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IMMUNOLOGICAL AND PATHOLOGICAL STUDIES ON *OREOCHROMIS NILOTICUS* FED ON CINNAMON AND OXYTETRACYCLINE IN DIET AND CHALLENGED WITH *CLOSTRIDIUM PEREFRINGES*.

Walaa El-Houseiny*; Alshimaa A. Khalil*
and Rehab E. Mowafy**

**Dept. of Fish Diseases, Faculty of Veterinary Medicine, Zagazig University*

*** Dept. of Pathology, Animal Health Research Institute (Zagazig Branch).*

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Abstract

The present study was aimed to study the effect of adding cinnamon and oxytetracycline (OTC) to fish diet as a control strategy to illustrate their impact on immunity, growth and histopathological changes of *Oreochromis niloticus*. A total number of 90 of *Oreochromis niloticus* with an average body weight of 24.5 ±0.4g were used. The fish were randomly divided into three equal triplicate groups (Each replicate contained 10 fish). Fish were primarily divided into two experimental studies. Experiment I (pre challenged experiment) in which fish was fed with 0.5% cinnamon and 0.5% OTC. The fishes were fed for 2 months and then applied the experiment II (post challenged experiment) the groups after that kept for 15 days observation period. The obtained results revealed that the fish fed diet contained o.5 % cinnamon had a higher final body weight, body gain and body gain % as compared to fish fed OTC dietary treatment. Immunological parameters estimated in this work (IgM and lysozyme) were significantly improved due to supplementation of the cinnamon in compared with that of OTC and control groups. Congestion of most internal organs, deposition of melanomacrophages and hemosiderin, leucocytic cells infilterating most of the parenchymatous organs and some degenerative changes were the obivious pathological changes in post infected control group, these lesions were less demonstrated in both treated groups with cinnamon and oxytetracyclin after challenged with bacteria.

INTRODUCTION

Fish are considered one of the most widely accepted and valuable food in most countries. Tilapia is possibly the most important freshwater aquaculture species worldwide. The increasing scale of production demands new accurate

and efficient tools to screen and monitor the health status of these fish. The presence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture (El-Shorbagy *et al.*, 2012).

Intensification and commercialization of production, aquaculture industry faces major problems with bacterial diseases, and vast quantities of chemical and antibiotic products are frequently used. During the last decades, antibiotics were used as traditional strategy for fish diseases management but also for the amelioration of growth and efficiency of feed conversion. On the other hand antibiotics inhibit or kill beneficial micro biota in the gastrointestinal (GI) ecosystem (Kashem *et al*., 2014).

Rizkalla *et al*., (2004) decided *Clostridium perfringens* bacteria caused a high mortality rate among the cultured fish, while the drastic effect of the mild bacterial infection was inflected more on the fish physiological status and as a consequence decreases the farm production. Either mild or severe bacterial infection should receive an immediate eye inspection.

Large proportion of the world's antimicrobial industrial production is used as prophylactics and as growth promoters that far outweigh their use as therapeutics. Especially, antibiotics are still used until now as growth promoters in some of the fish farms in Egypt (Reda *et al*., 2013). Sanchez-Martinez *et al*. (2008) studied the effect of supplementing channel cat fish (*Ictalurus punctatus*) feeds with oxytetracycline. Treated fish exhibit a significant increase in weight gain suggesting a growth promotion action of this antibiotic agent. OTC is a tetracycline broad-spectrum antibiotic with bacteriostatic action produced by *Streptomyces* spp. fungi, used to treat systemic bacterial infections of fish (Jerbi *et al.*, 2011).

Even today, due to high cost of effective antibiotics and the predicament of antibiotic resistance microbial strains worldwide, about 60-85% of the population of developing world relies either on herbal or on indigenous forms of Complementary and Alternative Medicine (CAM) medicines for their various general health related issues and countering several diseases/disorders(Dhama *et al.*, 2014). Historically, cinnamon has been used

for centuries worldwide by various societies to combat infectious diseases. It has been reported to inhibit the growth of several antibiotic resistant strains of bacteria also, which is high in antioxidant and antimicrobial activity (Shivendu Ranjan *et al*., 2013). So, the main goal of the study was: Evaluate the potential effect of cinnamon and oxytetracycline in enhancing growth and immunostimulant effect in *O. niloticus* challenged with *C.perfringens*.

