Clinicopathological studies on some antibiotics used in Nile tilapia infected with *Streptoccocus iniae*

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Abstract: This study was carried out to investigate the effect of infection of fish with *S. iniae* and their treatments on the hemato-biochemical, immunological and pathological studies. A One hundred and fifty Nile tilapia (*Oreochromis niloticus*) of 50 g average body weight were randomly divided into 5 equal groups. The 1st gp. was kept as a normal control. The 2nd, 3rd and 4th gps. were infected intraperitoneally (IP) with 25μl of *S. iniae* (1.2×10^8 CFU/ ml). Following the infection by 48 hours, the 3rd gp. was treated with florfenicol (10 mg/kg B.W /day) for 10 successive days through medicated feed, while the 4th gp. treated with dry leaves of *R. officinalis* which ground to fine powder and mixed with grained commercial fish feed in a ratio of 3:17 w/w for 10 days. The 5th gp. was fed dry leaves of *R. officinalis* (prepared as in gp. 4) for 5 days, then infected IP with 25μl of *S. iniae* (1.2×10^8 CFU/ ml), and after 48 hours from the infection, it fed dry leaves of *R. officinalis*, for 10 days. The hematological results showed a significant decrease in RBCs counts, Hb content and PCV (gps.2, 3, 4&5). The fish of gps. (2&3) showed a leukocytosis with neutrophilia. In addition, significant changes were recorded in the immunological and biochemical parameters. Antioxidant analysis revealed a significant increase in the hepatic level of malondialdhyde (MDA) in gps. (2, 3&4) with insignificant change in gp. (5).The hepatic catalase (CAT) level revealed a significant decrease in gps. (2, 3&4), while it showed insignificant change in gp. (5).

1. Introduction

Streptococcal infections have severe economic consequences on fisheries in many areas of the world, with mortality rate between 30% - 50% over a period of 3–7 days (Eldar *et al.,* 1997; Shoemaker and Klesius, 1997). *Streptococcus iniae* (*S. iniae*) has become one of the most serious aquatic pathogens in the last decade, causing large losses in wild and farmed fish world-wide (Agnew and Barnes, 2007), especially tilapia (*Oreochromis niloticus*) and the hybrid of *O. niloticus* and *O. aureus* (Al-Harbi, 1994; Shoemaker *et al.,* 2000 and Raissy *et al.,* 2012), barramundi Lates calcarifer (Bromage *et al.,* 1999) and red drum *Sciaenops ocellatus* (Eldar *et al.,* 1999). The economic losses caused by this bacterium in aquaculture farms worldwide are estimated to be over US$ 100 million per year (Shoemaker *et al.,* 2001). In addition to its importance in aquaculture, *S. iniae* has recently emerged as a threat to public health due to its zoonotic agent during handling fresh infected fish (Sun *et al.,* 2007).

Florfenicol is a broad-spectrum antibacterial and currently being registered for use in food fish in the United States. Florfenicol (d-(threo)-1-(methylsulfonylphenyl) 2- dichloroacetamide-3-fluoro-1-propanol) is a synthetic antibacterial compound, a fluorinated analogue of thiamphenicol and chloramphenicol (Lundén *et al.,* 1999).

Florfenicol, closely related to chloramphenicol, is effective against chloramphenicol-resistant bacterial isolates and also lacks the functional group associated with human toxicity (Yanong and Curtis, 2005).

The increased public awareness of the negative effects caused by overexposure to synthetic chemicals led to the search for "green solutions", such as organic and synthetic chemical-free food products. To enable organic fish production, it is essential to develop antibacterial treatments that are based on materials from natural sources. *Rosmarinus officinalis* (*R. officinalis*) L. is the most important and new one in the folk medicine.

R. officinalis (common name, rosemary; family Labiatae) is known to be a rich source of active metabolites (Karamanoli *et al.,* 2000) and is used in traditional medicine (Al-Sereiti *et al.,* 1999). It has been reported to possess a number of therapeutic applications in folk medicines in curing or managing of a wide range of diseases such as Diabetes mellitus, respiratory disorders, stomach problems and inflammatory diseases, (Erenmemişoğlu *et al.,* 1997, Al-Sereiti *et al.,* 1999 and Kültür, 2007). Rosemary has long been recognized as having antioxidant molecules, such as rosmarinic acid, carnasol and rosmaridiphenol (Dorman *et al.,* 1995).

