Egyptian Journal of Animal Health

P-ISSN: 2735-4938 On Line-ISSN: 2735-4946
Journal homepage: https://ejah.journals.ekb.eg/

Evaluate the efficacy of Doxycycline and some essential oils in controlling *Pseudomonas* infection in *Oreochromis niloticus* (Nile tilapia)

Thoria A. Hamed[*], Hanan A. Fahmy[**], Heba A. Ewis [***], Shaimaa Nabil El_Tahawy, Dalia Ibrahim Mohamed[*], Mona M. El-Azzouny[****], Sally H. Abou-Khadra[****]

*Biochemistry Department, (Zagazig Branch), **Biotechnology Department, Pathology and Clinical Pathology Department (Zagazig Branch), ****Microbiology Department, (Zagazig Branch), Animal Health Research Institute (AHRI), Agricultural Research Center (ARC) Egypt,

Received in 11/2/2025
Received in revised from 2/3/2025
Accepted in 24/3/2025

Keywords:

Pseudomonas
Fish
Antioxidant
essential oils
AST.

ABSTRACT:

This study investigated the occurrence and antimicrobial susceptibility of *Pseudomonas aeruginosa* in *Oreochromis niloticus* and evaluated the antibacterial effect of essential oils (Mintodin®) in vitro and in vivo. A total of 100 samples of apparent health *Oreochromis niloticus* collected from fish markets and examined bacteriologically for *Pseudomonas aeruginosa* which isolated from24/100 (24%) of the samples tested and confirmed via PCR, yielding a 956 bp product specific to 16S rDNA gene. Antibiotic susceptibility testing showed high resistance rates, with 95.8% resistant to amoxicillin and 87.5% to erythromycin. Moreover, 83.33% of isolates were multidrug-resistant (MDR), and 16.66% exhibited extensive drug resistance (XDR). An experimental infection study was conducted on apparently healthy 150 Nile tilapia were divided into five groups: G1 (healthy control), G2 (infected, untreated control), G3 (infected , doxycycline-treated), G4 (infected, essential oil treated), G5 (pretreated with essential oil, then infected and doxycycline-treated). The infected untreated group (G2) showed significant reductions in RBC count, Hb, PCV, lymphocytes, and monocytes, alongside increased serum ALT, AST, urea, creatinine, and glucose, with decreased antioxidant enzyme activity (catalase, GSH-Px). In post mortem examination, signs of septicemia were observed and histopathological changes of different organs of infected fishes were described. However, treatment with doxycycline (G3), essential oils (G4), or a combination of pre-treatment with essential oils followed by doxycycline (G5) resulted in notable improvements in blood parameters, oxidative stress markers, inflammatory responses, and histopathological alterations. Notably, G5 showed the most promising results, with upregulated antioxidant genes expression (*SOD*, *CAT*) and metabolic gene expression (*Tfam*), while downregulating the pro-inflammatory cytokine IL-6. These findings suggest that

Corresponding author: Thoria A. Hamed, Biochemistry Department, Zagazig Provincial Lab., Animal Health Research Institute, Agriculture Research Center (ARC), Egypt.
Email address: thoria77@yahoo.com
DOI: 10.21608/ejah.2025.424294

essential oils could enhance the therapeutic efficacy of antibiotics and serve as a protective strategy against *P. aeruginosa* infections in aquaculture.

INTRODUCTION:

Nile tilapia (*Oreochromis niloticus*) are typically subjected to a variety of biological, physical, environmental, and chemical stresses as a result of increased production, which can harm their health, lower their general performance, and make them more susceptible to illnesses. **(Ibrahim et al. 2022).**

Bacterial infections are considered the most serious problem faced by the Egyptian aquaculture industry during the intense farming of fish by this method **(Abdel-Rahman et al. 2020).**

The world's most popular fish is the Nile tilapia (*Oreochromis niloticus)*, which is prized for its flavor, profitability, and ability to withstand hazardous farming conditions **(Mengistu et al. 2020**).

The Nile tilapia (*Oreochromis niloticus*), the most economically valuable fish, has been lost to Pseudomonas sp. infection **(El-Saadony et al. 2022).** Pseudomonas species are one of the most aggressive bacterial infections that infect fish and cause ulcerative conditions **(Algammal et al. 2020).** One of the primary reasons of nosocomial infections is the opportunistic, Gram-negative bacterial pathogen *Pseudomonas aeruginosa* **(Crone et al. 2020).**

Pseudomonads are commonly found in aquatic environments and are a natural component of the gut flora in healthy fish. They trigger outbreaks when environmental conditions shift, leading to symptoms such as fin rot, petechial hemorrhages, detached scales, skin darkening, abdominal ascites, and exophthalmia (**Khalil et al. 2010).** Additionally, Pseudomonas (most commonly *Ps. aeruginosa*) can harm human consumers by causing illnesses linked to healthcare. **(Zilberberg and Shorr, 2009).**

Oxytetracycline and doxycycline are part of the broad-spectrum antibiotic class known as tetracyclines. Tetracyclines are widely accessible due to their low cost and effectiveness in treating a variety of infectious illnesses. The drugs that are most frequently provided in several African countries, including Egypt, are oxytetracyclines **(Alsayeqh et al. 2021).**

Doxycycline a broad-spectrum antibiotic has more permeability and lipophilicity than its analogs, which leads to a lengthy elimination half-life and widespread tissue disposition of the medication to effectively eradicate infections. **(Riviere and Papich, 2018).**

Because of its many qualities (such as anesthetic, antioxidant, and anti-bacterial), essential oils generated from plants have been the subject of aquaculture research. These features have been demonstrated to lessen endocrine and biochemical changes and, as a result, to enhance the state of welfare **(Souza et al. 2019).** Furthermore, aquaculture research have employed essential oils generated from plants due to their various qualities that might enhance the welfare, growth, and health of animals. **(Souza et al. 2018a).**

The incorporation of plant essential oils (EOs) as dietary supplements has demonstrated positive effects across various farm animal species in numerous studies. These additives have the potential to enhance fish growth, improve animal welfare, optimize feed utilization, boost disease resistance, and prevent disease outbreaks. these nutritional substitutes have recently been assessed and documented in production of fish **(Sutili et al. 2017).**

Due to their positive effects on growth performance, overall well-being, gut microbiota, and digestion, essential oils (EOs) present a promising alternative to antibiotic growth promoters in animal feed, offering a new generation of solutions for animal nutrition and health **(Zeng et al. 2015).** EOs have been shown to have antimicrobial and immunomodulatory qualities, making them a potentially useful agent for fish health. Therapeutic use of essential oils has demonstrated antimicrobial effects against various fish contaminants and pathogens, including *Pseudomonas* spp., *Vibrio* spp., *Aeromonas* spp., *Enterobacter* spp., etc

(Da Cunha et al. 2018). Significant effects of essential oils (EOs) on fish growth, immunity, and antioxidant responses **(Brandão et al. 2021)**

The present study was conducted to assess the effectiveness of doxycycline and some essential oils (Mintodin®), both individually and in combination, in managing *Pseudomonas* infections in Nile tilapia (*Oreochromis niloticus*).

MATERIALS and METHODS

Ethical approval

The approval number from the Ethical Committee of the Animal Health Research Institute is ARC/AHRI/164 /24, and the local committee of the ARC-IACUC committee approved this study. The Animal Health Research Institute recommendations and the OIE criteria for the use of animals in research and education were followed in all methodological aspects.

Collection of samples:

One hundred *O. niloticus* apparent health samples were collected from fish markets. Every sample was gathered and delivered to the lab in an icebox as soon as possible. Every sample underwent a bacteriological analysis.