MATERIAL AND METHODS

C. perfringens strains:

Clostridium perfringens and with standard known biochemical and pathogenicity profiles were kindly obtained from the microbiological archive of the department of Microbiology, Animal Health Research Institute, Dokki, Egypt (Isolates were originally retrieved from clinically affected *O. niloticus* during a previous microbiological survey that encountered tilapias from different earthen ponds located within the scope of Giza province). Bacterial isolates were reconstituted and aliquoted in 2ml microfuge tubes containing glycerol- phosphate buffered saline (pH 7.4 – volume 1: 1).

Fishes used for experimental studies:

Nile tilapia "*O. niloticus*" with average weight 24.5 ±0.4g were obtained from the Central Laboratory for Aquaculture Research. Abbassa. Sharkia., Egypt. Fishes were acclimated before the experimental initiation for 2 weeks and then randomly distributed in glass aquaria of 80 liter capacity at rate of 10 fish /aquarium. Each aquarium was supplied with compressed air pumps and the temperature adjusted thermostatically controlled heaters. Water was partially changed by siphoning method to remove the excreta of fish daily.

Fish Diets and Feeding:

Diets were prepared at Fish Research Unit, Faculty of Veterinary Medicine, Zagazig University with 30% crude protein. The dietary treatments included a basal control diet, diet contains 0.5% cinnamon and the other diet contains OTC by a rate of 0.5%. The fish were fed diets 2 times daily at total

rate of 3% of body weight for 2 months (Table 1). The ingredients and calculated composition of the experimental diets was done according to Badawi *et al.* (2014).

Experimental studies:

Fish were primarily divided into two experimental studies as following:

Experiment I (pre challenged experiment): Fish divided into three triplicate groups (10 fish/aquarium) 1st group was kept as control which was fed with basal diet. The 2nd group was fed with 0.5% cinnamon incorporated diet Sivagurunathan and Xavierinnocent, (2014) and the 3rd group was fed with OTC by a rate of 0.5% Koh *et al*. (2014). The fishes were fed with their respective feed for 2 months.

Experiment II (post challenged experiment): After feeding of fish for a period of 2 months, fish after that were experimentally infected with 0.3 ml of the culture of ($3X10^7$cell/ml*) C. perfringens* by intraperitonial injection and the groups kept for 15 days observation period.

Growth Performance Parameters:

The fish were weighted at the start and every two weeks till the end of the experiment I. Average body weight was calculated by dividing the total weight of fish by the number of fish in each group. The values of the fish weights changes were used to assess the growth performance of the fish which are Specific Growth Rate (SGR), Average body weight, Weight Gain and Percentage Weight Gain as described by pouomonge and Ombredane (2001).

Blood Sample Collection:

Blood samples were collected from all groups at the end of experimental periods. Blood was collected from caudal vessels into Eppendorf tubes without anticoagulant in syringe to centrifuged (3000 rpm for 10min) for serum collection. The serum samples were collected and stored immediately in deep freezer (-20 °C) until use.

Immunological Assessment:

After the expiration of the time limit for each experiment, blood samples were collected and immunological assessment carried out.

Serum lysozyme activity assay determination:

Serum lysozyme activity was measured according to the method of (Ellis, 1990).

Immunoglobulin M (IgM) Determination:

Immunoglobulin M (IgM) was determined using ELISA Kit. Catalog No. CSB-E12045Fh (96k test). CUSABIO BIOTECH CO., Ltd. according to (Siwicki and Anderson, 1993).

Histopathological examination:

Eighteen *O. niloticus* were necropsied (three fish from each group at the end of the experiment I) and (three fish from each group at the end of the experiment II). Specimens from livers, spleens, kidneys, intestines, gills and muscles were collected and fixed in 10% neutral buffered formalin solution. Paraffin sections of 5 µ thickness were prepared and stained with haematoxylin and eosin for histopathological examination (Bancroft and Stevens, 1996).

Statistical analysis:

Data were analyzed using one-way analysis of variance (ANOVA) through the general linear models (GLM) procedure of the Statistical Package for Social Sciences version 21.0 (SPSS for Windows 21.0, Inc., Chicago, IL, USA). The comparison of means was carried out with Duncan's multiple range tests (DMRT). Results were recorded as mean ± standard deviation (SD). The value of $P < 0.05$ was used to indicate statistical significance (Tamhane and Dunlop, 2000).

