Pathological and biochemical effects of pumpkin seed oil and florfenicol on *Clarais species* fish challenged with *Aeromonas hydrophila.*

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ABSTRACT

The goal of the current study was to look into the immunological and histopathological effects of pumpkin seed oil against *Aeromonas hydrophila* in fish of the Clarias species. In the experiment, fifty fish were split equally into five groups. Group 1 (G1) normal healthy fish non-infected, and non-treated (negative control). Fish from Group 2 (G2) were inoculated intraperitoneally with 0.2 mL of 24h broth cultures of *Aeromonas hydrophila* and kept untreated (positive control), while fish from Groups 3, 4, and 5 (G3, G4, and G5) were inoculated intraperitoneally with 0.2 mL of 24h broth cultures of *Aeromonas hydrophila* (2.5×10^6 mL) and then fed on a diet supplemented with florfenicol (G3), pumpkin seed oil (40 mg/kg diet) **(**G4) and pumpkin seed oil plus a therapeutic dose of florfenicol (G5), respectively. The obtained results showed that the erythrocytic count, Hb concentration, and packed cell volume significantly decreased in the *A. hydrophila* (G2)-infected and untreated fish. The leucocytic count and lymphocytes, however, significantly increased during the two study periods (1st and 10 days post-treatment). Fish that were experimentally infected with *A. hydrophila* and left untreated (Group 2) demonstrated a considerable rise in glucose, T. cholesterol, TG, HDL, LDL, and VLDL levels throughout the course of the two experimental periods. In contrast, the infected group that got pumpkin seed oil just (Group 4) and pumpkin seed oil combined with florfenicol (Group 5) displayed an improvement in liver and kidney markers, glucose, and lipid profiles, as well as an elevation of anti-inflammatory, antioxidant, and immunological parameters (phagocytic ratio and index) at the two examined periods. They also displayed histopathological changes in all examined organs.

Keywords: Pumpkin seed oil, *Aeromonas hydrophila*, Catfish, Florfenicol

Introduction

With an average annual growth rate of 8.9% since 1970, aquaculture is currently the fastest-growing food-producing industry worldwide **(Bondad-Reantaso et al., 2005)**. However, severe issues including illness and environmental circumstances are present in aquaculture farming. bacterial infections have caused significant losses in the fish industry, including high morbidity and mortality, limiting both economic and social development, slowing growth, and raising the cost of chemicals for both treatment and prevention **(Lafferty et al., 2015)**.

Aeromonas hydrophila is a bacterium that is commonly found in aquatic habitats and is an opportunistic pathogen for fish, reptiles, amphibians, and humans **(Janda and Abbott, 2010)**. It causes hemorrhagic septicemia and skin ulceration in aquatic species as well as diarrhea in mammals **(Nielsen et al., 2001; Peatman et al., 2018)**.

Due to their antibacterial and anti-parasitic effects on animal digestive systems, medicinal plants and their extracted essential oils are becoming significant growth promoters in animal farming and aquaculture. The use of herbs and plants as medicines has been described in several studies, some of which cite information from other scientific literature. More than 30% of fish are affected by endoparasite infections including protozoa, nematodes, trematodes, and cestodes, which can be treated with medicinal herbs like pumpkin seed oil **(Wink, 2012)**.

A member of the Cucurbitaceae family of angiosperms, the pumpkin. The pumpkin fruit contains a wide variety of nutrients, such as pumpkin polysaccharides, active proteins, vital amino acids, carotenoids, and minerals **(Fokou et al., 2004)**. Pumpkin seeds are very nutrient-dense and contain high-quality oil that has antimicrobial, anti-diabetic, antifungal, antibacterial, anti-inflammatory, and antioxidant properties **(Wang and Ng, 2003)**. The effects of adding pumpkin oil, a naturally occurring growth-promoting ingredient, to fish diets and how it affects helminth parasites in the intestine and abdomen **(Hajati et al. 2011)**.

The most important and dispensable strategies in animals for the prevention and treatment of bacterial infections, and also as growth promoters are antibiotic drugs **(Cabello et al., 2013)**. However, the excessive use of antibiotic drugs in the aquaculture industry has led to the development of antibiotic resistance, thereby reducing antibiotic efficacy **(Hatha et al., 2005; Stratev and Odeyemi, 2016)**.

Florfenicol is a broad-spectrum antibiotic in the phenicol class approved for use in a wide variety of fish species in 25 countries around the world with proven efficacious in a wide variety of fish-related bacteria specially *Aeromonas spp*. To ensure that florfenicol is present in plasma concentrations that surpass the highest MIC values for Aeromonas bacteria for longer than the inter-dose interval, repeat daily dosing at 10 mg florfenicol/kg bw for 10 days (as per the label's instructions) is necessary **(Gaunt et al., 2012)**.

The purpose of this study is to determine the best combination of the natural antimicrobial (pumpkin oil) and the synthetic antibiotic, florfenicol, to treat *Aeromonas hydrophila*-infected *Clarias species*. Particular attention was paid to the hematological, biochemical, and pathological effects in fish.

Material and Methods

Bacteriological examination:

Fifty Nile catfish (*Clarias gariepinus*) were collected from various locations in Sharkia Governorate. Fish were brought alive to the Animal Health Research Institute, Zagazig branch. Fish were clinically examined in glass aquariums filled with aerated, chlorine-free tap water. Tissue samples were taken, including lesions that appeared to be pathological in the gills, kidney, liver, and intestine. These samples were made on tryptic soya broth (TSB; Oxoid, England), which was incubated at 28°C for 24 hours. Loopful from each broth sample was streaked over Aeromonas selective agar-base, ASA (Biolife, Italy), and then incubated at 28°C for 24 hours, where green colonies with dark cores were presumed to represent Aeromonas **(Austin and Austin, 2012; Markey et al., 2013)**.

Phenotypic and biochemical identification:

Phenotypic characterization was done according to **Austin and Austin, (2016)** and was dependent on a series of morphological, biochemical, and metabolic activities, these tests included gram staining, oxidase test, catalase test, indole production, methyl red, Voges Proskauer, Simmon's citrate, TSI, Motility test, and H2S production, were performed to ensure *A. hydrophila*.

Sensitivity test:

The disc diffusion technique was used following **Bauer et al. (1966)**. Cefotaxime 30 mg, ciprofloxacin 5 mg, florfenicol 30 mg, gentamycin 10 mg, norfloxacin 10 mg, oxacillin 30 mg, oxytetracycline 15 mg, and sulphamethoxazole Trimethoprim (1.25/23.75) mg were the antibiotics contained in the antibiotic discs. This method was carried out in accordance with the National Committee for Clinical Laboratory Standards guidelines **(NCCLS, 1994)**.

Preparation of the inoculum:

Preparation of the *A. hydrophila* inoculum (bacterial suspension), whose oxytetracycline sensitivity had previously been established using an *in vitro* susceptibility test was done according to the following steps. After being freshly grown on a TSA (tryptic soy agar) plate for 48 hours at 25°C, *A. hydrophila* colonies were combined with sterile physiological saline (6×10^6 cfu/ml) and adjusted using McFarland's standards **(McFarland, 1907)**. In an experiment, 0.5 ml of the produced inoculum was administered intraperitoneally to the fish to infect them **(Emeish et al., 2018)**. Care was used when administering injections to prevent internal organ punctures. All of the injected fish were subsequently moved to aquariums, where daily records of clinical symptoms, postmortem lesions, and mortalities were made for ten days (experimental period). Experimentally infected moribund fish during the experimental period had their internal organs tested for *A. hydrophila* re-isolation. Traditional bacteriological methods were utilized to validate

the positive culture to assure that the re-isolated strains were the same as the isolates used in the experimental infection.

Determination of MICs by dilution method (Owuama, 2017)

The inoculum preparation:

The inoculum was prepared for each bacterial isolate, adjusted to a turbidity equivalent to 0.5 McFarland standards, and diluted by sterile saline to give a concentration of 6×10^6.