P. aeruginosa Isolation and Identification:

Following the collection of internal organs a loopful sample was directly streaked onto Cetrimide agar and MacConkey's agar (Oxoid, UK), followed by incubation for 24 hours at 37°C under aerobic conditions. Yellowish-green colonies luminous pigment synthesis is frequently linked to pseudomonads. (**Lamont and Martin, 2003**). All suspected colonies were subsequently purified. In order to determine their biochemical and phenotypic traits. In summary, Gram's stain was used for morphological identification of the isolates, and a range of biochemical assays, such as urease, catalase, oxidase, indole, methyl red, Voges Proskauer, citrate utilization, H2S generation, mannitol fermentation, and gelatin hydrolysis, were used for biochemical identification. Additionally, the isolates' motility was evaluated using the hanging drop technique **Mac Faddin (Williams & Wilkins, 1985).**

Molecular identification of *P. aeruginosa:*

Presumed colonies were incubated overnight in tryptone soy broth (Oxoid, USA). Following the manufacturer's guidelines, bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Using the Polymerase Chain Reaction Technique (PCR), the bacterial DNA is molecularly identified. PCR was conducted following **Spilker et al. (2004)** amplified 956 bp (position and size relative to 16S rDNA sequence of P. aeruginosa AT2 (AB091760)) using PA-SS-F GGGGGATCTTCGGACCTCA, situated at locations 189–206, and PA-SS-R TCCTTAGAGTGCCCACCCG, located at locations 1124–1144. Initially, the thermal cycling protocol involved an initial denaturation step at 95°C for 2 minutes, followed by 25 cycles consisting of 20 seconds at 94°C, 20 seconds at 58°C, and 40 seconds at 72°C. This was concluded with a final extension phase at 72°C for 10 minutes. A 1.5% agarose gel in TBE buffer, stained with Ethidium Bromide, was employed to visualize the PCR product, and images were captured using a BioRad Gel Documentation system.

Antibiotic Susceptibility of *P. aeruginosa* Isolates

An in-vitro sensitivity test was performed on the isolated P. aeruginosa using the Kirby-Bauer disc diffusion method to evaluate its susceptibility to different antibiotics. **Bauer et al. (1966)** using different antimicrobial agents (Oxoid, UK): doxycycline (Do; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), streptomycin (S; 10 µg), amoxicillin (Ax; 10 µg), gentamycin (CN; 10 µg), imipenem (IPM;10 µg),), erythromycin (E; 15 µg) and sulfamethoxazole/trimethoprim (SXT; 25 µg). The results were analyzed following the **CLSI (2023)** guidelines. Multidrug resistance (MDR) Indicators were computed using the methodology outlined by **Tambekar et al. (2006).**

In-vitro antimicrobial efficacy of Mintodin® essential oils

To test the antimicrobial efficacy of Mintodin® against *P. aeruginosa*, the isolate with the highest level of antimicrobial resistance profile was selected. The antibacterial efficacy of Mintodin® were evaluated using the Agar Well Diffusion Method as follows: The bacterial culture was grown in sterile saline and titrated to 0.5 Macfarland (1.5×1^8 CFU/mL) optical density. A sterile cotton swab was used to evenly distribute the bacterial suspension on Mueller Hinton (MH) agar (Oxoid Ltd., England. In each inoculated agar plate, 8 mm wells were made, and 10 µL of Mintodin® at a 100% (v/v) concentration was added to each well. The agar plates were then incubated at 37 °C for 24 hours. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zones in millimeters, with all experiments conducted in triplicate.

Gene Expression Analysis for Quantification of Host Response

QIAamp RNeasy Mini Kit (Qiagen, Germany) was used to extract total RNA from tissue samples.On a StepOneTM real-time PCR system, real-time PCR was carried out using the HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK). B-actin was employed as an internal control gene to assess host responses, such as metabolic, oxidative stress, and immune responses. Table 1 lists the primer sequences and PCR conditions. 10 µL of 2x HERA SYBR® Green RT-qPCR Master Mix, The RT Enzyme Mix (1 µL) (20X), 0.5 µL of each primer (20 pmol), 3 µL of water, and 5 µL of RNA template were combined to create a 20 µL reaction mixture. Every PCR run included a no-template control. According to **Yuan et al. (2006**), we utilized the 2-ΔΔCt method to calculate relative gene expression levels.

Antibiotic: Doxybiotic® Doxycycline hyclate obtained commercially from Memphis Company, Egypt. By dose 20 mg /Kg BW **(Ibrahim et al. 2020).**

Essential oils: Mintodin® obtained commercially from Pro pharma company, Egypt. Composed of (Oregano oil, Mint oil, Eucalyptus oil, Ginger oil and Curcmin oil) by dose 1-2 ml/ Kg diet.

Pseudomonas aeruginosa: *P. aeruginosa*, the field isolate with the highest level of antimicrobial resistance profile was used for experimental infection by dose 0.2 ml of trypticase soy broth containing $3x10^7$ CFU/ml intraperitoneal **(Eissa et al. 2010**).

Experimental design:

A total of apparently healthy 150 Nile tilapia (*Oreochromis niloticus*) , each weighing approximately 35.8 ± 0.5 gram, were sourced from the El-Abassa Fish Hatchery in Sharkia, Egypt. These fish were then transported to the Fish Research Unit at the Animal Health Research Institute, located in the Zagazig branch of Egypt. Fish were fed the control food and allowed to adjust to the laboratory-rearing conditions for two weeks before the experiment started. Fish were bacteriologically examined before experiment to confirm that they were free from *P. aeruginosa* infection. Fish were divided into five equal groups (15 for each with one replicate) in a well-aerated glass tank and dechlorinated tap water was added to each aquarium. All aquariums were maintained under consistent rearing conditions during the acclimatization and experimentation phases. Group 1 (G1) healthy normal fish fed on ration without any supplement (control), Group2 (G2) were experimentally infected by *Pseudomonas aeruginosa* field isolate by dose 0.2 ml ($3x10^{7)}$ CFU/ml intraperitoneal and non-treated (control positive). Group 3 (G3) infected by *Pseudomonas* and treated by doxycycline at dose 20 mg /Kg BW for 1 week 48 hours post infection. Group 4 (G4) infected by *Pseudomonas* and supplemented by essential oils by dose 1-2 ml/ Kg diet for 1 week 48 hours post infection. Group 5 (G5) supplemented by essential oils for 1 week and then infected with *Pseudomonas* then treated with selected antibiotic (doxycycline) by the same dose 48 hours post infection (the experiment time was 3 weeks).

Blood samples:

Under aseptic conditions, blood samples (50) were taken from fish caudal veins. One

milliliter of blood was drawn on EDTA as the initial sample for hematological analysis. The second sample of blood was taken without anticoagulant. It was left to coagulate at room temperature and rotated at 3000 rpm for ten minutes. Serum was collected for biochemical analysis, properly labeled, transferred into clean, dry tubes with covers, and stored frozen at -20°C.

Hematological studies:

Red blood corpuscles (RBCs), concentration of hemoglobin (Hb), and total leukocytic counts were determined according to the hematological procedures routine outlined by **Feldman et al. (2000).**

Biochemical studies:

Each biochemical parameter was measured using commercial kits, and the manufacturer's instructions were followed for each parameter's technique. The activity of the liver transferases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were calculated using **(Murray. 1984).** According to **Kaplan (1984),** serum urea was measured and serum creatinine was approximated using (**Henry, 1974).** The procedure outlined by **Lott and Turner (1975)** was used to measure serum glucose. Glutathione peroxidase (GSH-Px) and catalase (CAT) activities were assessed after following **(Aebi, 1984).**

Histopathological Investigation:

Samples from liver, gills, muscle, intestine were taken from all experimental fish group at the end of this experimental period then fixed in 10% neutral buffered formalin, the samples were dehydrated using ascending degrees of ethyl alcohol, cleared with xylene, and embedded in paraffin wax. Thin sections of 5 micron were prepared on glass slides and stained with hematoxylin and eosin for examination. Then stained sections were inspected and captured on camera using a microscope with a digital camera according to (**Suvarna et al. 2018).**

Statistical analysis:

The statistical analysis employed the analysis of variance (ANOVA). At a significant threshold of 0.05, Duncan's Multiple Range was employed to identify changes in the treatment groups. The SPSS application was used on a PC to run all statistics **(SPSS, 2004).**

RESULTS

Occurrence and molecular identification of *Pseudomonas aeruginosa*

Based on their distinctive blue-green colonies on Pseudomonas agar and their large irregular greenish-blue colonies with a distinctive grape-like odor on nutrient agar, the bacteriological analysis showed that 24 out of 100 fish samples were bacteriologically positive with *P. aeruginosa*, with a total prevalence of 24%. All isolates showed positive biochemical reactions to arginine dihydrolase, nitrite reduction, catalase, oxidase, and ornithine decarboxylase, but negative reactions to methyl red, indole, Gram stain, and Vogues-Proskauer (VP). These isolates were identified molecularly using a species-specific gene (16S rDNA gene) PCR, which showed a distinctive band size of 956 bp.

Antimicrobial Susceptibility results

Antimicrobial susceptibility testing revealed that *P. aeruginosa* isolates exhibited high sensitivity to ciprofloxacin (87.5%) and doxycycline (83.33%) and moderate sensitivity to both trimethoprim/sulfamethoxazole and gentamycin (58.33%). However, high rates of resistance (95.8%) were noticed for amoxicillin, and (87.5%) for erythromycin.