RESULTS AND DISCUSSION

Growth parameters:

The effect of dietary treatments on growth performance of *Oreochromis niloticus* is showed in (Table 2). Which illustrated that, final body weight, BW gain and BW gain % are significantly difference ($P<0.05$). The specific growth rate exhibited an increasing trend in the experimental groups; however it was significantly higher in cinnamon incorporated diet. This result was similar to that obtained by Sivagurunathan and Xavierinnocent (2014) who reported that the incorporation of cinnamon in the diet might have an improved effect on palatability, digestion and absorption of nutrients. Also results was in accordance with that of Chang *et al. (2001*) mentioned that Cinnamaldehyde possess strong antibacterial activity against nine strains of bacteria including *E.coli, P.aeruginosa, E.faecalis, S.aureus, S.epidermidis, Klebsiella pneumonia,* Salmonella *species* and *Vibrio parahaemolyticus*. Thus cinnamon can be incorporated in fish feed to promote growth.

There was no significant difference in final body weight, BW gain and BW gain % recorded amongst the fish fed the experimental diets treated with OTC and control diet. Our results were in harmony with Omoregie (2001) who approved that no statistically significant differences were detected in the growth rate and feed utilization in the group of fish fed diets incorporated with 0.625% of OTC. However, at levels above 2.5%, growth performance was not as encouraging as with lower levels. This indicates signs of impairment in the physiology of the fish, hence the significantly lower content of protein, lipid and ash in the tissues of the fish fed with level more than 2.5%. Also, Hafiz *et al.* (2008) indicated that OTC can be used as a growth promoters under controlled condition for Nile Tilapia at high protein level (40%), but not when the protein level of the diet was low (25%). Lawal *et al.* (2012) observed that fish fed diets (0.2% OTC/100 g feed) recorded better feed conversion ratio compared to fish in the control group. Reda *et al.* (2013) observed that oxytetracycline treated *O. niloticus* at rate of 100 mg/ kg diet exhibited a significant increase in weight gain to values higher than those of controls,

suggesting a growth promotion action of this antibiotic agent. Also Gaskins *et al*. (2002) demonstrated that the effect of OTC in growth promotion depends on the reduction of the gastrointestinal tract bacteria. The conflicting results between these results may be due to different conditions used in various studies beside fish species, temperature and dose of OTC which affect drug absorption and utilization.

Immunological Parameters:

The results of Table (3) illustrated that the Immunoglobulin M (IgM) and lysozyme activity in fish fed with diet containing 0.5% cinnamon for 2 months prior the infection showed the highest significance rate ($P < 0.05$), also after challenged with bacteria these levels remain significant high in comparison to control and oxtetracycline groups. It may be due to that cinnamon contains different components that enhance the immunity and health status of fish. this result in harmony with that mentioned by Villupanoor *et al.* (2008) who reported that the dried bark of cinnamon contains 59.5% carbohydrate, 20.3% fiber, 9.9% moisture, 4.6% protein, 2.2% fat and 3.5% total ash. It also contains 1.6% calcium, 0.05% phosphorous, Vitamin-A (175 IU), Vitamin-B1 (10-14mg/100gm), Vitamin-B2 (0.21mg/100gm), Vitamin-C (39.8mg/100gm) and niacin (1.9mg/100gm). These results also agreed with Sivagurunathan and Xavierinnocent *(*2014) who mentioned that cinnamon (Cinnamaldehyde) diet fed fishes exhibited significant increase in total leucocyte counts (TLC) and lymphocyte counts in infected fishes and maintenance of a normal neutrophil population, which can be considered as a sign of improvement in both nonspecific immune and specific immune response. Also, it was noticed that IgM and lysozyme were significantly increased after infection than non infected fish which indicate antibacterial and immunostimulatory effect cinnamon. Hand with hands with Faikoh *et al.* (2014) who demonstrate that cinnamaldehyde exhibits antimicrobial activity against aquatic pathogens, even antibiotic-resistant bacterial strains and immune-stimulating effects to protect the host's defenses against pathogen infection in bacteria-infected zebrafish by increased endogenous interleukin (IL)-1β, IL-6, IL-15, IL-21, tumor necrosis factor

(TNF)-α, and interferon (INF)-γ. These results suggest that cinnamaldehyde could be used as an antimicrobial agent and immunostimulant to protect bacteria-infected fish in aquaculture.