Serial dilution of pumpkin seed oil

In Muller Hinton broth, two-fold serial dilutions of pumpkin seed oil were made from a stock solution containing 1.024 mg/ml DMSO. To ascertain the MICs of florfenicol and pumpkin seed oil for Aeromonas isolates, the dilution method was modified. In test tubes filled with sterile nutritional broth, pumpkin seed oil was diluted into various quantities of 1000, 500, 250, 125, and 6.25 g/ml. A loopful (10 µl) of the *Aeromonas hydrophila* culture, together with 0.5 McFarland standards **(Eucast, 2003)**, were inoculated into test tubes containing 2 ml of the various concentrations of pumpkin seed oil in nutrient broth using a conventional wire loop (Merck). These tubes were kept at 37°C for 18 to 24 hours, following which growth or turbidity was checked. A loopful of broth from each test tube that did not demonstrate growth was then injected into a plate of nutritional agar **(CLSI, 2012)**. The MICgenus90 and MIC90 values of the four most abundant species for the phenols class (chloramphenicol and florfenicol), gentamicin, and temocillin were not significantly different, with a maximum of one dilution step variation **(Baron et al., 2017)**.

Experimental design:

A private fish farm in Sharkia governorate provided fifty Nile catfish (*Clarias garipeinus*), each weighing between 55 and 70 g and measuring between 23 and 30 centimeters. They spent two weeks acclimated to drinking dechlorinated tap water while being maintained in a well-ventilated glass aquarium. Each tank had an air pump, and the water's temperature was set at 27 ±2 °C with a pH range of 7-8.5. Fish were fed commercial pelleted food at a rate of 2% of body weight once each day. They were divided into five equal groups: Group 1 (G1) consisted of healthy, normal fish that weren't treated (a "negative control"); Group 2 (G2) consisted of fish that had been intraperitoneally inoculated with a single dose of 24-hour broth cultures of Aeromonas hydrophila ($6x10^6$ CFU/ml obtained from Animal Health Institute Dokki, Cairo); Groups 3, 4, and 5 (G3, G4, and G5) were inoculated intraperitoneally with 0.5 mL of 24-hour broth cultures of *Aeromonas hydrophila* (6×10^6 CFU/ml), and then (G3) fed on a diet supplemented with florfenicol (Floricol® each ml contains 100 mg of florfenicol, Pharma Swede-EGYPT) at 10 mg/kg diet for 10 successive days **(Gaikowski et al., 2003)**. G4 was provided a diet that included pumpkin seed oil supplements (Purchased from Shana company) **(El-Mosallamy et al., 2012)**. G5 was fed on a diet supplemented with pumpkin seed oil along with a therapeutic dose of florfenicol. The diet was prepared in the college of veterinary medicine (Zagazig university) for a week before infection and a month following infection.

Blood sampling:

After 1 and 10 days post-treatment, three blood samples from each group were taken from the caudal vein in an aseptic environment. The initial blood sample for hematological analysis was drawn on EDTA (1 mL). The second blood sample was taken in a sterile plastic tube containing heparin to be used for phagocytic activity investigation (2 mL). While the third blood sample was taken without anticoagulant in a clean, dry centrifuge tube (3 mL), allowed to clot at room temperature, and centrifuged at 3000 rpm for five minutes. For biochemical analysis, serum was drawn out, labeled, put in dry, clean tubes with caps, and frozen at -20°C.

Hematological studies:

According to **Feldman et al. (2000),** erythrocytic count (RBCs), hemoglobin concentration (Hb g/dl), packed cell volume (PCV%), total leukocytic, and differential counts were all evaluated.

Biochemical studies

The following parameters were estimated colorimetrically using a spectrophotometer utilizing test kits from Spinreact. The activities of the hepatic transferases (alanine aminotransferase "ALT" and aspartateaminotransferase "AST") were estimated according to **Murray (1984)**. According to **Young (1990)**, ALP was measured using a colorimetric approach. **Sanders et al. (1980)** provided the method for measuring serum uric acid, and **Henry, (1984)** provided the method for estimating serum creatinine. Total cholesterol was measured following **Artiss and Zak (1997)**, triglycerides were assessed in accordance with **Stein and Myers (1995)**, HDL cholesterol was assessed in accordance with **Lopes-Virella et al. (1977)**, LDL cholesterol was assessed according to **Friedewald et al. (1972)**, and VLDL cholesterol was assessed following **Wilson et al (1981)**. According to **Trinder (1969)**, serum glucose was tested.

Inflammatory markers:

IL6 and TNF- α assays were determined according to **Dowlati et al. (2010)**.

Antioxidant activity:

Glutathione (GSH) activity was measured according to **Paglia and Valentine, (1967)**.

Phagocytic activity and phagocytic index:

A. Separation of peripheral blood mononuclear cells:

Peripheral blood mononuclear cells (PBMC) were isolated according to the method described by **Goddeeris et al. (1986).**

B. Phagocytic Assay:

We applied 0.25 ml of adjusted viable leukocyte suspension to 0.25 ml of heat-inactivated *C. albicans* in sequential plastic tubes to measure the cell phagocytic activity. The tubes were incubated in a humidified CO2 incubator at 37°C for 30 minutes. After 5 minutes of centrifugation at 2500 rpm, the supernatant was withdrawn from the tubes using a Pasteur pipette, leaving a drop in which the sediment was re-suspended. From the deposit, smears were made, dried in the air, and dyed with Leishman's stain.

C. Evaluation of phagocytic activity:

Hundred phagocytic cells were counted at random over ten microscopic areas using a light microscope with an oil immersion lens. To determine the phagocytic cell activity in each of the tested groups, the number of yeast cells that were consumed by each phagocyte was counted. By using a microscope field, the phagocytic activity is calculated as the proportion of phagocytic cells. The average number of *C. albicans* that one phagocytic cell consumes is known as the phagocytic index.

Histopathological examination:

Specimens from the gills, liver, heart, kidneys, and spleen were fixed in 10% formalin for 48 hours, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, embedded in paraffin, and cut with a microtome to a thickness of 5 m before being stained with hematoxylin-eosin for histopathological analysis **(Suvarna et al., 2018)**.

Statistical analysis:

The analysis of variance was used in the statistical analysis (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 and Discussion

Fish and its products play a significant role in the global human diet and offer numerous health benefits **(Darlington et al., 2001)**. Nonetheless, pathogenic bacteria that are present in aquatic settings naturally, are produced from polluted waterways, or are contaminated after capture may be transmitted through fish **(Huss et al., 2003)**.

Serious issues are brought on by the genus Aeromonas in a number of fish and shellfish species. Septicemia and high mortality rates are the disease's defining symptoms **(Noga, 2010)**. The most prevalent species thought to infect *C. gariepinus* in Egypt belongs to the genus Aeromonas **(Emeish et al., 2018)**. Bacteriological examination of the collected samples revealed recovery of 29 *A. hydrophila* out of 50 examined *Clarias gariepinus*, with a total recovery rate of 58% (29/50). Each isolate was from a different fish, regardless of the number of examined organs, and the recovered isolates had grown well on tryptic soya agar, producing white, round, creamy colonies, and pale colonies on MacConkey agar, whereas it produces green colonies with dark center on Aeromonas agar and finally on blood agar it produces large grayish glistening colonies mostly surrounded by ß- hemolysis. Biochemical and morphological characterization of presumptively identified *A. hydrophila* were summarized in **Table 1**. The prevalence rates of *A. hydrophila* in catfish were previously reported as 50% and 55%, respectively **(Radu et al., 2003; Emeish et al., 2018)**. While **Kusdarwati et al. (2017)** showed that 95% of the analyzed catfish in their study were infected with *A. hydrophila*, while **El-Barbary and Hal (2016**) and **Fowoyo and Achimugu (2019)** reported lower recovery rates of *A. hydrophila* from catfish with incidence rates of 21.8% and 30%, respectively. The presence of *A. hydrophila* in fish skin, muscles, kidney, gut, and liver is consistent with other investigations that isolated this organism from the same isolation locations **(Fowoyo & Achimugu, 2019)**. The high prevalence of contaminated *C. gariepinus* may indicate that *A. hydrophilla* is a part of the normal intestinal flora of freshwater fish and under certain conditions it became pathogenic; as a result, it is regarded as an opportunistic fish pathogen. Some researchers believe that *A. hydrophila* is a primary fish pathogen, while others consider it to be a secondary invader as immune suppressive in fish **(Plumb, 1999)**. The acquired isolates' biochemical profile, Gram staining, and morphological traits were all remarkably consistent with the findings of other studies **(Sabur, 2006)**.