The Resistance profiles showed that 83.33 % (20 out of 24) of the examined isolates showed MDR pattern However, only 16.66% (4 out of 24) among the isolates were XDR pattern. The MAR index for *P. aeruginosa* examined isolates were ranged from 0.33 to 0.88.

Table 1. resistant profile of *P. aeruginosa* isolates (n=24)

Antibiotic agent	Sensitivity %	Resistant %
Doxycycline	20(83.3%)	4(16.66%)
Chloramphenicol	12(50%)	12(50%)
Ciprofloxacin	21(87.5%)	3(12.5%)
Streptomycin	10(41.66%)	14(58.3%)
Amoxicillin	1(4.16%)	23(95.8%)
Gentamycin	14(58.33%)	10(41.66%)
Imipenem	4(16.66%)	20(83.3%)
trimethoprim/sulfamethoxazole	14(58.33%)	10(41.66%)
Erythromycin	3(12.5%)	21(87.5%)

% calculated according to the total number of samples examined

In vitro Antimicrobial Action of *Mintodin®*

The antimicrobial action of the *Mintodin®* against the multi-drug resistant *P. aeruginosa* isolate was evaluated utilizing the agar well diffusion technique. Mintodin® with concentration 100% demonstrated strong antibacterial activity, with a 28 mm inhibition zone diameter (figure 1).

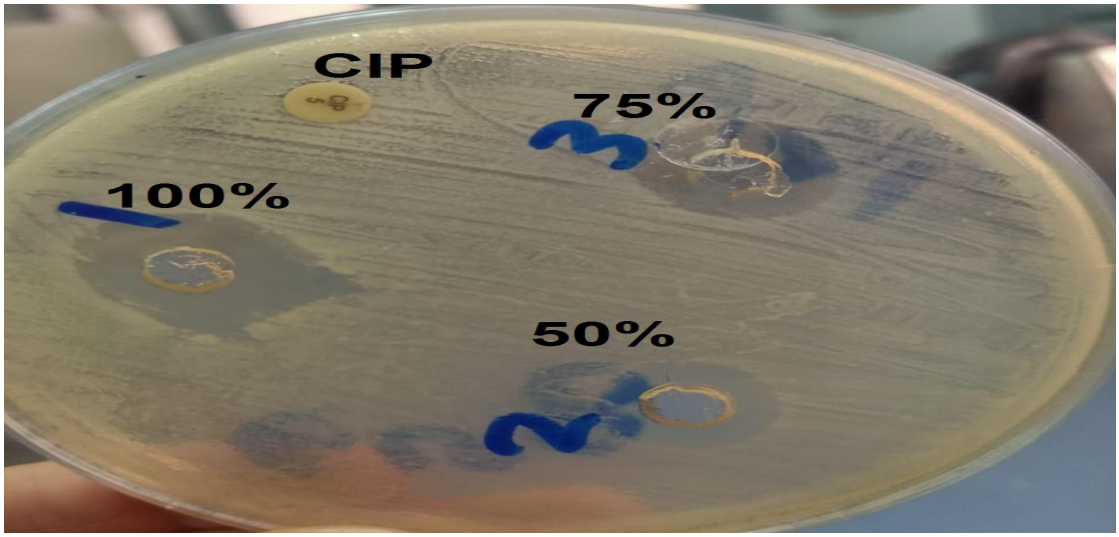

figure 1: The antimicrobial activity of the *Mintodin®* against MDR *P. aeruginosa*

Experimental study findings

Hematological findings

The changes in Erythrogram and leukogram parameters were demonstrated in table (4) showed that the infected non treated group (G2) revealed a significant decrease in RBCs count, Hb, PCV, lymphocytes and monocytes count with a significant increase in TLC and heterophils count compared with negative control group. A significant improvement in RBCs count, PCV, Hb content and leukogram in infected treated groups (G3, G4 and G5) compared to positive control group at 7 and 14 days post treatment.

Biochemical findings

The untreated infected group (G2) demonstrated a significant increase ($p < 0.05$) in serum levels of ALT, AST, urea, creatinine, and glucose, alongside a marked reduction in catalase and glutathione peroxidase (GSH-Px) activities at Days 7 and 14 after infection compared to the negative control group (G1). However, groups treated with doxycycline and/or essential oils (G3, G4, and G5) showed significant improvements in these biochemical parameters relative to the positive control group (G2) during the same periods. (table5).

Table 2. Effect of antibiotic and or some essential oils on Erythrogram and leukogram of healthy and experimentally infected *Oreochromis niloticus* with *Pseudomonas aeruginosa* at 7 and 14 days post treatment (M±S.E) (n=5).

Groups	7 days post treatments					14 days post treatments				
	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
RBCs x10^6 /µl	2.66^{a} ±0.02	1.75^{c} ±0.03	2.20^{b} ±0.06	2.15^{b} ±0.02	2.59^{a} ±0.4	2.87^{a} ±0.02	1.97^{c} ±0.04	2.28^{b} ±0.03	2.20^{b} ±0.02	2.82^{a} ±0.03
Hb (gm/dl)	9.13^{a} ±0.37	6.66^{e} ±0.20	7.92^{c} ±0.30	7.70^{d} ±0.25	8.10^{b} ±0.42	9.56^{a} ±0.27	6.78^{c} ±0.25	8.18^{b} ±0.26	8.27^{b} ±0.28	8.37^{b} ±0.20
PCV %	33.75a ±2.02	25.53^{d} ±1.70	27.59^{c} ±1.50	27.14^{c} ±1.07	29.88^{b} ±2.50	34.40^{a} ±2.30	26.28^{e} ±2.08	28.68^{d} ±1.93	27.94^{c} ±1.90	31.45^{b} ±2.02
TLCx10^3 /µl	10.81^{d} ±1.36	12.36^{a} ±1.20	11.98^{b} ±2.15	11.59^{c} ±1.04	11.00^{d} ±1.17	10.77^{c} ±1.51	12.17^{a} ±1.04	11.95^{a} ±1.70	11.17^{b} ±2.00	11.12^{b} ±1.46
Heterophilsx 10^3 /µl	3.50^{e} ±0.77	6.30^{a} ±0.90	5.20^{b} ±0.27	4.29^{c} ±0.70	3.79^{d} ±0.63	3.74^{e} ±0.31	5.79^{a} ±0.20	5.22^{b} ±0.43	4.02^{c} ±0.45	3.95^{d} ±0.23
Lymphocytesx 10^3 /µl	4.73^{a} ±0.36	3.96^{c} ±0.28	4.36^{b} ±0.17	4.83^{a} ±0.39	4.50^{a} ±0.29	4.56^{a} ±0.60	3.86^{c} ±0.25	4.24^{b} ±0.57	4.63^{a} ±0.44	4.69^{a} ±0.45
Monocytesx 10^3 /µl	1.78^{ab} ±0.04	1.25^{d} ±0.03	1.62^{c} ±0.03	1.70^{bc} ±0.08	1.80^{a} ±0.06	1.77^{a} ±0.02	1.58^{d} ±0.07	1.66^{c} ±0.06	1.73^{ab} ±0.08	1.70^{bc} ±0.05
Eosino-philsx10^3 /µl	0.70 ±0.02	0.75 ±0.03	0.69 ±0.03	0.74 ±0.02	0.70 ±0.02	0.65 ±0.03	0.70 ±0.04	0.72 ±0.02	0.69 ±0.02	0.68 ±0.03

The presence of different letters in the same row indicates a significant change at $p<0.05$

Table 3. Effect of antibiotic and or some essential oils on some biochemical parameters of healthy and experimentally infected *Oreochromis niloticus* with *Pseudomonas aeruginosa* at 7 and 14 days post treatment (M±S.E) (n=5).