The table (3) also, showed that significant increase in IgM levels in OTC incorporated diet than control one. The results were agree with El-Sayed *et al*. (2014) who mentioned that the level of IgM after 4 weeks and 8weeks was significantly increased in groups treated with OTC in comparison with control untreated group. These findings disagree with Yonar *et al.* (2011) who concluded that OTC had a suppressive effect on total plasma protein and immunoglobulin levels of rainbow trout (*Oncorhynchus mykiss*). While lysozyme activity had a significant decrease in the previous group than control or cinnamon incorporated diet. The results were disagree with Reda *et al.* (2013) and El-Sayed *et al.* (2014) who mentioned that, the lysozyme activity of OTC in diet with a ratio 1gm/kg diet showed a significant increase in comparison with control untreated group.

Histopathological results:

Macroscopical examinations:

The fish appeared normal without any gross lesions in untreated control group and both treated groups with cinnamon and oxytetracyclin till the end of experiment I, while Fifteen days post inoculation, the infected fishes showed darkness of the body, congestion of most internal organs, intestinal gases were also observed .These signs were mild in oxytetracyclin treated group and milder in cinnamon treated one. The macroscopical results as darkness of the body, congestion of most internal organs, intestinal gases were in partial agreement with those obtained by (Rizkalla, 2004)

Microscopical examinations:

Microscopical results in experimentally infected *O. nilotica* with *C.perfringes* showed severe congestion of hepatic blood vessels with lecuocytic cells infilteration (Fig.1) periductal edema of hepatopancreatic acini were also noticed (Fig. 2). |Congestion of splenic blood vessels with deposition

of melanomacrophages and hemosiderin (Fig. 3 & 4) .Vertebral column showed numerous melanomacrophage between bone and spinal cord (Fig. 5 & 6). While, gills showed desquamation of secondary lamellae. congestion of branchial blood vessels and desquamation of 2ndry lamellae (Fig.7). Destruction of cartilage core of primary lamellae and telangectasis of secondary lamellae. Hyperplasia of pillar cells with edema in secondary lamellae (Fig.8). Gills suffered from complete absence of secondary lamellae (Fig. 9), Necrosis of some chondrocyte of cartillagenous part of gill arch. Interstitial edema of intestinal submucosa also observed (Fig. 10). Congestion of submucosal blood vessels was noticed .Some cases showed destruction of intestinal serosa. Mucinous degeneration of intestinal villi with presence of numerous goblet cells (Fig. 11). Kidneys showed focal subcapsular haemorrhage in the renal cortex (Fig.12), interstitial edema in the muscle fiber was remarkable lesion of the muscle (Fig. 13).Cinnamon treated groups pre infection showed congestion of hepatic blood vessels (Fig. 14), which became mild post infection ,thickening of intestinal submucosa also observed, pale esinophilic structurless material of mainly haemolysed RBCs with slight diffuse fatty change (Fig. 15), while oxytetracyclin treated groups showed edema beneath intestinal submucosa (Fig. 16).Spleen showed slight deposition of melanomacrophages and haemosidrin in the parenchyma (Fig. 17).Congestion of renal blood vessels with leucocytic cells infilterating the parenchyma and cystic dilation of some renal tubules were also detected (Fig. 18). Microscopical signs were in complete agreement with those obtained by (Alshaimaa, 2007) who attributed these systemic signs to its toxins which leads to dermonecrotic effect (necrosis of skin dermal layer) and septicemic signs. While, microscopically, our hepatic lesions were similar to those described by (Marzouk *et al*. 2005) and (Alshimaa, 2007).

Conclusion and Recommendation:

In conclusion, it has been shown that Oxytetracycline (OTC) is one of antibiotics currently available and approved as a chemotherapeutic agent and is widely used in the aquaculture industry. Even though they give positive effects,

they cannot be recommended due to their residual and other side effects also suppress some parameters of the non specific immunity. The alternative cinnamon herbal bio-medicinal products in the aquacultural operations, have the characteristics of growth promoting ability and improve the immune system of *O. niloticus* infected with *Clostridium perfringens*. Thus from the present study it was inferred that incorporation of cinnamon in fish feed formulations may not only act as immunostimulator but also as growth Promoter.

Table 1. Ingredients and calculated composition of the experimental diets.