The recovered *A. hydrophila* was susceptible to florfenicol with a distinct zone of inhibition (18 mm). The sensitivity test indicated that florfenicol is the best medication to use. The MIC for the same pathogen in cation-adjusted Mueller-Hinton broth after 24 to 48 hours at 28°C was consistently 4 g/ml, which is within the permitted quality control limit for this organism **(CLSI, 2006)**. Therefore, 0.125 mg/ml, or 4 g/ml, of pumpkin seed oil is the recommended dose based on MIC **(Tables 2, 3)**. Sensitivity testing of the recovered *A. hydrophila* agreed with previous reports **(Aravena-Román et al., 2012; Kusdarwati et al., 2018)** that demonstrated sensitivity of *A. hydrophila* to florfenicol. Antibiotics are commonly used for prevention and control of infectious agents as *A. hydrophila* **(Smith and Reynard, 1992).**

Table (1) Biochemical and morphological characterization of presumptively identified *A. hydrophila*.

Test	Reaction
Gram's stain	Gram –ve bacilli
Motility	Motile
Oxidase	Positive
Catalase	Positive
H_2S	Positive
Simmons Citrate	Positive
Indole	Positive
M. R.	Negative
V. P.	Positive
T. S. I	Y/Y (gas)

M.R.: methyl red **V. P.:** Voges Proskauer
T.S.I: triple sugar iron **Y/Y:** yellow slant, yellow butt

Table (2) *In-vitro* anti-microbial sensitivity test for *Aeromonas hydrophila* isolates.

Antimicrobial agents	Symbols	Disk concentrations	Sensitive		Intermediates		Resistant	
			No.	%	NO.	%	NO.	%
Cefotaxime	CTX/30	30µg	3	10.34	6	20.7	20	%
Ciprofloxacin	CIP/5	5µg	26	90	2	6.89	1	3.44
Florfenicol	FFC/30	30 µg	27	93	1	3.44	1	3.44
Gentamicin	CN/10	10 µg	24	83	4	14	1	3.44
Norfloxacin	NOR/10	10 µg	26	90	2	6.89	1	3.44
Oxacillin	OX/1	1µg	0	0	3	10.4	26	89.6
Oxytetracycline	T/30	30 µg	3	10	5	17	21	72
Sulfamethoxazole Trimethoprim/	SXT/25	(1.25/23.75) µg	7	24.1	12	41.4	10	34.5

No; number of isolates A.A.: antibiogram activity.
% Percentage in relation to total number of isolates (29)

Table (3): MIC (mg/ml) pumpkin seed oil of 10 *Aeromonas hydrophila* isolates.

pumpkin seed oil (µg/ml)	**Frequency (numbers)**
>256	-
128	-

64	-
32	1
8	2
4	9
2	2
1	1
0.5	4
0.25	3
0.125	6
0.062	-

Table (4): The effect of pumpkinseed oil and florfenicol (mean ± SE) on erythrogram of clinically healthy and infected *Clarias garpennius* with *Aeromonas hydrophila*

Group	1st day post treatment			10th days post treatment		
	RBCs (Million/µl)	Hb gm/dl	PCV%	RBCs (Million/µl)	Hb gm/dl	PCV%
G1	1.63±0.07^{a}	8.65±0.24^{a}	38.80±0.68^{a}	1.59±0.05^{a}	8.60±0.18^{a}	40.40±0.50^{a}
G2	1.26±0.13^{b}	7.50±0.17^{b}	36.40±0.64^{b}	1.17±0.05^{b}	7.75±0.30b^{c}	36.40±0.64^{b}
G3	1.24±0.12^{b}	7.40±0.13^{b}	35.60±0.76^{b}	1.24±0.09^{b}	7.38±0.20^{c}	36.20±0.48^{a}
G4	1.30±0.11^{b}	7.40±0.35^{b}	37.00±0.60^{ab}	1.55±0.04^{a}	8.58±0.18^{a}	39.60±0.40^{a}
G5	1.40±0.09^{ab}	7.60±0.23^{b}	37.60±0.55^{ab}	1.43±0.03^{a}	8.24±0.17^{ab}	39.20±0.42^{a}

Different letters at the same column means that there was a significant difference at p<0.05.

Table (5): The effect of pumpkin seed oil and florfenicol (mean ± SE) on leukogram of clinically healthy and infected *Clarias garpennius* with *Aeromonas hydrophila*

Gr	1st day post treatment						10th days posttreatment					
	Total and absolute differential leucocytic count x (10^3 µl)						Total and absolute differential leucocytic count x (10^3 µl)					
	WBCs	Lymphocyte	Neutrophil	Monocyte	Eosinophil	Basophil	WBCs	Lymphocyte	Neutrophil	Monocyte	Eosinophils	Basophil
G1	16.68±0.24^{b}	10.08±0.18^{a}	5.15±0.11^{c}	0.96±0.17	0.33±0.01	0.16±0.05	16.55±0.30^{b}	9.15±0.17^{c}	6.02±0.07^{b}	0.95±0.03	0.30±0.13	0.13±0.13
G2	17.79±0.35^{a}	9.84±0.20^{ab}	6.52±0.24^{b}	0.95±0.17	0.32±0.01	0.16±0.06	17.34±0.30^{ab}	9.65±0.20b^{b}	6.35±0.12^{a}	0.92±0.03	0.31±0.01	0.11±0.05
G3	17.78±0.12^{a}	9.85±0.16^{ab}	6.52±0.20^{b}	0.95±0.17	0.30±0.00	0.16±0.04	17.48±0.22^{a}	9.60±0.17^{b}	6.47±0.29^{a}	0.95±0.01	0.33±0.00	0.13±0.04
G4	18.15±0.40^{a}	9.75±0.18^{b}	6.92±0.25^{a}	0.98±0.28	0.35±0.01	0.18±0.04	17.70±0.53^{a}	9.54±0.25^{b}	6.81±0.18^{a}	0.91±0.02	0.32±0.01	0.12±0.04
G5	16.80±0.13^{b}	10.19±0.13^{a}	5.22±0.78^{c}	0.92±0.29	0.32±0.01	0.15±0.04	17.54±0.28^{a}	10.25±0.20^{a}	5.93±0.20^{a}	0.93±0.02	0.31±0.01	0.12±0.05

Different letters at the same column means that there was a significant difference at p<0.05.

Table (6): The effect of pumpkin seed oil and florfenicol (mean ± SE) on some biochemical parameters of clinically healthy and infected *Clarias garpennius* with *Aeromonas hydrophila*