Groups	7 days post treatments					14 days post treatments				
	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
ALT U/L	5.48± 0.23^{e}	21.79± 0.88^{a}	11.79± 0.37^{c}	15.48± 0.31^{b}	7.23± 0.20^{d}	5.29± 0.26^{d}	18.48± 0.34^{a}	8.47± 0.24^{c}	13.09± 0.12^{b}	6.10± 0.41^{d}
AST U/L	145.34 ±0.38^{d}	367.38± 1.06^{a}	206.40± 1.70^{b}	202.16± 4.06^{b}	168.61± 1.84^{c}	144.56± 0.49^{d}	300.53± 2.29^{a}	177.01± 3.35^{b}	167.01± 3.09^{c}	151.63± 1.84^{d}
Urea mg/dl	1.66± 0.06^{e}	3.18± 0.02^{a}	2.48± 0.05^{c}	2.65± 0.02^{b}	1.83± 0.03^{d}	1.52± 0.03^{c}	2.55± 0.10^{a}	1.96± 0.06^{b}	2.01± 0.14^{b}	1.62± 0.02^{c}
Creatinine mg/dl	0.16± 0.00^{e}	0.34± 0.00^{a}	0.24± 0.01^{c}	0.27± 0.00^{b}	0.19± 0.00^{d}	0.13± 0.00^{c}	0.29± 0.00^{a}	0.17± 0.00^{b}	0.19± 0.00^{b}	0.13± 0.00^{c}
Glucose mg/dl	68.40± 1.43d	116.88± 0.47a	98.78± 0.90b	88.40± 1.61c	71.09± 0.91cd	68.26± 1.00d	102.88± 1.25a	87.71± 0.98b	74.44± 1.07c	70.53± 0.35d
Catalase U/L	131.29 ±1.64^{a}	46.31± 1.73^{e}	79.14± 2.94^{d}	94.83± 2.69^{c}	113.35± 1.14^{b}	134.55± 1.17^{a}	65.24± 3.09^{d}	91.69± 0.87^{c}	110.53± 0.49^{b}	129.93± 0.91^{a}
GSH-Px µmoL/mg	126.65 ±1.27^{a}	56.83± 0.89^{e}	92.11± 0.46^{d}	99.53± 0.86^{c}	117.78± 1.39^{b}	126.52± 0.65^{a}	66.09± 1.53^{d}	103.32± 1.28^{c}	111.86± 0.87^{b}	123.53± 0.49^{a}

The presence of different letters in the same row indicates a significant change at $p<0.05$

Gene expression findings (Quantitative assessment of *Mintodin®* efficiency using the qRT-PCR)

The findings of antioxidant-related genes' mRNA expression levels (super oxide dismutase: SOD and catalase: CAT) post experimentally infection with multi-drug resistant *P. aeruginosa* was shown in (figure 2). SOD, and CAT genes were upregulated in group 5 (increased by 2.81and 2.80-fold change respectively, vs. the control group2). However, the expression of pro-inflammatory cytokine, IL-6 was significantly downregulated in group (G5) compared to group (G2) by 1.39- fold (**Figure 3**). The expression of metabolic genes, TFAM, was considerably elevated in G5. compared to the control group (G2) by 4.01-fold, respectively (**Figure 4**).

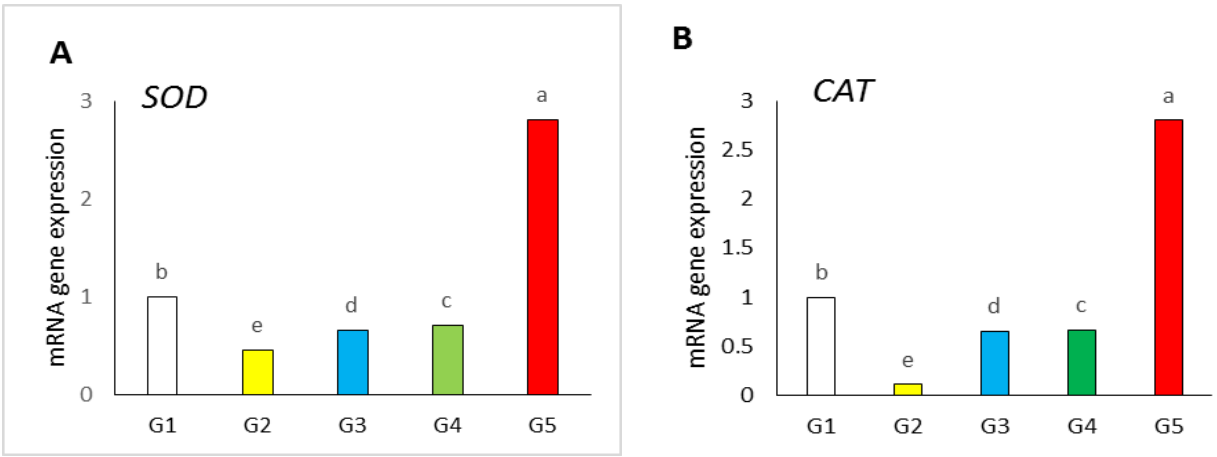

Figure 2: Relative gene expression of oxidative-related genes *SOD* (A), *CAT* (B) in Mintodin® treated fish. Bars with distinct letters show significant differences ($p < 0.05$)

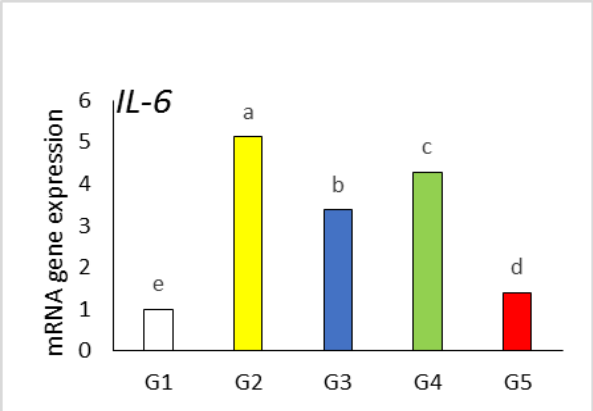

Figure 3: Relative gene expression of Immune-related gene *IL-6* in Mintodin® treated fish. Bars with distinct letters show significant differences ($p < 0.05$).

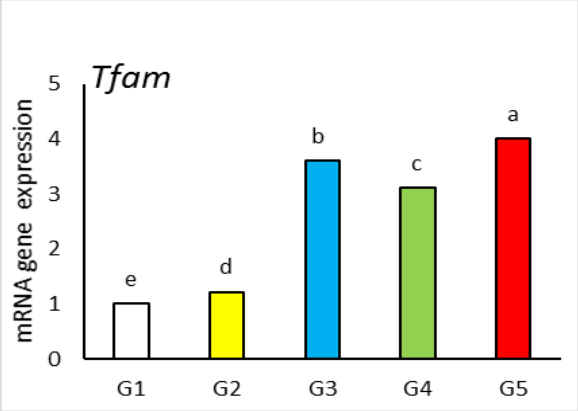

Figure 4: Relative gene expression of metabolic-related gene *Tfam* in Mintodin® treated fish. Bars with distinct letters show significant differences ($p < 0.05$).

Histopathological findings

In comparison with organs of (G1) Nile tilapia fish which revealed normal tissue architecture and cellular details in gills, liver, muscle and intestine tissues, the microscopical examination of fish of group (G2) (challenged-non treated) gills revealed lamellar fusion (Fig.5A). Diffuse telangiectasis (Fig.5B), congestion and hemorrhage was also seen (Fig.5C). Muscle appeared with discontinuity of some muscle fibers with interfibrillar inflammatory cell infiltration (Fig.5D). Intestine showed diffuse vacuolation of submucosal glands (Fig.5E), with destruction of upper parts of lamina propria (Fig.5F) and over branching of some intestinal villi were also observed. (Fig.5G).While liver exhibited congestion in the hepatic blood vessels along with widespread hepatic vacuolation. (Fig.5I). Vacuolation with coagulative necrosis of some hepatocytes represented in pyknosis . Organs of infected fish and treated with doxycycline group (G3) showed gills with normal lamellae (Fig.6A), muscle appeared with normal muscle fiber and vasculature (Fig.6B), intestine was also with normal tissue architecture (Fig.6C). Congestion of hepatic blood vessels with hepatocytes vacuolation (Fig.6D). Organs of infected fish and supplemented with essential oils 48 hours post infection group (G4) The gills displayed congestion in the lamellar blood vessels along with hyperplasia of the lamellar epithelium. (Fig.7A), muscle showed interfibrillar edema (Fig.7B) ,while intestine showed destruction of villus tips and submucosa (Fig.7C). Liver appeared with hyperplasia of melanomacrophage center (Fig.7D). Organs of fish supplemented with essential oils for 1 week and then infected and treated with (doxycycline) group (G5) revealed gills with apparently normal lamellae (Fig.8A),and muscles were apparently normal(Fig.8B).Intestine tissue appeared normal (Fig.8C) .While liver appeared with few inflammatory cells infiltration (Fig.8D)