Ingredient	**Control**	**Cinnamon**	**OTC**
Yellow corn	35	34.5	34.5
Cinnamon	-	0.5	-
OTC	-	-	0.5
Wheat flour	10	10	10
Soybean meal, 44%	18	18	18
Fish meal, 60%	16	16	16
Poultry by-product meal	14	14	14
Vegetable oil	5.5	5.5	5.5
*Vitamin and mineral mixture	1.5	1.5	1.5

The calculated composition of diet: DM % 84.28, CP % 30.79, CF % 2.40, Ash % 7.09 * Vitamin and Mineral mixture (Alfakema): Each 1 kg contains: Vit. A 580000 I.U, Vit. D3 8600 I.U, Vit. E. 720 mg, Vit. K3 142 mg, Vit C 0.1 mg, Vit B1 58 mg, Vit B2 34 mg, Vit. B6 34 mg, Vit.B12 58 mg, Folic acid 86 mg, Pantothenic acid 8 mg, Manganese sulfate 65 mg, Zinc methionine 3000 mg, Iron sulfate 2000 mg, Copper sulfate 3400 mg, Cobalt sulfate 572 mg, Sodium selenite 25 mg, Calcium iodide 25 mg, Calcium carbonate (Carrier substance) till 1000 g.

Table 2. The effect of dietary supplementation with cinnamon and OTC on growth performance of *O. niloticus*.

Group / **Parameters**	**Control**	**Cinnamon**	**OTC**
Initial body weight (g)	24.5 ± 0.23^{a}	24.5 ± 0.57^{a}	24.5 ± 0.11^{a}
Final body weight (g)	29.43 ± 0.03^{bc}	34.37 ± 0.01^{a}	29.93 ± 0.28^{bc}
Weight gain (gm)	4.53 ± 0.33^{bc}	9.83 ± 0.01^{a}	5.03 ± 0.28^{bc}
Weight gain %	17.80 ± 0.22^{bc}	36.11 ± 1.76^{a}	20.20 ± 1.97^{bc}
Specific growth rate (SGR)	7.55 ± 0.05^{bc}	16.35 ± 0.03^{a}	8.38 ± 0.47^{bc}

Means within the same row carrying different superscripts are sig. different $P< 0.05$ based on Duncan's multiple range tests (DMRT).

Table 3. Effect of oxytetracycline (OTC) and cinnamon in both pre infected and post infected Tilapia on Ig M (g/L) and Lysozyme (u/ml).

Groups	Ig M	Lysozyme
Control	0.096 ± 0.03^{d}	0.214 ± 0.01^{d}
Effect of Cinnamon pre infection Experiment I	0.234 ± 0.02^{a}	0.312 ± 0.02^{a}
Effect of OTC pre infection, Experiment I	0.198 ± 0.01^{b}	0.127 ± 0.01^{e}
Effect of Cinnamon post infection, Experiment II	0.177 ± 0.01^{c}	0.290 ± 0.02^{b}
Effect of OTC post infection, Experiment II	0.102 ± 0.03^{d}	0.100 ± 0.03^{c}

Means within the same column carrying different superscripts are sig. different $P< 0.05$ based on Duncan's multiple range tests (DMRT).