Variables	1st day post treatment					10th days post treatment				
	Groups					Groups				
	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
AST (U/L)	45.34±1.93^{d}	95±1.61^{a}	81.80±3.92^{b}	86.80±3.30^{ab}	64±4.30^{c}	45.56±1.91^{e}	132±3.39^{a}	110.80±3.33^{b}	86.40±3.38^{c}	61.40±4.23^{d}
ALT (U/L)	187.20±8.10^{d}	329±13.82^{a}	230±3.53^{b}	218±8.00b^{c}	201.60±5.04^{cd}	185.60±8.85^{d}	393±9.16^{a}	284±9.27^{b}	217±7.68^{c}	199.40±3.48^{cd}
ALP(U/L)	9.32±0.41^{c}	15.61±0.20^{a}	14.22±0.23^{b}	10.19±0.24^{c}	10.05±0.35^{c}	9.07±0.39^{c}	15.25±0.24^{a}	15.32±0.36^{a}	11.94±0.16^{b}	12.04±0.32^{b}
Urea (mg/dl)	10.44±0.71^{d}	15.42±0.19^{a}	13.60±0.27^{b}	13.20±0.25^{bc}	12.06±0.33^{c}	9.88±0.38^{d}	23.90±0.78^{a}	21.32±0.81^{b}	13.32±0.39^{c}	12.30±0.25^{c}
Creatinine (mg/dl)	0.77±0.03^{c}	1.46±0.15^{a}	1.10±0.08^{b}	1.03±0.04^{bc}	0.89±0.03^{bc}	0.81±0.04^{c}	2.14±0.09^{a}	1.71±0.05^{b}	0.98±0.009^{c}	0.92±0.02^{c}
Glucose (mg/dl)	155.84±10.10^{c}	254.30±14.90^{a}	172.42±14.70^{b}	159.18±5.16^{c}	157.80±18.80^{c}	147.50±13.03^{b}	230.92±14.79^{a}	168.84±11.59^{b}	156.50±10.26^{b}	133.20±20.91^{b}
Cholesterol (mg/dl)	310.07±3.45^{d}	377.14±10.36^{a}	347.66±6.07^{b}	326.77±12.39^{c}	327.26±10.41^{c}	323.78±11.00^{d}	370.32±17.42^{a}	355.20±10.25^{b}	334.93±10.77^{c}	330.39±8.51^{c}
Triglycerides (mg/dl)	122.28±6.80^{b}	150.34±2.87^{a}	139.98±1.69^{a}	128.30±2.07^{b}	122.42±2.37^{b}	118.60±1.67^{c}	155.40±1.86^{a}	130.80±1.39^{b}	123.00±1.45^{c}	122.20±1.46^{c}
HDL (mg/dl)	44.61±1.59^{b}	54.58±1.32^{a}	52.48±4.30^{a}	43.98±1.38^{b}	46.74±1.82^{b}	46.68±1.60^{b}	53.52±0.42^{a}	53.00±1.09^{a}	48.44±1.12^{b}	49.39±0.78^{b}
LDL (mg/dl)	241.00±1.22^{d}	292.49±1.04^{a}	267.19±5.04^{b}	257.13±1.78^{c}	256.04±1.86^{c}	253.38±1.85^{d}	285.72±1.89^{a}	276.04±1.58^{b}	261.89±1.41^{c}	256.56±1.75^{d}
VLDL (mg/dl)	24.46±0.65^{c}	30.07±0.97^{a}	27.99±0.35^{b}	25.66±0.82^{a}	24.48±10.41^{c}	23.72±0.43^{c}	31.08±0.63^{a}	26.16±0.67^{b}	24.60±0.55^{c}	24.44±0.84^{c}

Different letters at the same column means that there was a significant difference at $p < 0.05$.

Table (7): The effect of pumpkin seed oil and florfenicol (mean ± SE) on some anti-inflammatory and antioxidant parameters, phagocytic% and phagocytic index of clinically healthy and infected *Clarias garpennius* with *Aeromonas hydrophila*.

Variables	1st day post treatment					10th days post treatment				
	Groups					Groups				
	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
INF- α (pg/ml)	1118.2±31.30^{c}	714.7±20.69^{e}	865.8±29.56^{d}	1334.2±27.71^{b}	1348.6±27.85^{a}	1177±22.48^{c}	877.4±20.45^{e}	928±24.28^{d}	1319±19.23^{a}	1311±23.87^{b}
IL6 (pg/ml)	334.00±13.87^{b}	245.60±12.50^{d}	305.40±12.72^{c}	368.80±13.22^{a}	369.40±12.88^{a}	356.20±21.54^{b}	266.40±22.49^{d}	312.40±18.05^{c}	377.80±21.07^{a}	373.80±18.27^{a}
GSH (mmol/L)	70.08±3.42^{a}	58.50±0.96^{b}	63.30±1.56^{ab}	59.50±3.01^{b}	69.2±2.78^{a}	72.05±2.36^{a}	61.88±1.74^{b}	68.60±1.81^{b}	63.10±2.08^{b}	71.80±1.93^{a}
Phagocytic ratio	73.62±0.20^{d}	71.80±0.24^{e}	74.26±0.15^{c}	77.26±0.22^{a}	75.62±0.20^{b}	75.60±0.17^{b}	75.42±0.25^{c}	69.30±0.37^{d}	74.28±0.53^{c}	78.26±0.42^{a}
Phagocytic index	2.13±0.04^{d}	2.02±0.03^{d}	2.13±0.04^{d}	2.52±0.01^{a}	2.41±0.02b^{c}	2.47±0.01^{a}	2.29±0.03^{b}	2.07±0.03^{c}	2.48±0.02^{a}	2.47±0.02^{a}

Different letters at the same column means that there was a significant difference at $p<0.05$.

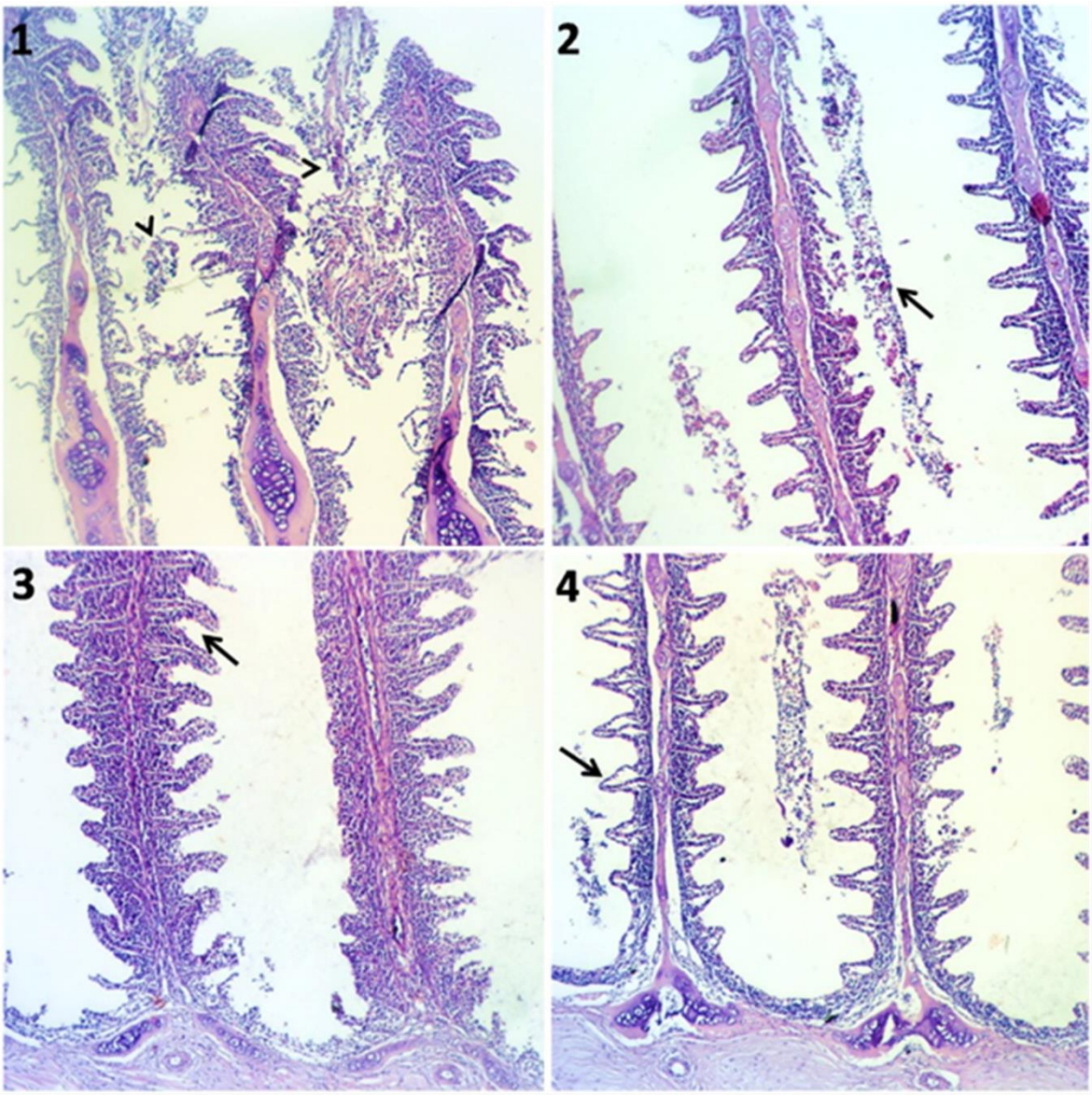

Fig. 1: Photomicrograph of **gills** showing A) fusion of some adjacent secondary lamellae at filament tips **(arrow)** and inflammatory cells and mucous secretions admixed with denuded necrotic epithelium between primary filaments **(arrowheads)** within the infected group of catfish with *Aeromonas hydrophila*. (H&E x 100). **B)** focal desquamated epithelium of secondary lamellae accompanied with mucous secretion between primary filaments **(arrow)** in the treated group with florfenicol (H&E x 100). C) hyperplastic of some gill filaments with lymphocytic infiltration in the treated group with pumpkin H&E x 100). **D)** edema of some secondary lamellas **(arrow)** in the treated group with a combination of florfenicol and pumpkin (H&E x 100).

