitivity of our fungal isolates to 5 antifungal drugs showed that *A.Fumigatus* isolates showed 100 % resistant to Fluconazole, 67% resistant to both Nystatin and Amphotericin B while 50 % resistant to Itraconazole and only sensitive to Ketoconazole with 67%. Our results agreed to some extend with those reported by Ziółkowska *et al.* (2014) and Tartor and Hassan (2017) in case of Fluconazole, Amphotericin B and Itraconazole, except in case of ketoconazole who found that most strain of *A.fumigatus* was resistant to it.

Our isolates of *A.flavus* showed 100 % resistant to Fluconazole, Itraconazole, Nystatin and Amphotericin B, while 100% susceptible to Ketoconazole. These results were totally in agreement with Kumar *et al.* (2010) in case of Fluconazole and Ketoconazole but totally disagreed in case of Itraconazole and Amphotericin B, this may be due to the difference in countries.

Aspergillus.niger strains isolates showed 100 % resistant to Fluconazole, Itraconazole, 50 % resistance to Nystatin and Amphotericin B while only susceptible to ketoconazole with 83%. These results accepted with those mentioned by Tokarzewski *et al.* (2012). It can be assumed that as in the case of other *Aspergillus* species, the effectiveness of antifungal agents may depend not only on the type of fungus, but also on the site of isolation (environment, patient), and even the species of the host (human, animal, bird) (Tokarzewski *et al.*, 2012).

In vitro sensitivity of our fungal isolates to 6 EOs showed that in case of *A.fumigatus*: cinnamon oil, tea tree oil and clove oil displayed antifungal activity for *A.fumigatus* isolates, with the highest efficacy by cinnamon oil, which showed the highest inhibition zone diameter (27 ± 2 mm) followed by tea tree oil (13 ± 2 mm), and clove (12.5 ± 1 mm). Garlic oil has moderate activity (10 ± 1 mm), thyme oil and chamomile oil did not exhibit any antifungal sensitivity for *A.fumigatus* isolates. These results

were in agreement with that reported by Singh *et al.* (1995); Inouye *et al.* (2000); Carmo *et al.* (2008); Rana *et al.* (2011); Srivastava (2015) and Tartor and Hassan (2017).

Concerning to in vitro sensitivity of *A. Flavus* to tested EOs: cinnamon oil, garlic oil and clove oil displayed antifungal activity for *A.flavus* isolates, with the highest efficacy by cinnamon oil, which showed the highest inhibition zone diameter (27.8 ± 3 mm) followed by garlic oil (12.3 ± 2 mm), and clove oil (10 ± 2 mm), while chamomile oil, thyme oil and tea tree oil did not exhibit any antifungal sensitivity for *A.flavus* isolates.

These results were similar to that mentioned by Singh *et al.* (1995); Carmo *et al.* (2008); Gutiérrez *et al.* (2010); Rana *et al.* (2011) and Srivastava (2015).

Our result in vitro sensitivity of *A. niger* to tested EOs: cinnamon oil, clove oil and garlic oil displayed antifungal activity for *A.niger* isolates with the highest efficacy by cinnamon oil, which showed the highest inhibition zone diameter (26.8 ± 1 mm) followed by clove oil (14.5 ± 1.5 mm), and garlic oil (11.8 ± 2 mm). Chamomile oil has weak activity (9.7 ± 2 mm) while thyme oil and tea tree oil did not exhibit any antifungal sensitivity. These result were in agreement with that illustrated by Tansey and Appleton, (1975); Singh *et al.* (1995); Carmo *et al.* (2008); Rana *et al.* (2011) and Srivastava (2015).

General and respiratory signs of aspergillosis in experimentally infected birds were observed two days after the inoculation in group 1 and in some chicks in experimentally infected and treated birds in group 2. Clinical signs included ruffled feathers, gasping, dyspnea, dullness, green watery diarrhea, loss of weight, cloudy eye and anorexia. These signs were similar to those described by Atasever and Gümüşsoy, (2004); Gümüşsoy *et al.* (2004). All chicks in infected untreated group (group 1) died within 15 days after the inoculation, this was similar to that found by Fadl Elmula *et al.* (1984) and agreed to certain degree with Peden and Rhoades, (1992); Le Loc'h *et al.* (2006). While in infected treated birds (group 2), 10 chicks (66.7%) survived and no death or clinical signs occurred after day 8 post inoculation ,so the cinnamon oil reduced the mortality rate between the chicks, while in only treated birds (group 3) or control groups (group 4) neither death nor clinical signs of aspergillosis were observed. These was going hand by hand with that mentioned by Symeon *et al.* (2014) who found that cinnamon oil at the selected concentrations (0.5 and 1 ml/kg feed) may not have the potential to influence mortality rates and Atasever and Gümüşsoy (2004) who found that the controled group don't have any clinical sign or mortality.

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دور الزيوت الأساسية في التحكم في مرض الاسبرجولس في الدجاج المعدى تجريبياً

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تهدف الدراسة الى تقييم تأثير بعض الزيوت الطبيعى على بعض انواع الفطريات ومقارنتها ببعض الادويه المضاده للفطريات معمليا وقد لوحظ أن الكيتوكونازول له اكبر تأثير على جميع انواع الفطريات التي خضعت لاختبار الحساسيه مقارنة مع كل من الفلوكونازول والامفوتريسين ب والنيسنتاين والاتراكونازول. قد وجود تأثير مضاد للفطريات لبعض الزيوت التي خضعت لاختبار الحساسيه وخصوصا زيت القرفة يليه زيت القرنفل والثوم عن طريق اختبارهم معمليا وقد وضح تقييم تأثير زيت القرفة على الكتاكيت المصابة تجريبياً بالاسبيرجولس فيوماجيتس أن استخدام زيت القرفة كعامل مضاد للفطريات على العلف له تأثير كبير في العلاج ضد هذا الفطر من خلال انخفاض معدل الوفيات بنسبة (٦٦.٧٪) مقارنة بالمجموعة غير المعالجة التي وصلت نسبة الوفيات بها الى ١٠٠٪.