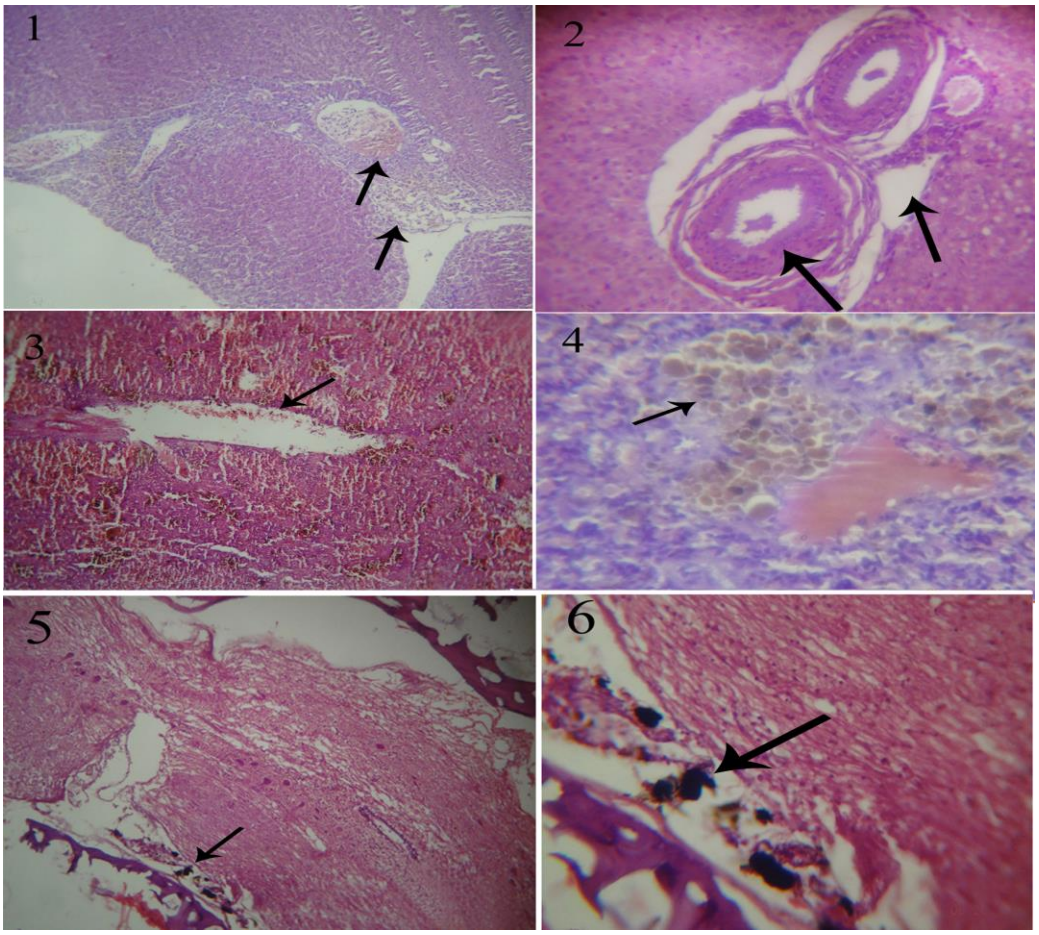

Plate 1. (Fig. 1-6). Photomicrographs of experimentally infected tilapia nilotica with c.perfringes showing.

Fig. 1. Photomicrograph of liver at 15th day PI (post infection) showing moderate congestion of hepatic blood vessels with lecuocytic cells infilteration . (H&E x 120).

Fig. 2. Photomicrograph of liver at 15th day PI showing per ductal fibrosis and per ductal edema of bile duct. (H&E x 300).

Fig. 3. Photomicrograph of spleen at 15th day PI showing congestion of splenic blood vessels with deposition of melanomacrophages and hemosiderin. (H&E x 120).

Fig. 4. Photomicrograph of spleen at 15th day PI showing more obvious melanomacrophages and hemosiderin in c.perfringes infected fishes (H&E x 300).

Fig. 5. Photomicrograph of vertebral column at 15th day PI showing numerous melanomacrophage between bone and spinal cord. (H&E x 120).

Fig. 6. High power of the previous figure to show the melanomacrophage between bone and spinal cord (H&E x 300).