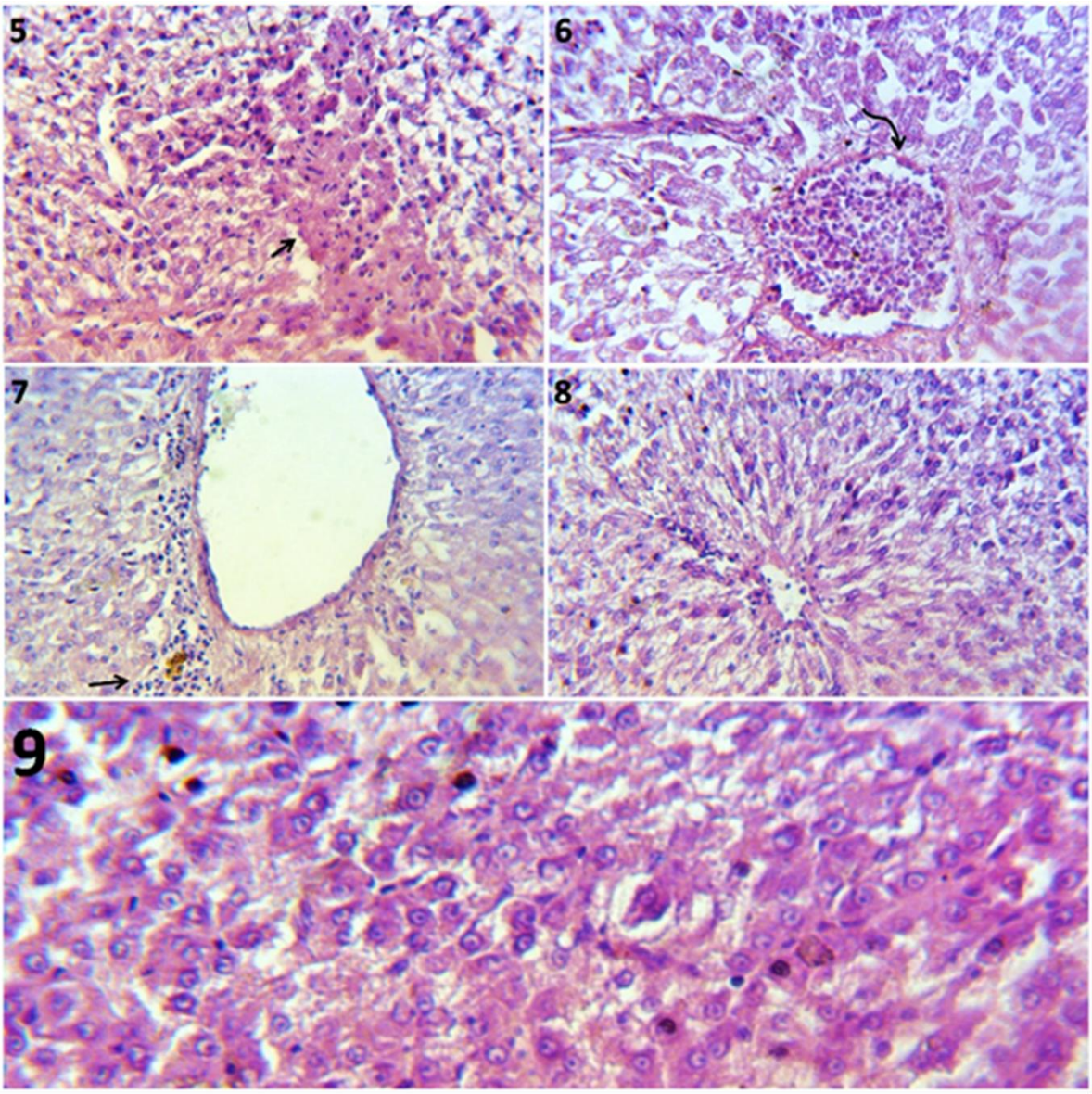

Fig. 2: Photomicrograph of **liver** showing A, B) vacuolated hepatic cytoplasm, area of necrotic hepatocytes **(arrow),** and congested hepatic blood vessel **(curved arrow)** within the infected group of catfish with *Aeromonas hydrophila* (H&E x 400). C) presence of a few numbers of inflammatory cell infiltrations and melanomacrophages within the portal area **(arrow)** in the treated group with florfenicol (H&E x 400). D) mild degenerative changes within some hepatocytes in the treated group with pumpkin (H&E x 400). **E)** preserved histomorphological structures of hepatic parenchyma in the treated group with a combination of florfenicol and pumpkin (H&E x 400).