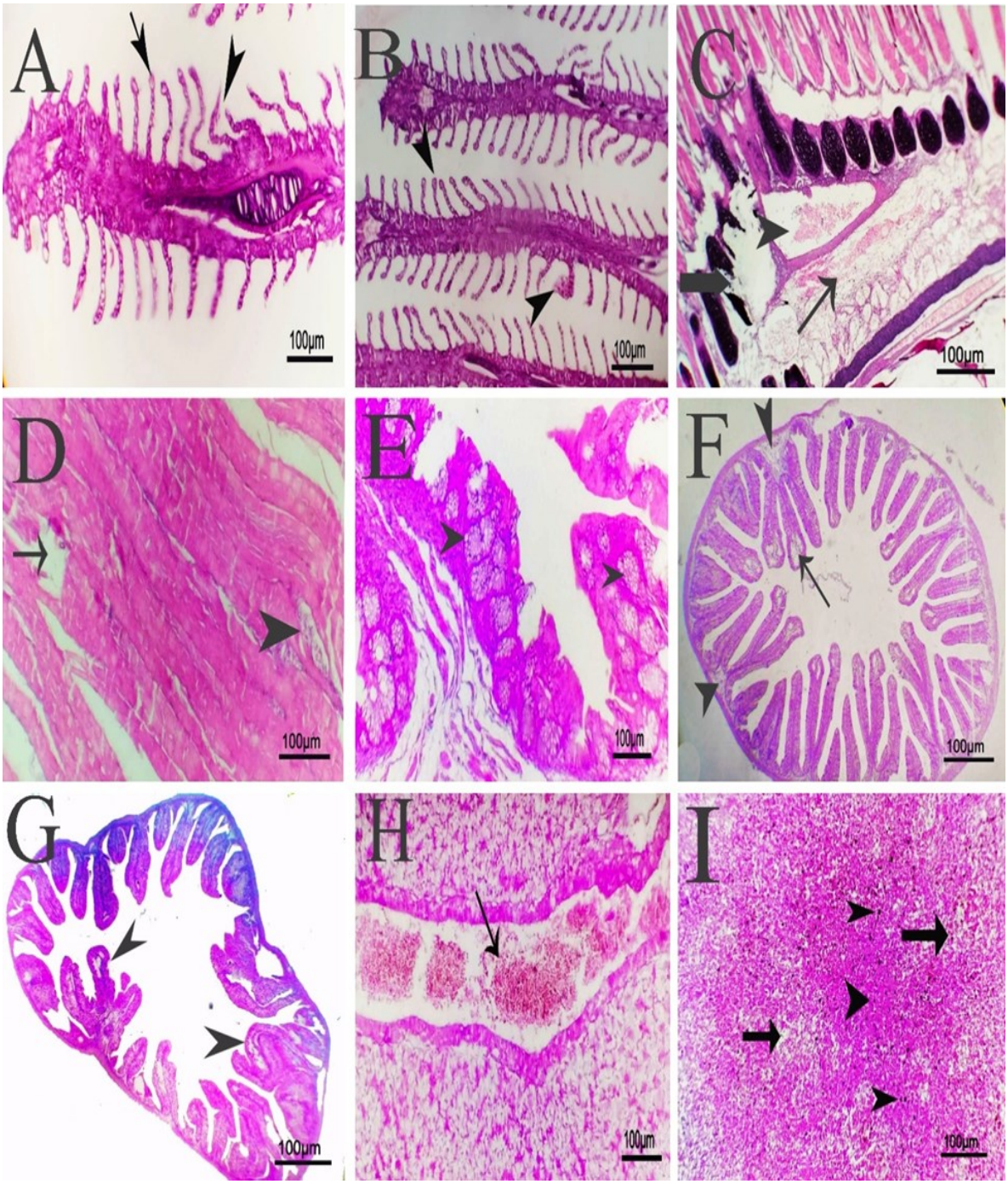

Figure (5): Photomicrograph of H&E sections of group (2) experimental infected fish by Pseudomonas: A) gills showing curved, bent secondary lamellae with almost fusion (arrow head) and telangiectasis (arrow). B) gills with diffuse telangiectasis (arrowhead). C) gills with severe congestion (arrow head) and mild hemorrhage (arrow). D) muscle with discontinuity of some muscle fibers (arrow) and inter-fibrillar few inflammatory cellular infiltration (arrowhead). E) intestine with diffuse vacuolation of submucosal glands (arrowhead). F) intestine with destruction of upper parts of lamina propria . G) intestine with over branching of some villi (arrowhead). H) liver with extensive hepatic blood vessel congestion (arrow) and diffuse hepatic vacuolation. .I) liver with coagulative necrosis (arrows head) and vacuolation of some other hepatocytes (arrows). (Scale bar =100µm)

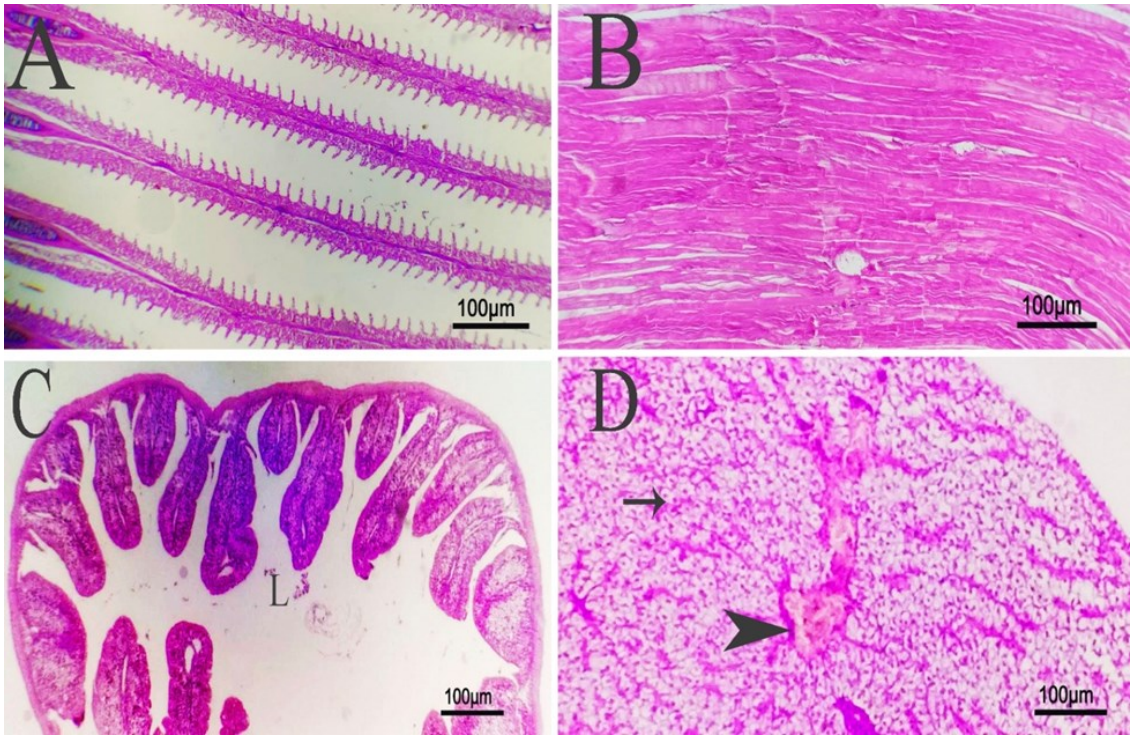

Figure (6): Photomicrograph of H&E sections of group (3) experimental infected fish with Pseudomonas and treated by doxycycline showing: A) gills with apparently normal primary and secondary lamellae . B) muscle with apparently normal muscle fiber and vasculature. C) intestine with normal mucosa , muscularis mucosa and serosa (L= lumen). D) liver revealing congestion of some blood vesseles (arrowhead) and vacuolation of hepatocytes (arrow). (Scale bar =100µm)