The present work is aimed to study the role of florfenicol and *R. officinalis* in treatment of infected

Nile tilapia (*Oreochromis Niloticus*) with *S. iniae* through investigation of some hematological, biochemical, immunological and pathological studies.

2. Material and methods
2.1. Experimental animals

A total number of one hundred and fifty apparently healthy Nile tilapia (50g average body weight) were purchased from the Central Laboratory for Aquaculture Research, Abbassa, Sharkia province, Egypt. The fish were transported to the laboratory in plastic bags filled with oxygenated water. They were fed commercial fish ration *ad-libitum*. The fish were kept in glass aquaria for 3 weeks to be acclimatized before start of the experiment.

2.2. Bacterial strain

Streptoccocus iniae was obtained from the Animal Health Research Institute, Dokki, Cairo, Egypt. It was subcultured on brain heart infusion agar (BHI) and incubated at 27 ℃ for 24 hours. A 100 ml of BHI broth was inoculated with 50 µl of the frozen isolate. The cultures were shaken (100 rpm) at 27 °C for 24 hrs and centrifuged 5000 rpm at 15 °C for 10 min. The pellet was resuspended in sterile saline (NaCl 0.85%) then adjusted to 0.5 McFarland barium sulfate standard solutions corresponding to 1.2×10^8 CFU/ml (Abutbul *et al.,*2004).

2.3. Florfenicol

Floricol was obtained from Pharma Swede – EGYPT Company.

2.4. R. officinalis

R. officinalis was obtained from cultivated plants, Damietta, Egypt. Leaves were shade- dried. Dry leaves of *R. officinalis* were ground to fine powder and mixed with grained commercial fish feed in a ratio of 3:17 w/w. Distilled deionized water was added to the feed (1.2 ml/g) and mixed until a homogenous mixture was obtained. The mixture was passed through a minced-meat processing machine, producing extruded strings, which were dried at 30 °C for 24 hrs and then broken down to about 2-mm-long pellets (Abutbul *et al.,*2004).

2.4. Experimental design

One hundred and fifty Nile tilapia (*Oreochromis niloticus*) were randomly divided into 5 groups (30 fish in each group). The 1st gp. was kept as a normal control. The 2nd gp. was infected IP with 25µl of *S. iniae* ($1.2x10^8$ CFU/ ml) (Abutbul *et al.,*2004). The 3rd gp. was infected IP with 25µl of *S. iniae* ($1.2x10^8$ CFU/ ml), then treated with florfenicol (10 mg/kg B.W/day) for 10 successive days through medicated feed (Gaunt *et al.,*2010). 4th was infected IP with 25µl of *S. iniae* ($1.2x10^8$ CFU/ ml), then treated with dry leaves of *R. officinalis* (dry leaves of *R.*

officinalis which ground to fine powder and mixed with grained commercial fish feed in a ratio of 3:17 w/w for 10 days. The 5th gp. was fed dry leaves of *R. officinalis* (prepared as in gp. 4) for 5 days, then infected IP with 25µl of *S. iniae* ($1.2x10^8$ CFU/ ml), then fed dry leaves of *R. officinalis* for 10 days (Abutbul *et al.,*2004). The treatments were 48 hours after the infection by *S. iniae* in gps. (3, 4&5).

2.5. Blood sampling

Blood samples were collected from the caudal vessels of fish after 2&4 weeks of treatment. The 1st sample was taken in clean Wasserman tubes containing dipotassium salts of EDTA to be used for hematological examination. The 2nd sample was collected in heparinized syringe for immunological studies. The 3rd blood sample was collected without anticoagulant for serum separation for biochemical analysis.

2.6. Hematological studies

The erythrocytic and leukocytic counts were performed using an improved neubauer hemocytometer and special diluting fluid (Natt & Herrick's solution) for fish blood as described by Stoskopf (1993). The PCV, hemoglobin (Hb) concentration (using the cyanmethemoglobin colorimetric method), erythrocytic indices (MCV, MCHC and MCHC) and blood smears were performed using standard methods according to Stoskopf (1993).