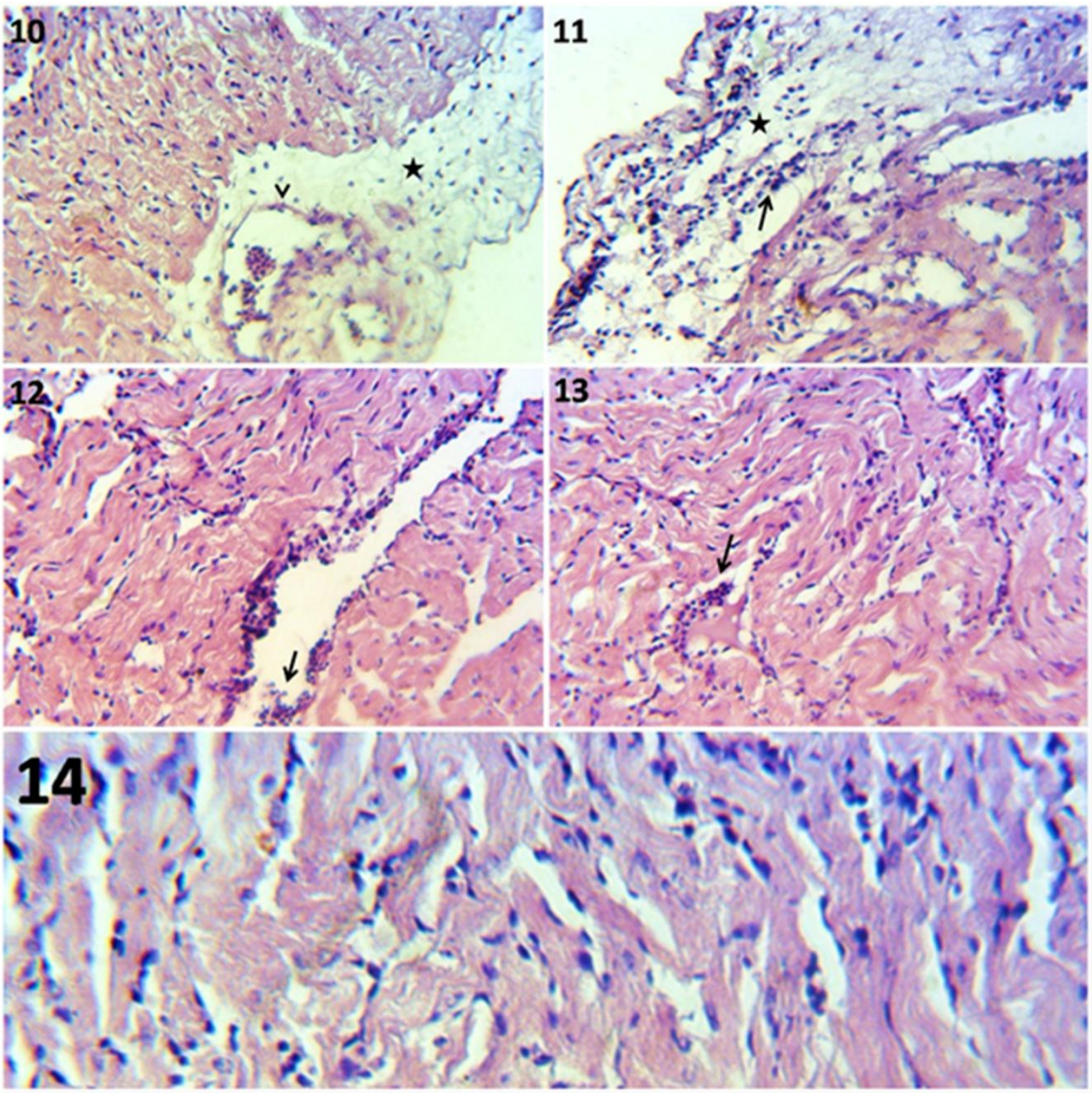

Fig. 3: Photomicrograph of the **heart** showing: Lanes 10, 11) pericarditis with dilated capillary **(arrowhead)**, edema **(stars),** and aggregations of inflammatory cells **(arrow)** in the infected group of catfish with *Aeromonas hydrophila* (H&E x 400). C) inflammatory cells aggregate adhered to the endocardium **(arrow)** in the treated group with florfenicol (H&E x 400). D) interstitial edema and focal aggregate of inflammatory cells between muscle fibers in the treated group with pumpkin **(**H&E x 400). E) normal cardiomyocytes in the treated group with a combination of florfenicol and pumpkin (H&E x 400).

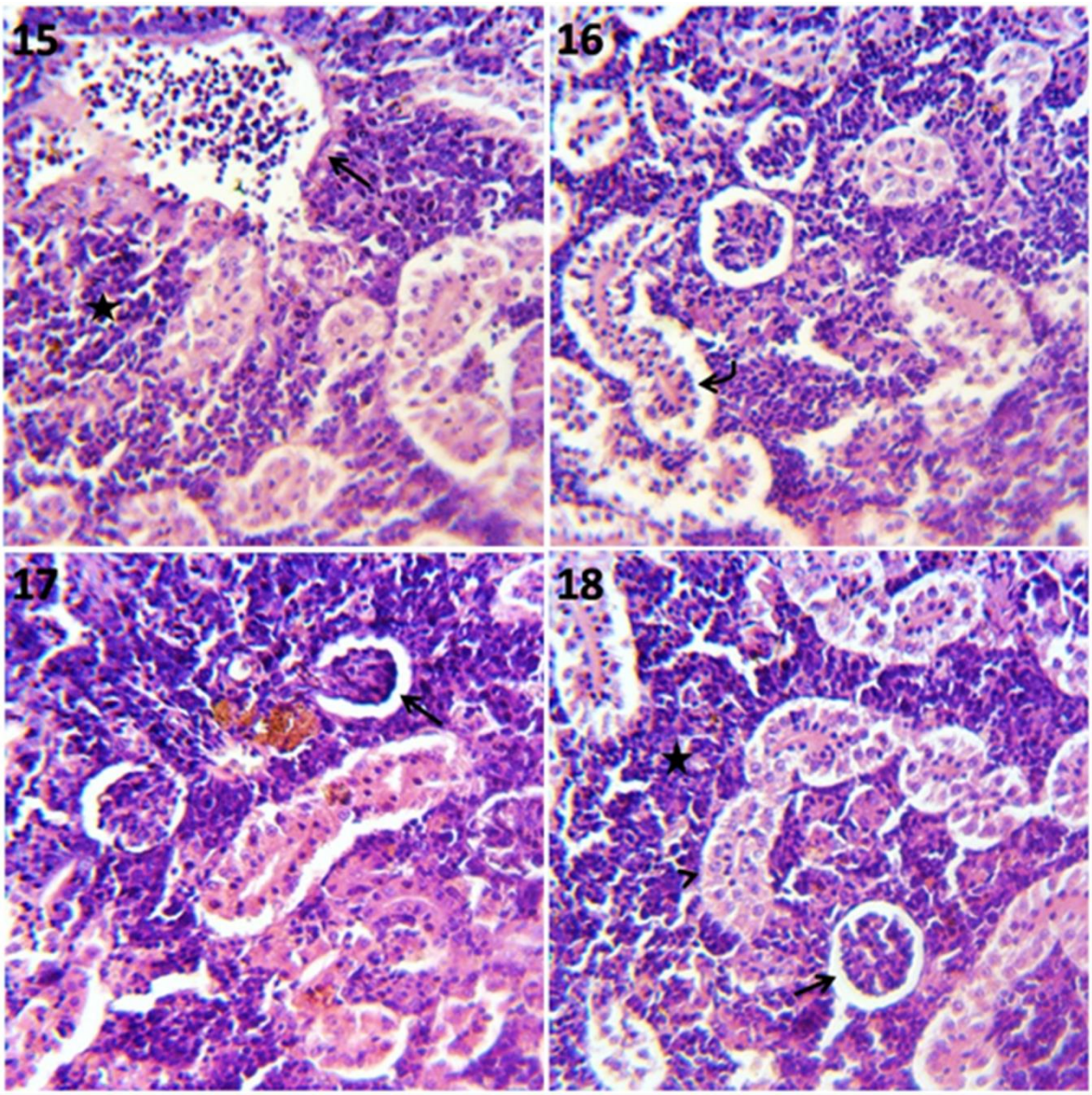

Fig. 4: Photomicrograph of a **kidney** showing: **Lane 15)** impacted renal blood vessel with inflammatory cells **(arrow),** degenerative and necrotic changes of the majority of renal tubular epithelium and obvious hematopoietic elements between renal tubules and glomerular tufts **(star)** in the infected group of catfish with *Aeromonas hydrophila* (H&E x 400). **Lane 16)** pyknotic nuclei of tubular epithelium a few tubules **(curved arrow)** in the treated group with florfenicol (H&E x 400). **Lane 17)** shrinkage of some glomerular tufts **(arrow)** and aggregation of melanomacrophage cells between tubules in the treated group with pumpkin (H&E x 400). **Lane 18)** normal renal tubules **(arrowhead)**, glomerular structures **(arrow),** and renal hematopoietic tissue between them **(star)** in the treated group with a combination of florfenicol and pumpkin (H&E x 400).