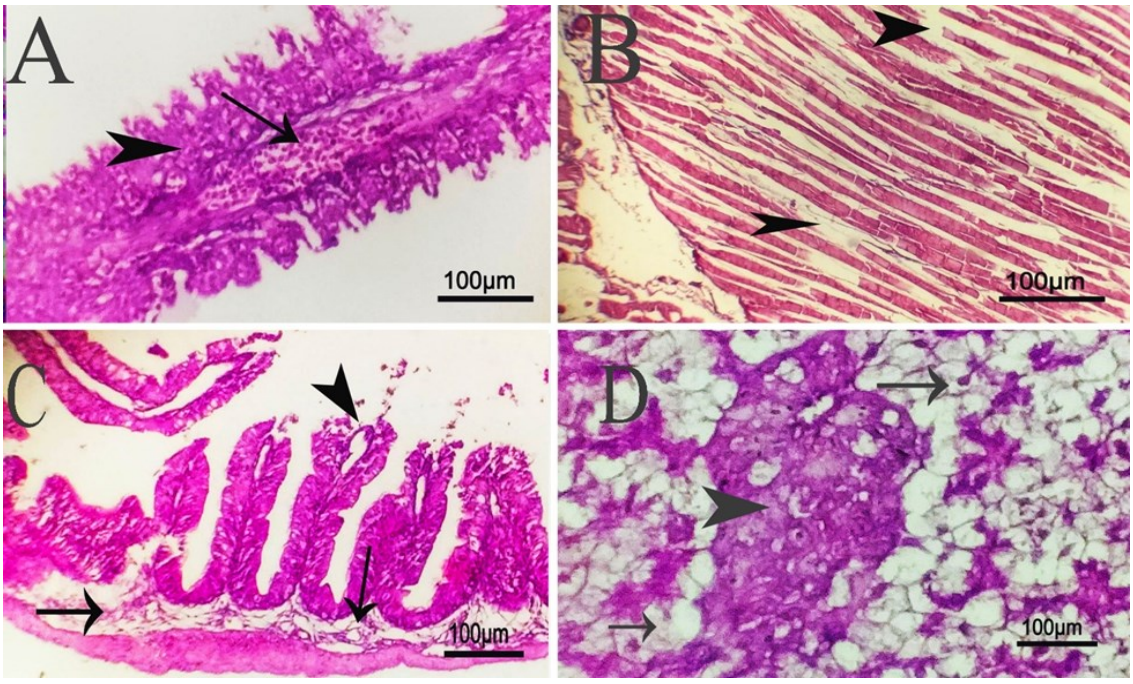

Figure (7): Photomicrograph of H&E sections of group (4) experimental infected fish with Pseudomonas and supplemented with essential oils showing : A) gills with congestion of lamellar blood vessels (arrow) and hyperplasia of lamellae epithelium (arrow). B) muscle with interfibrillar edema (arrows head). C) intestine with destruction of villus tips (arrowhead) with submucosal edema and destruction (arrow). D) liver with hyperplasia of melanomacrophage center (arrowhead). (Scale bar =100µm)

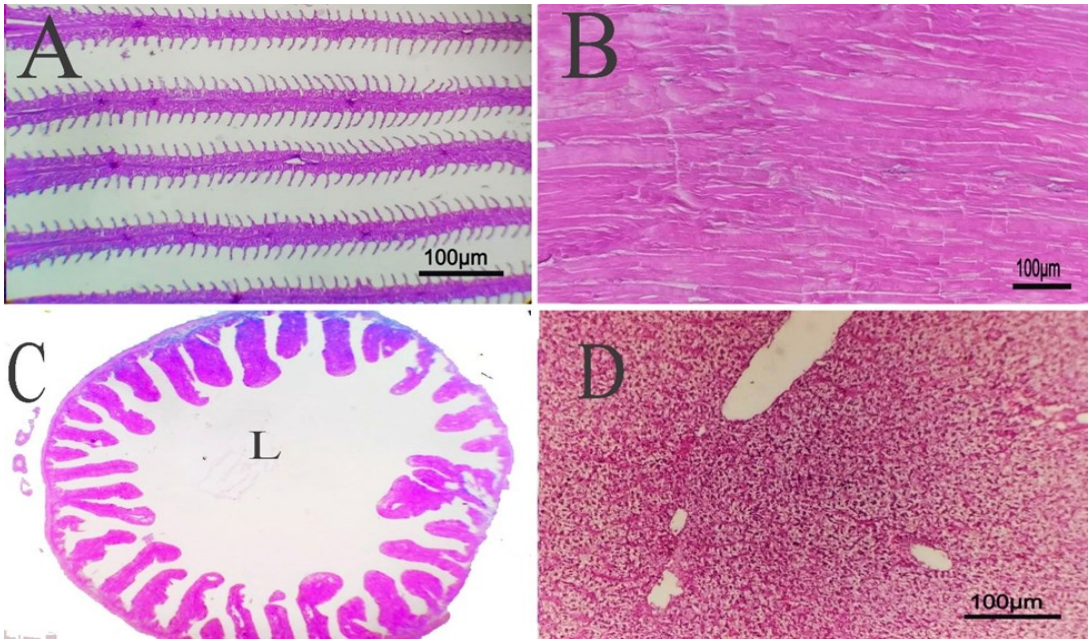

Figure (8):Photomicrograph of H&E sections of group (5) fish supplemented with essential oils for 1week and then infected with Pseudomonas then treated with selected antibiotic (doxycycline) showing: A) gills with apparently normal primary and secondary lamellae . B) Muscle with apparently normal muscle fiber and vasculature . C) intestine with normal mucosa, muscularis mucosa and serosa (L= lumen). D) liver showing few inflammatory cells infiltration in the hepatic tissue . (Scale bar =100µm).

DISCUSSION:

The majority of fish species are vulnerable to *Pseudomonas* species, which causes moderate to significant financial losses **(Somsiri and Soontornvit, 2002**). Freshwater fish septicemia is primarily caused by *Pseudomonas aeruginosa*, which causes enormous financial losses for the industries that produce fish all over the world (**Roberts, 2012**). For the bacteriological analysis of *Pseudomonas aeruginosa*, 100 fish samples (O. niloticus) were randomly selected for the investigation. and the occurrence rate was 24%. Almost identical outcomes were noted by **Eissa et al. (2010)**. Meanwhile, disagreed with others who recorded lower incidence, **EL-Hady and Samy, (2011**) and **Elham et al. (2017**) who recorded higher prevalence. **Shahrokhi et al. (2022)** reported a lower prevalence of Pseudomonas aeruginosa in freshwater fish (5%), whereas **Mohamed et al. (2023)** and **El-Tarabili et al. (2023)** isolated the bacteria in freshwater fish at 29% and 32 %, respectively. Prevalence variations may be caused by host susceptibility, environmental variables, geographic distribution, and sample collection season (**Algammal et al. 2020).** Gene specific to a species even for rare isolates, (16S rDNA) provides clear information, making it a useful initial gene for *P. aeruginosa* detection **(Abu-Elala et al. 2016**).

According to our PCR results, this gene was found at 956 bp in all identified *P.aeruginosa* strains.

Long-term use of antibiotics to treat Pseudomonas infections causes R-plasmids to spread, creating multidrug-resistant strains in aquatic environments (**Abd-El-Maogoud et al. 2021**). *Pseudomonas aeruginosa* has become resistant to several antibiotics, including β-lactams, quinolones, and aminoglycosides **(Hancock and Brinkman 2002)**. In this study, *P. aeruginosa* isolates exhibited increased susceptibility to ciprofloxacin (87.5%) and doxycycline (83.3%) which was contradicting the results of **Benie et al. (2017)** who documented that *P. aeruginosa* isolates exhibited primary resistance to ciprofloxacin. While the isolates were resistant to amoxicillin (95.8%), and erythromycin (87.5%) which, like another

result by **Abd El-Baky et al. (2020),** documented that the isolates of *P. aeruginosa* showed total resistance to clavulanic acid and amoxicillin.

The Resistance profiles showed that 83.33 % (20 out of 24) among the isolates were multidrug resistance (MDR) pattern. However, only 16.66% (4 out of 24) of the examined isolates showed an extreme drug-resistant (XDR) pattern. These results closely align with the findings of **Abou Elez et al. (2024)** who noted that when *P. aeruginosa* tested isolates were examined, 23.1% showed XDR and 61.5% showed MDR. The MAR index for *P. aeruginosa* examined isolates were ranged from 0.33 to 0.88. The results of **Darwish et al. (2023)** in Egypt were in agreement with this study's MAR of more than 0.2. The frequency of MDR *P. aeruginosa* ranges from low in Saudi Arabia (0–7.3%) to high in Egypt (50–75%) due to differences in data gathering methods and sample sizes **(Al-Orphaly et al. 2021)**. The absence of pan drug resistant (PDR) Pseudomonas in this investigation was consistent with **Abd El-Baky et al. (2020)** and **Abou Elez et al. (2024**). Additionally, because fish infections are become increasingly resistant to conventional antimicrobial therapy, study on the antibacterial properties of natural materials like essential oils (EOs) is required **(Kluga et al. 2021).**

The result of antimicrobial activity of the Mintodin® EOs against the multi-drug resistant *P. aeruginosa* isolate showed that Mintodin® demonstrated notable antibacterial efficacy, producing an inhibition zone measuring 28 mm in diameter which higher than previous recorded with **Shehata et al. (2013**) who found that T. vulgaris EOs had antibacterial activity with a 13 mm inhibition zone diameter on *P. aeruginosa*.