2.7. Immunological studies
2.7.1. Phagocytosis and phagocytic index
a- Preparation of E. coli

E. coli was grown into macConky s agar medium for 24 hrs at 37℃. The *E. coli* were counted in an improved Newbaur's counting chamber and resuspended in buffer peptone water and matching with 0.5 McFarland standard (Bauer *et al.,*1966), to give a concentration of 1.5×10^8 cell/ml.

b- Preparation of leukocytic suspension

Peripheral blood leukocytes suspension was prepared according to Wilkinson (1977) and Lucy and Larry (1982). A 3 ml venous blood (pooled blood sample) collected on heparin (50 IU/ml) to which 0.5 ml of the dextran (dextran 250, Sigma Co.) was added. This causes rapidly sedimentation of red cells leaving an upper layer of leukocytes with the plasma. The sample was allowed to stand in 37 ℃ incubators for 45 minutes to enhance red cell sedimentation. Leukocytes rich plasma was removed by its centrifugation at 1500 rpm for 15-20 minutes. The deposited cells were taken and washed twice in phosphate buffered saline. Sedimented cells were suspended in 1ml of the Roswell Park memorial institute (RPMI) media containing 1% Foetal calf serum. The neutrophil cell count was adjusted to 2×10^6 cell /ml in phosphate buffered saline.

c- Preparation of pooled serum of fish

One ml of blood was collected from each fish in a sterile wasserman tubes without addition of anticoagulant. It was left to clot and separate clear serum after centrifugation. The serum from 3fish were pooled together and stored at -20 ºC until used.

d- Evaluations of phagocytic activity:

This method based on the uptake of *E. coli* by neutrophils over a certain period of time.

Under a light ordinary microscope using oil immersion lens, 10 fields each containing about 10 phagocytes were examined. The phagocytic percent (P %) and phagocytic index (PI) were used to evaluate phagocytic activity (Sarah, 2007).

P%: Number of neutrophils containing *E. coli* × 100/ Total number of counted neutrophils.

PI: Number of *E. coli* ingested by 100 neutrophils or total number of *E. coli* in 100 neutrophils.

2.7.2 Lysozyme activity:

Serum lysozyme activity in Nile tilapia serum was determined according to a modified method described by Yildirim *et al.* (2003). Preparation of agarose plates and lysozyme assay were made according to Schultz (1987). Lysoplates were prepared by dissolving 0.01 % agarose in 0.067 M PBS at pH 6.3 completely at 100°C. The Agarose is cooled at 60-70 °C, after which a 500 mg uniform suspension of *Micrococcus lysodeikticus* in 5 ml saline is added to 1 liter of agarose and mixed well. Plates were poured at thickness of 4mm depth and left to cool down and wells 2 mm in diameter in 4 × 4 rows, 15 mm apart were cut in the agarose. At the end of the assay, plates and samples were brought to room temperature. The wells were filled with volume of 25 μl of serum samples as quickly as possible. The plates were covered tightly and incubated at room temperature on a level surface for 12- 18 hours. At the end of the incubation period, the clear zone ring diameters were measured to the nearest 0.1 mm. For each lysoplate, the lysozyme concentrations in the samples were determined from a plotted standard curve against the corresponding clear zone ring diameter on the linear axis.

2.8. Biochemical studies

The serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) *(*Reitman and Frankel, 1957) and alkaline phosphatase, (Kind and King, 1954) were determined. The serum levels of bilirubin (total and direct), (Jendrassik, 1938), indirect bilirubin (calculated by subtracting the obtained direct bilirubin level from total bilirubin level), total protein (Henry, 1974), albumin (Doumas *et al.,* 1971), globulin (Doumas and Biggs, 1972), urea (Patton and Crouch, 1977) and creatinine (Henry, 1974) were determined.

2.9. Antioxidant enzymatic activities in tissue homogenate.

The supernatant obtained after centrifugation of liver homogenates was used for the determination of Catalase (CAT) (Aebi, 1984) and lipid peroxidase activities expressed by Malondialdhyde (MDA) (Satoh, 1978).

2.10. Histopathological studies

Specimens collected from the liver and kidneys were fixed in 10% neutral buffered formalin.

Paraffin   sections of 5 μ thickness were prepared , stained by hematoxylin and eosin (H&E)   and examined microscopically (Bancroft *et al.,*1996).