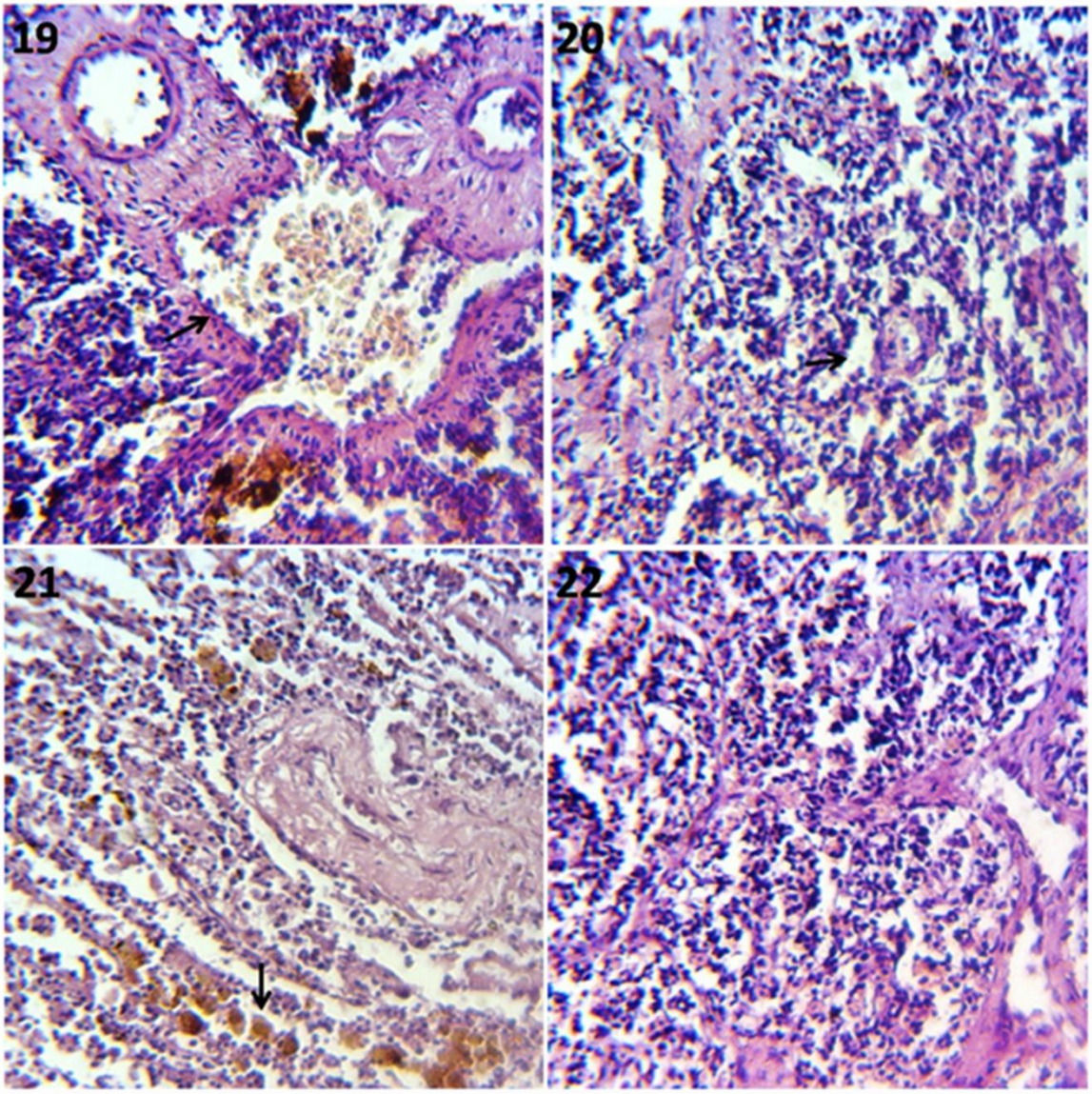

Fig. 5: Photomicrograph of **spleen** showing: **Lane 19)** congested splenic blood vessels **(arrow),** depleted lymphoid aggregates with the present number of melanomacrophagesin infected group of catfish with *Aeromonas hydrophila* (H&E x 400). **Lane 20)** mildly depleted white pulp around ellipsoids arterioles **(arrow)** and filled red pulp with RBCs and lymphocytes in the treated group with florfenicol (H&E x 400). **Lane 21)** abundant sinusoidal phagocytic cells **(arrow)** with clearly visible hematopoietic tissue in the treated group with pumpkin (H&E x 400). **Lane 22)** normal white and red pulp in the treated group with a combination of florfenicol and pumpkin (H&E x 400).

The current investigation showed that the erythrocytic count, Hb concentration, and packed cell volume significantly decreased in A. hydrophila (G2)-infected non-treated fish. On the other hand, at two experimental periods (1st and 10th day post treatment), there was a considerable rise in the leucocytic count and lymphocyte **(Table 4, 5)**. The current findings are consistent with those of **Ahmed (2000), Amer et al. (2009), and Sawsan et al. (2013)**, who reported that infected fish with *A. hydrophila*

displayed a significant decrease in total erythrocytic count, hemoglobin concentration, and packed cell volume while there is a significant increase in total leucocytic count in the first, seventh-, and fourteenth-days post treatment. These alterations are the result of the *A. hydrophila* pathogenesis, which has been reported to involve a variety of extracellular products and enzymes with biological activity, such as cytotoxins, hemolysis, proteases, and enterotoxins, which are thought to be linked to the virulence of *A. hydrophila* **(Allan and Stevenson 1981)**. **Coles (1986)** also discovered that bacterial infection-induced antigenic stimulation is responsible for the rise in total leucocytic count.

Florfenicol treatment improved the negative effects of *A. hydrophila* infection on the hematological parameters of Nile catfish, according to **Dalia (2013)**. However, fish that were experimentally infected and treated with florfenicol (G3) showed a significant increase in RBCs count, Hb concentration, and PCV% when compared with the infected non-treated group. In contrast, when compared to the infected non-treated group, the infected fish treated with pumpkin seed oil (G4) and pumpkin seed oil combined with florfenicol (G5) both demonstrated improvements in the majority of hematological parameters. The chemical composition of pumpkin seed oil, which is rich in mono- and poly-unsaturated fatty acids, minerals, vitamins, pigments, phytosterols, phenolic compounds, and pyrazine derivatives, may be to blame for this. Given everything, it generates curiosity as a potential nutraceutical oil **(Aktas et al., 2018)**.

Fish that were experimentally infected with *A. hydrophila* but not treated (G2) displayed a significant improvement in liver and kidney functions (AST, ALT, urea, creatinine), glucose, total cholesterol, TG, HDL, LDL, and VLDL at the two experimental periods (1st and 10th day posttreatment) in comparison to G1. Similar findings were made by **Ahmed (2000), Amer et al. (2009), and Sawsan et al. (2013)**, who reported that *A. hydrophila*-infected fish showed a substantial increase in AST, ALT, urea, and creatinine compared with the control group. According to **Halliwell (1981)**, the rise in enzymatic activity was brought on by liver damage via the effects of infectious agent's toxins, which led to a high level of these enzymes escaping into the serum. Our findings also showed improvement in serum enzymes in the treated infected groups (4 and 5), which is consistent with **Gharreb's (1999)** observation that *A. hydrophila* infection of *Oreochromis niloticus* led to an increase in blood urea and creatinine levels. He also stated that the bactericidal properties of florfenicol, which restrict the toxic and damaging effects of *A. hydrophila* in the liver as well as the regenerating process that occurs in the liver cell, may be the cause of the improvement in liver function.

Both the infected group that received pumpkin seed oil alone (G4) and the infected group that received pumpkin seed oil combined with florfenicol (G5) demonstrated improvements in their lipid and glucose profiles. These improvements in biochemical parameters may be attributable to pancreatic beta-cell qualification, insulin release, and the effects of phenolic antioxidant compounds (trigonelline, D-chiro-inositol, and nicotinic acid) of pumpkin seed oil, which are including trigonelline and nicotinic acid found in pumpkin seeds, have been shown to increase the activity of the enzymes glucokinase and glucose-6-phosphatase, which are involved in the metabolism of glucose. According to **Caili et al. (2006)** the wide molecular weight range (3–60 kDa) of pumpkin seeds may stabilize blood insulin levels and lower blood sugar. Also, according to **Patel et al. (2012)**, pumpkin seed oil contains significant amounts of sterols and phytoestrogens such secoisolariciresinol and lariciresinol. According to **Makni et al. (2011)**, feeding

hypercholesterolemic rats pumpkin seed oil has significant hepatoprotective and anti-atherogenic effects. The authors added that phytoestrogen chemicals have a critical function in suppressing cardiovascular issues and regulating blood cholesterol levels.

A strong pro-inflammatory cytokine that is essential to inflammation is TNF-α. It interacts with its cellular receptor, TNF receptor 1, which sets off signaling cascades that activate transcription factors called NF-B and activator protein 1 (NF-BAP1). So, by activating NF-B and creating a positive feedback mechanism that amplifies the inflammatory process, proinflammatory cytokines like IL-1 and TNF may aid in the propagation of the expansion of a local or systemic inflammatory process **(Sonis, 2002)**.