To evaluate the anti-oxidant efficacy of Mintodin® post experimentally infection with the multi-drug resistant *P. aeruginosa* we investigated the modulatory effect of Mintodin® on expression of antioxidant-related genes (super oxide dismutase: *SOD* and catalase: *CAT*). SOD, and CAT genes were upregulated in group 5 (increased 2.81and 2.80 – fold change respectively, vs. the control group2). SOD is an antioxidant enzyme that is essential to the first line of defense because it detoxifies H2O2 and free radicals **(Fridovich 1995).** CAT, another essential antioxidant defense enzyme, is believed to be a key biomarker for evaluating oxidative stress by observing changes in activity **(Kim et al. 2017)**.

Moreover, the result displayed decreased the expression levels of the pro inflammatory related gene *IL-6* un like the control group 2 (up to 1.39 fold change respectively). In line with previous studies, the high expression levels of inflammatory related genes in G2 justified by **Drolia et al. (2018)** who proposed that an infection could affect the permeability of the gastrointestinal epithelium by increasing the IL-6 and TNF-α genes expression.

However, the transcription of metabolism related gene *Tfam* significantly decreased in G5 compared to the control group2(decreased by 4,01 – fold change respectively). According to certain reports, TFAM regulates cell migration, proliferation, and survival (**Yoshida et al. 2005).** A transcription factor found in the mitochondria, TFAM controls the biogenesis and function of the mitochondria **(Xu et al. 2023**).

Since blood is a component of the majority of mobile tissues, the cell composition and protein pattern of serum can be used as a trial indicator of diseases or changes and accurately depict the immunophysiological state of a fish organism. The hematological parameters changes in infected non-treated group (G2) may be due to the pathogenic effect of pseudomonas which cause anemia as a result of disruption in the production of RBCs and the destruction of hematopoietic organs whose explained the RBCs, Hb and PCV reduction and that agree with **(El Azzy et al. 2020, Zafar et al. 2023 and Alemu et al 2024).** Group 3, 4 and group 5 which administrated antibiotics or essential oils showed a significant improvement in the hematological parameters. **El Azzy et al. (2020)** and **Salah et al. (2022a)** indicated that supplementation of essential oil (*Origanum vulgare L.*) revealed a notable en-

hancement in hemoglobin, red blood cells and white blood cells count due to improvement oxygen carrying capacity. Hemoglobin may show how much oxygen is in the blood, and under stress conditions, anoxia can happen, which stops the body's other energy-synthesis method, oxidative phosphorylation **(Ramesh et al. 2018). Oliveira et al (2022)** stated that**,** fish treated with doxycycline exhibited no significant changes in erythrocyte counts ($P > 0.05$).

Leukocytosis and heterophilia observed in the infected group in our study could be attributed to antigen stimulation caused by bacterial infection. This, in turn, may trigger an inflammatory response, leading to the activation of the immune system **(Nasr El –Deen et al, 2019)**. Treated birds with a combination of antibiotics and essential oils showed improvement in the leukogram compared to non-treated group. Our results were supported by **Gehan et al. (2011)** who recorded that, volatile oils and volatile oils co-administered with oxytetracycline resulted in an improvement in blood picture which returned to its control level. **Oliveira et al. (2022)** who said that during treatment with this tetracycline, no changes in the quantity of circulating leukocytes were seen, indicating that therapy with doxycycline's low toxicity does not cause inflammatory alterations. The reduction in monocyte and neutrophil counts on the 7th day following 20 mg of doxycycline treatment supports the results of **(Maklakova et al. 2011)** in rainbow trout (*Oncorhynchus mykiss*) treated with five doses of oxytetracycline at 20 mg/kg.

Regarding to the recorded results of serum biochemical parameters. Infected non-treated group revealed significant rise in ALT, AST, urea, and creatinine levels in the blood. The same results obtained by **EL-*Wafai et al.* (2024)**, who investigated that *Pseudomonas aeruginosa* infection in Nile Tilapia show significant increase in AST, ALT, urea and creatinine. *P. aeruginosa*-induced infection stress causes liver and renal damage, as seen by abnormalities in the ALT, AST, urea, and creatinine levels. **Magdy et al. (2014)** and **Soror., (2020)** observed same outcomes in *P. aeruginosa*-infected African catfish and common carp.

Fish infected with *Pseudomonas aeruginosa* and non-treated showed significant increase in serum glucose level. **Abd- Allah et al. (2015)** recorded an increase in glucose level in African catfish (*Clarias gariepinus*) Infected with *Pseudomonas florescence*. The treated groups (G3, G4 and G5) which received doxycycline and or essential oils showed significant improvement in the mention parameters. **Ibrahim et al. (2020)** recorded that doxycycline could improve serum ALT, AST and creatinine levels in *Clarias gariepinus* challenged by *Aeromonas hydrophila.* This improvement could be attributed to the bacteriostatic effect of the drug **(Flower et al. 2012).** This helps minimize the harmful and toxic effects on the liver and kidneys. In addition, the group that received essential oils before infection recorded the best results. Oregano essential oil's high carvacrol and thymol concentration gives it antibacterial, anti-inflammatory, antioxidative, and immunological-modulating properties in aquatic species **(Hernández Nava et al 2020).**

The antibacterial qualities of essential oils have applications in alternative medicine, pharmaceuticals, and the food industry. **Tarek et al. (2014**). **Ghafarifarsani et al. (2022)** found that thyme essential oil significantly lower creatinine, glucose and urea in common carp. Otherwise **Zeppenfeld et al. (2017)** found that the glucose level of silver catfish fed a meal fortified with 2.0 mL per kilogram of EOs for 21 days is unaffected. It is advised to add 2% dietary oreganum essential oil to the diet of Nile tilapia in order to enhance blood biochemical parameters and indicators of liver and kidney function **(Salah et al. 2022b).** Among the challenged groups, the fish with the lowest serum levels of ALT, urea, and creatinine were those exposed to Pseudomonas aeruginosa and treated with a combination of florfenicol and origanum oil **(Rehan et al. 2024).**

Oxidative stress is closely linked to the onset and progression of disease, and pathological conditions may result from an imbalance in the body's antioxidant system **(Song et al. 2023).**

The present study revealed significant decrease in antioxidant markers (Catalase and GSH-Px) in infected non-treated group. A rise in reactive oxygen species (ROS) release in the cell membrane due to *Pseudomonas aeruginosa* toxins may result in oxidative damage and a compromised antioxidant system. **Suntres et al. (2002). Abdel Rahman et al. (2024)** detected a significant decrease in antioxidant markers in *Oreochromis niloticus* challenged by *Pseudomonas putida.* Also **Alzahrani et al. (2023)** shown that *Pseudomonas putida* inhibits the activity of antioxidant indicators, such as catalase and SOD. The treated groups showed a significant improvement in catalase and GSH-Px activities. **Xu et al. (2023)** demonstrated that doxycycline increase the activity of GSH-Px in soft coral *Sarcophyton trocheliophorum.* Silver catfish exposed to Melaleuca alternifolia essential oil have higher glutathione-S-transferase activity **(Souza et al. 2018b**). The oxidative state of freshwater fish was often enhanced by dietary supplementation with various essential oils. Catalase and superoxide dismutase levels were elevated in channel catfish (Ictalurus punctatus) fed 0.5 mL per kg EO of Origanum vulgare in comparison to fish fed a control diet **(Zheng et al. 2009).** Compared to silver catfish fed a control diet, those fed diets supplemented with EO of L. alba (linalool chemotype, approximately 0.5 –2.0 mL per kg) showed increased glutathione peroxidase, catalase, and superoxide dismutase activity **(Saccol et al. 2013).** *Pseudomonas aeruginosa* is among the most common bacteria known to adversely affect fish **(Ndi and Barton, 2012; Shahrokhi et al. 2022).**

In our study, the clinical signs and postmortem lesions observed in the infected fish were loss of balance, fin erosion and erection of tail, loose scales, darkening with red spots on the body surface, mucus secretions on the skin and gills, accompanied by ascites and a slightly protruding reddish vent, and some fish showing slight exophthalmia and congested gills, congested liver these results partially similar to several studies **(Hossam et al. 2015; Khairnar et al. 2013). Eissa et al. (2010)** reported typical clinical signs of *Pseudomonas* septicaemia of the infected tilapia including hemorrhages over body surface, loosening scales with abdominal distending, cloudiness of eyes, and congested gills, congested liver similar results run parallel with those obtained by ***Algammal* et al. *(2020) and Osman* et al. *(2021)*** . *P. aeruginosa*-infected Nile Tilapia had off-food, tail erosion, , exophthalmia and ascites, fish lost escape reflex and swam at the water surface these result noted by **Aboyadak et al. (2024)**. These clinical signs and postmortem lesions might be caused by the virulence factor of bacteria and its toxin production according to *pseudomonus* pathogenicity. Pseudomonades is characterized by haemorrhagic spots all over the body, skin ulcer, tail rot and exophthalmia with congested gills and liver were reported by **El-Keredy and Naena., (2020)**.