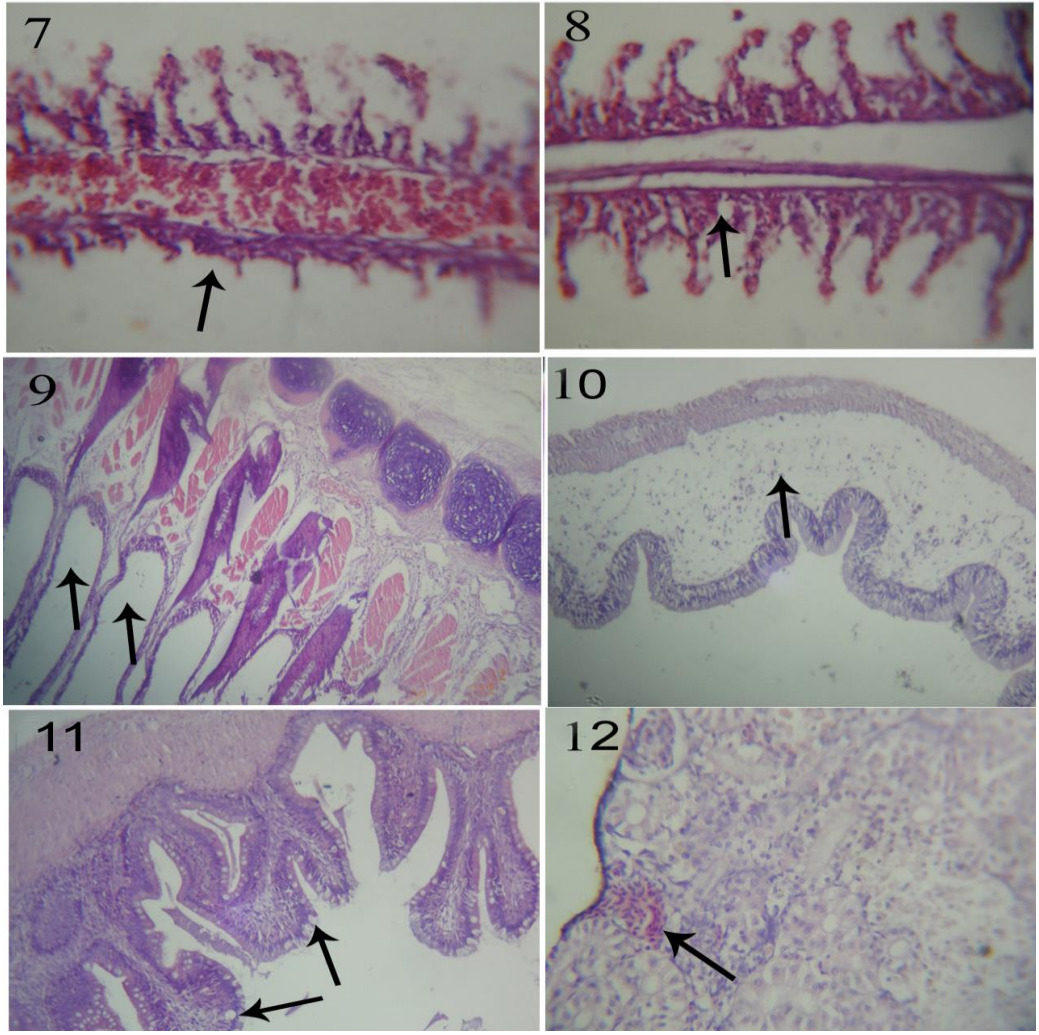

Plate 2. (From Fig. 7-12). Photomicrographs of experimentally infected tilapia nilotica with c.perfringes showing:

Fig. 7. Photomicrograph of gills at 15th day PI showing congestion of branchial blood vessels and desquamation of 2ndry lamellae.(H&E x 1200)

Fig. 8. Photomicrograph of gills at 15th day PI showing hyperplasia of pillar cells with edema in secondary lamellae (H&E x 1200)

Fig. 9. Photomicrograph of gills at 15th day PI showing complete absence of secondary lamellae (H&E x 300)

Fig. 10. Photomicrograph of intestine at 15th day PI showing interstitial edema of intestinal submucosa (H&E x 300)

Fig. 11. Photomicrograph of intestine at 15th day PI showing mucinous degeneration of intestinal villi with presence of numerous goblet cells (H&E x 300)

Fig. 12. Photomicrograph of kidneys at 15th day PI showing focal subcapsular haemorrhage in the renal cortex (H&Ex300).