The fish treated with pumpkin seed oil showed an increase in anti-inflammatory, antioxidative, and immunological parameters (phagocytic ratio and index) at both times in G4 and G5 **(Table 4)**. These are in line with **Amara et al. (2008)**, who found that zinc content of pumpkin seeds can prevent the production of free radicals or directly occupy the copper or iron-binding sites of proteins, lipids, and DNA molecules. As a result, they are regarded as potent antioxidants. Although selenium is incorporated into proteins to generate selenoproteins, which are thought to be essential antioxidant enzymes, **Rampersaud et al. (2005)** also noted that the selenium in pumpkin seed oil promotes the glutathione antioxidant system. According to **Sharma et al. (2013)**, the presence of carotenoids, phenolics, flavonoids, and saponins in the ethanolic extract of pumpkin seeds significantly reduced total cholesterol (TC), triacylglycerol (TAG), low density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C), while also increasing HDL-C in experimental mice. Simvastatin, an antihypercholesterolemic synthetic drug, and pumpkin seed oil have been shown by **Al-Zuhair et al. (1997)** to have synergistic effects that significantly reduce the aortic contractile response to norepinephrine and lessen the severity of hypercholesterolemia in rabbits fed a high-fat diet. These synergistic effects have been attributed to simvastatin's therapeutic action as well as its antioxidant capacity. According to several researchers, pumpkin seed oil stimulates the production of cytokines like IL-1 and TNF-α by peritoneal macrophages and modifies non-specific immunity through macrophage activation **(Damiano et al., 2016; Gossell-Williams et al., 2006)**. According to **Sedigheh et al. (2011)** and **Al-Okbi et al. (2014)**, supplementing rats with pumpkin seed powder or oil caused significant decreases in CRP and TNF-α levels. The flavonoids in this plant, which are antioxidant substances, are what give it its hypolipidemic and anti-inflammatory properties. According to **El-Mosallamy et al. (2012)**, pumpkin seed oil's high linoleic and oleic unsaturated fatty acid content is what gives it its anti-inflammatory properties. Arachidonate cyclooxygenase products are produced less as a result of linoleic and linolenic acids' competition with arachidonate for oxidative enzymes. It has been shown that a diet rich in linolenic acid has actions similar to nonsteroidal anti-inflammatory agents in reducing the production of prostaglandin E2 and leukotriene B4 generated during inflammation. The fact that selenium-containing pumpkin seeds and selenium supplementation reduced the ICAM-1 and VCAM-1-mediated monocyte adhesion produced by microparticles of resuscitated patients, according to **Fink et al. (2015),** provides further evidence that selenium has anti-inflammatory effects. The current study found that the fourth and fifth groups that were given pumpkin seeds had a significant increase in cellular immune response via a high increase in phagocytic indices because pumpkin seeds are a rich source of zinc, an essential trace mineral that plays an important role in immune system protection. This result agreed with **Iwo et al. (2014)**

who stated that zinc in pumpkin seeds can activate Toll-like receptor 4 (TLR4) by daptomycin. The mean value of phagocytic indices in fish injected with *A. Hydrophila* was reduced dramatically in the infected group (second group) due to the effect of bacterial endotoxemia. This finding is consistent with **Al- Hashimi's (2005)** observation that bacteria continued to multiply in the tissue of the liver and spleen because these organs are rich in reticuloendothelial tissue.

Gross examination of group (2) revealed ulcer, sloughing epithelium, congestion of gills and all internal organs while in other treated groups revealed mild macroscopical changes.

Gills: Infected group of catfish with *A. hydrophila* (G2) showed detached epithelium of most secondary lamellae and fusion of some adjacent secondary lamellae at filament tips with inflammatory cells and mucous secretions admixed with denuded necrotic epithelium between primary filaments. Lymphocytic infiltration in the interstitial tissue of the primary gill filaments were also seen **(Fig. 1)**. Our results agree with the results obtained by **Dalia (2013)** who mentioned that gills are the target organ of *A. Hydrophila*, Infected treated group with florfenicol (G3) showed apparently normal most of epithelium lining filaments. However, focal desquamated epithelium of secondary lamellae accompanied with mucous secretion were present between primary filaments. Infected treated group with pumpkin seed oil (G4) showed apparent normal most gill structures but hyperplastic gill filaments with lymphocytic infiltration were detected. Infected treated group with combination of florfenicol and pumpkin seed oil (G5) showed apparently normal gill filaments and gill arch structures. However, edema of some secondary lamellas and a small number of detached secondary lamellae were seen.

Liver: Infected group of catfish with *A. hydrophila* (G2) showed septicemic lesions as vacuolated hepatic cytoplasm in addition to presence of some necrotic hepatocytes as well congested hepatic blood vessels **(Fig. 2)**. Our results were attributed to the liberation of bacterial toxins and other virulence factors inherent to Aeromonosis where it produces endotoxins and exotoxins, such as hemolysin and aerolysin, which cause the rupture of cellular membranes' enterotoxins (**Beaz-Hidalgo and Figueras, 2013)**, dermonecrotic factors, proteases, phospholipases and DNAses that causes tissue damage and facilitate bacterial invasion and multiplication in the hosts' cells. Infected treated group with florfenicol (G3) showed most of the hepatocytes were apparently normal most hepatocytes with presence few numbers of inflammatory cells infiltrations and melanomacrophages within portal area. Infected treated group with pumpkin seed oil (G4) showed apparently normal hepatic parenchyma but mild degenerative changes within some hepatocytes were seen. Infected treated group with combination of florfenicol and pumpkin seed oil (G5) showed preserved histomorphological structures of hepatic parenchyma.

Heart: Infected group of catfish with *A. hydrophila* (G2) showed pericarditis which represented by dilated capillaries of pericardium, edema, and aggregations of inflammatory cells as well as degenerative changes of some cardiomyocytes **(Fig. 3)**. Treated group with florfenicol (G3) showed inflammatory cells aggregate adhered to endocardium. Infected treated group with pumpkin seed oil (G4) showed interstitial edema and focal aggregate of inflammatory cells and edema between muscle fibers. Infected treated group with combination of florfenicol and pumpkin seed oil (G5) showed apparently normal cardiomyocytes.

Kidney: Infected group of catfish with *A. hydrophila* (G2) showed dilated renal blood vessels which impacted with inflammatory cells, degenerative and necrotic changes of the majority of renal tubular epithelium. Obvious hematopoietic elements between renal tubules and glomerular tufts **(Fig. 4)**. Treated group with florfenicol (G3) showed necrosis in a few numbers of tubules which demonstrated as pyknotic nuclei of tubular epithelium. Infected treated group with pumpkin seed oil (G4) showed shrinkage some glomerular tufts . and aggregation of melanomacrophage cells between tubules. Infected treated group with combination of florfenicol and pumpkin seed oil (G5) showed apparently normal renal tubules, glomerular structures, and renal hematopoietic tissue between them.

Spleen: Infected group of catfish with *A. hydrophila* (G2) showed congested splenic blood vessels, depleted lymphoid aggregates with presence of number of melanomacrophages **(Fig. 5)** which means activation of the immune system **(Agius and Roberts (2003)**. Treated group with florfenicol (G3) showed mildly depleted white pulp around ellipsoids arterioles and filled red pulp with RBCs and lymphocytes. The infected group with pumpkin seed oil (G4) showed abundant sinusoidal phagocytic cells with clearly visible hematopoietic tissue. Infected treated group with combination of florfenicol and pumpkin seed oil (G5) showed apparently normal white and red pulp .

Conclusion

This study indicates that the presence of *A. hydrophila* with high rate in catfish may be a major threat to public health. So, it is suggested that florfenicol can be an adequate treatment. On the other hand, it was found that pumpkin oil enhanced antibiotic activity of florfenicol as confirmed *in vitro* and *in vivo*. The co-administration of florfenicol and pumpkin oil improved the bactericidal efficacy of the treatment of *A. hydrophila* infection and greatly increased the survival rate of the infected fish. Therefore, pumpkin oil can be used as a new antibiotic adjuvant, which reduces antibiotic usage and facilitates the prevention and treatment of bacterial infections in the aquatic species.

Conflict of interest: none.

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