In the present study**,** histopathological examination of gills revealed lamellar fusion, congestion and hemorrhage similar result reported by **Tohamy, (2015) and Abdi et al. (2024)** who showed gills of infected Nile tilapia with *P.fluorescens*, with congestion and lamellaer fusion duo to basal hyperplasia and necrosis. **Hossam et al. (2015)** reported that the gills of experimentally *P. fluorescens* infected Nile tilapia revealed epithelial hyperplasia-induced branchial blood vessel congestion, lamellar lifting, stunting, and secondary lamellar fusion as a result of virulence factor producing by bacteria. **Magdy et al. (2014)** docomented that gills of African Catfish infected with *P.aeruginosa* revealed hypertrophy, epithelial hyperplasia of the secondary lamellae causing lamellar epithelial lifting, fusion, necrosis, and edema with lamellar epithelium desquamation similar lesions have been reported by **Devakumar et al. (2013)** who observed that degenerative changes in all tissues of crabs infected with P. aeruginosa **.** Our histopathological examination of organs of Nile Tilapia fish challenged with *Pseudomonas* revealed signs of congestion, inflammations, and degenerative changes of the gills, intestine, and liver similar to **(Saikia et al. 2018; Oh et al. 2018; Ali et al. 2021; Aboyadak et al. 2024)**. However, according to **(Lupia et al. 2024),** *P. aeruginosa*-infected tilapia fish showed severe signs such gill necrosis and hemorrhagic septicemia. In our result, muscle showed interfibrillar inflammatory cellular infiltration. **Abdi et**

al. (2024) demonstrated that muscle necrosis and mononuclear cell infiltration in experimental *P. Fluorescens* Infected Nile tilapia. In our result liver showed severe congestion of hepatic blood vessels and diffuse hepatic vacuolation with coagulative necrosis were agreed with **Elgohary et al. (2020b)** who recorded that congestion in hepatoportal blood vessels, vacuolar degeneration in hepatocytes, and necrosis in hepatocytes of Nile tilapia infected with Vibrio vulnificus. **Aboyadak et al. (2024)** demonstrated that hepatocellular vacuolation, mononuclear inflammatory cell infiltration, severe inflammation with necrotic foci in liver of O.niloticus fish experimental infected with *P. aeruginosa* . **Magdy et al. (2014**) stated congestion in portal blood vessels and degeneration of *P. aeruginosa* infection in African Catfish. Whereas focal areas of degeneration with necrotic hepatocytes of liver of Nile Tilapia expermintal infected with *P.putida* were demonstrated by **Abd-El-Rahman et al. (2024).** Moreover, vacuolization with mononuclear cell aggregation and congestion were seen in the liver of Nile tilapia infected with *P. fluorescens* **(Hal and M. 2020; Abdi et al. 2024**). Congestion of gills and hepatic sinusoid in *P. fluorescence* infected fish were noted by **Hossam et al. (2015)** who also reported congestion and hemorrhagic enteritis**.** Necrosis of the gills, liver, and intestine of the Nile Tilapia challenged with *P. aeruginosa* were observed by **Rehan et al. (2024).** Coagulative necrosis of hepatocyte, with thickening and proliferation f secondary lamellae of gills of infected O. niloticus with Vibrio alginolyticus were reported by **El-Gohary et al. (2020a)**

P.aeruginosa produce virulence factors, its toxic effects inducing oxidative stress and resulting in cellular death by interfering with different cellular processes this effect depend on its cocentration, modifying the expression and release of numerous cytokines these previous result reported by **Ran et al. (2003) and Muller., (2006).** In present study the histopathological finding of Nile tilapia fish organs challenged with Pseudomonas and treated with doxycycline showed improvement in the pathological changes of gills ,muscle, liver and intestinal tissue which were appeared normal. Doxycycline have significant effect in reducing the pathological changes as aresult of their bacteriostatic action recorded by **Flower et al. (2012). El-Gohary et al. (2020a)** recorded that improvement in liver of the infected *O. niloticus* with *Vibrio alginolyticus* treated with florfenicol then enrofloxacin and oxytetracycline respectively .Also improvement the toxic effecets and destructive changes in the liver of African catfish infectied with A. hydrophila as aresult of bacteriostatic action of Doxycycline were reported by **Ibrahim et al. (2020).** While **Aboyadak et al. (2024)** reported that enrofloxacin when combined with medicated feed protected Nile Tilapia from susceptible *P. aeruginosa* infection effectively. **Abd El-Tawab et al. (2019)** concluded that, most infections induce ulcer type disease with ulcerative and septicemic symptoms of fish, which include Aeromonas and Pseudomonas species, in particular isolates of A. hydrophila, A. caviae, Ps. aeruginosa, and Ps. fluorescens, showed tolerance to ciprofloxacin, norfloxacin, gentamycin, florphenicol, and meropenem, and can be employed as therapeutic agents..Meanwhile, Pseudomonas strains were intermediate sensitive to oxytetracycline partially same results were obtained by **El-Hady and Samy (2011),; Roy et al. (2014),; Abd El Tawab et al., (2016) and Abd El-Tawab et al. (2019).** In our study, organs of fish challenged with Pseudomonas and supplemented with essential oils, emerged improvement in pathological changes. Liver showed hyperplasia of melanomacrophage center that attributed to stimulation of liver with bacterial toxin and indicating the role of essential oils as immunostimulants and disease resistance. **Rehan et al. (2024)** domonstrated that Nile Tilapia fish infected with Pseudomonas aeruginosa and received *Origanum* oil showed marked improvement in the pathological changes with some focal lesions in the intestine and liver were observed.

Organs of fish supplemented with essential oils and treated with doxycycline in the present study showed normal tissue architecture and cellular details in gill, liver, muscle and intestine. Essential oils and their components have synergistic effects against bacteria when combined with antibiotics. **Rehan et al. (2024)** who stated that the infected fish received *Origanum* oil and florfenicol displayed a normal

histological architecture of gills, liver and intestine tissue. **Magouz et al. (2022**) recorded that improved intestinal health of Nile Tilapia fish supplemented with oregano essential oil demonstrated apparent branching of villi with increasing villi length and width. **Abdel-Latif et al. (2020)** documented that beneficial improvement in the histopathological finding of hepatic tissues of common carp fed oregano essential oil with different levels were noticed no pathologic lesions, the intestinal tissue was also revealed a significant improvement (when fed at a dose of 15 g/kg diet) without inflammation or degenerative changes. Plant essential oils have been shown to improve appetite in farm animals, and have significant antipathogenic activities in fish, It possess bioactive molecules which play a significant role in supporting optimum health and gut microbiate statue of fish **(Caipang, 2020).**

Essential oils such as oregano, thyme, and curcumin are commonly used in aquaculture and have been related to beneficial effects on fish health statue **(Aydın and Barbas, 2020)**. Essential oils when used as food additives combined with complementary derivatives improve fish welfare more effectively than using in an individual forms **(Ning et al. 2021)**.

Recently, many researchers are focusing on using of natural organic herbal as growth promoters to improve the gut functions, stimulate growth, and enhance animal health by adding different feed supplements to increase animal disease resistance and growth performance. Essential oils showed to improve the health and welfare of aquatic animals and increase their resistance to infectious diseases **(Alagawany et al. 2020; Dinardo et al. 2020)**.

CONCLUSION:

The obtained results suggested that the administration of essential oils (Mintodin®) before infection then the doxycycline against *P. aeruginosa* infection could modulate hematological, biochemical and pathological changes induced by *P.aeruginosa* infection in addition to enhancing the expression of antioxidant genes.

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