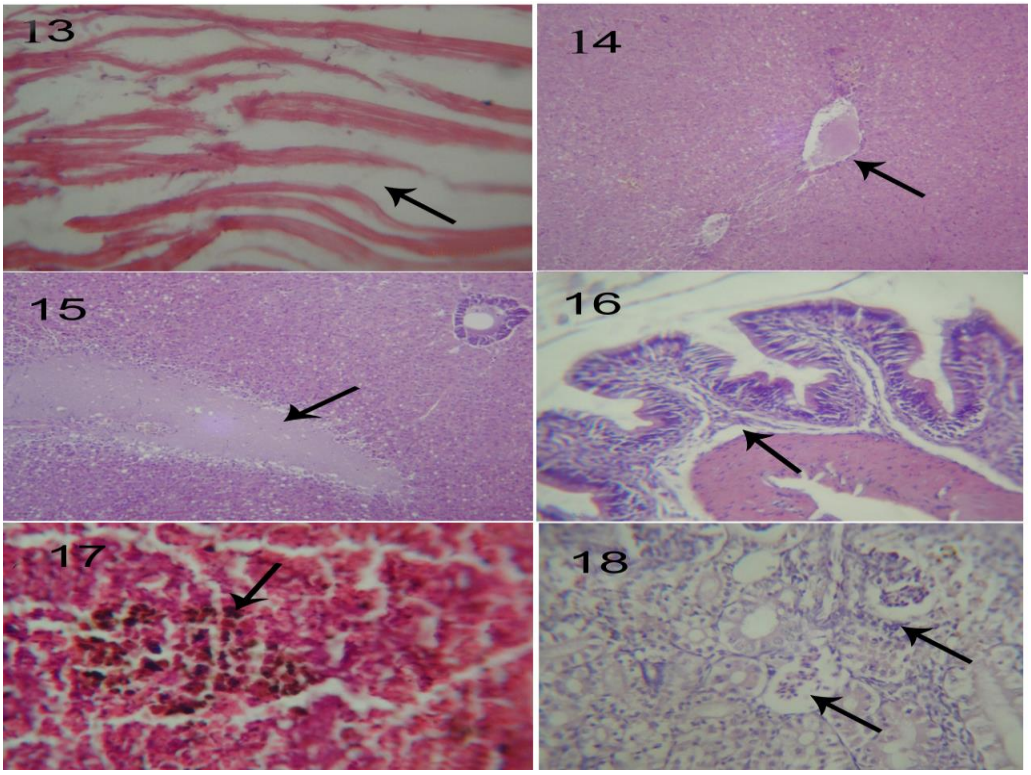

Plate 3. (Fig. 13). Photomicrographs of experimentally infected tilapia nilotica with c.perfringes**: and From Fig.(14-18):** photomicrographs of cinnamon***** &oxytetracyclin****** treated tilapia nilotica and experimentally infected with *C.perfringes* showing.

Fig. 13. Photomicrograph of intestine at 15th day PI showing interstitial edema between the muscle fiber (H&Ex300).

Fig. 14*. Photomicrograph of liver at 15th day PI showing congestion of hepatic blood vessels (H&E x 300).

Fig.15.** Photomicrograph of liver at 15th day PI showing pale esinophilic structurless material of mainly haemolysed RBCs with slight diffuse fatty change(H&E x 120)

Fig.16.** Photomicrograph of intestine at 15th day PI showing edema beneath intestinal submucosa (H&E x 300).

Fig.17.** Photomicrograph of spleen at 15th day PI showing deposition of melanomacrophages and haemosidrin in the parenchyma .(H&E x1200).

Fig.18.** Photomicrograph of kidney at 15th day PI showing slight congestion of renal blood vessels with leucocytic cells infilterating the parenchyma and cystic dilation of some renal tubules. (H&E x300)**.**

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دراسات مناعيه وباثولوجيه على أسماك البلطى النيلى المغذي علي عليقة تحوي القرفة والأوكسي تيتراسيكلين والمعدى تجريبيا بميكروب الكوليستريديم بيرفيرنجس

ولاء الحسينى*، الشيماء عبد العزيز خليل*، رحاب السيد موافى**

** قسم أمراض ورعايه الأسماك – كلية الطب البيطرى جامعة الزقازيق.*

***وحدة الباثولوجيا – معهد بحوث صحة الحيوان (الزقازيق).*

الملخص العربى

تم استخدام عدد تسعون سمكه بلطى نيلى بمتوسط وزن 0.4± 24.5 جرام قسمت بالتساوى لثلاث مجموعات حيث تم تغذيه الاولى على عليقه عادية لا تحوى اى اضافات بينما اضيفت القرفه الى عليقه المجموعه الثانيه وكذلك اضيف مركب الاوكسى تتراسيكلين لعليقه المجموعه الثالثه لمده شهرين لجميع المجموعات السابقة قبل العدوى حيث يهدف البحث لتقييم الحاله الصحية والمناعيه لاسماك هذه المجموعات وكذلك معدلات النمو كما تم احداث العدوى بميكروب الكوليستريديم بيرفيرنجس تجريبيا لاسماك هذه المجاميع حيث وضعت المجاميع تحت الملاحظه لمده ١٥ يوما وقد لوحظ ان معدلات النمو أعلي بين الاسماك المعامله بالقرفه مع تحسن للحاله المناعيه وانحسار للتغيرات الباثولوجيه الظاهره على اسماك المجموعه الاولى والتى سجلت اقل معدلات للنمو وتدهور للحاله المناعيه مع ظهور احتقان لمعظم الاعضاء الداخليه وانتشار للخلايا الملتهمه لصبغيات الميلانين وتغيرات اضمحلاليه وارتشاح للخلايا الدمويه البيضاء بينما اظهر مركب الاوكسى تتراسيكلين اثار ايجابيه ولكن بدرجه اقل من تلك التى ظهرت بين الاسماك المغذاه بالقرفه مع ظهور لبعض التغيرات الباثولوجيه بالكلى.