2.11. Statistical analysis

The obtained data from our experimental work were statically evaluated using one way ANOVA. $P \leq 0.05$ was considered to indicate statistical significance. All the results were expressed as mean ± SE for 5 fish in each group. Means at the same column followed by different small letters were significantly different and the highest value with a letter (a) (SAS, 1997).

3. Results and Discussion

Concerning the clinical signs, fish of gp. (2) showed hyperemia   (Fig. 1) all over the body, especially at the end of gill operculum, end of fins (pelvic and dorsal fins), ascitis (Fig. 2) and mortality rate 26.27 % during the 2nd weeks post infection. Ulcers formation (Fig. 3) and exophthalmia (Fig. 4) were the characteristic clinical signs observed in fish after the 4th week of infection. Similar results were obtained by Russo *et al.* (2006) and Raissy *et al.* (2012). Gp. (3) revealed few peticheal hemorrhages, depression, with mortality rate 6.67% during the experimental period. This indicated that florfenicol has bacteriostatic effects (Lim *et al.,*2010). Gp. (4) showed depression, with death of 2 fish (6.67%) during the experimental period. This indicated that *R. officinalis* increased the survival rate of fish challenged with *S. iniae* and in turn decreases the mortalities. Similar results were previously obtained by Abutbul et al. (2004) who recorded a significant reduction in the mortality rate of tilapia infected with *S. iniae* and fed a diet containing *R. Officinalis.* They attributed this reduction to the bacteriostatic effect of the main constituents of *R. officinalis* (α-pinene, limonene, 1, 8-cineol, camphor, terpineol-4-ol, and α-terpineol).

The hematological results (Tables 1, 2 & Figs. 5-8) showed a highly significant decrease in RBCs count, Hb concentration, PCV and leukocytosis with neutrophilia (gp. 2). This partially agrees with the results obtained by Mcnulty *et al.* (2003) who recorded a decrease in the value of RBCs, and they attributed the decrease in mean RBCs counts to the

hemolytic nature of *S. iniae*. Also these results partially agrees with Shoemaker *et al.* (2006) who noted significantly higher counts for RBCs and WBCs post challenge to Nile tilapia with *S. iniae* (low challenge 8.8×10^3, medium challenge 8.8×10^4 and high challenge 8.8×10^5). They also noted a significant decrease in Hb values in all injected treatments at day 14 and this may be a reflection of handling stress and/or anti-nutritional factors associated with the soy-based bacterial growth medium.

Nile tilapia infected with *S. iniae* and treated with florfenicol (gp. 3) showed highly significant decrease in RBCs count, Hb and PCV values, with microcytic normochromic anemia and leukocytosis with neutrophilia. By the end of the experiment, normocytic normochroic anemia, leucopenia and neutropenia were observed. This picture of anemia or leukocyte alterations may be attributed to depressive effect of florfenicol on the mitochondrial synthesis of protein in bone marrow. Inhibition of mitochondrial protein synthesis ultimately disrupts mitochondrial function, cellular function and cellular proliferation (Yunis, 1988). Our results were in agreement with Amer *et al.* (2009) who reported that florfenicol caused a decrease in all hematological parameters in catfish.

Treatment of infected tilapia with *R. officinalis* (gp.4) showed highly significant decrease in RBCs count, Hb and PCV values with macrocytic normochromic at first and normocytic normochromic by the end of the experiment, with normal leukocyte count. This means that *R. officinalis* after infection not improve the hematological picture.

Nile tilapia treated with rosemary before and after infection with *S. iniae* (gp. 5) showed a significant decrease in RBcs count, PCV and non significant decrease in Hb concentration with normocytic normochromic anemia. By the end of the experiment, these parameters return to normal, with normal leukocyte count, so this indicated that administration of *R. officinalis* improve the hematological picture. Unfortunately, there is no available literatures discussed our results for rosemary.

Fish have many non-specific and specific humoral and cellular mechanisms to resist bacterial diseases. Non-specific humoral factors like growth inhibiting substances including transferrin antiproteases and lysins such as lysozyme, C-reactive protein, bactericidal peptides and most importantly complement which has lytic, proinflammatory, chemotactic and opsonic activities thus make a link with non-specific phagocyte responses. These are primarily executed by neutrophils and macrophages. The phagocytes contain many hydrolytic enzymes and when stimulated by bacteria produce reactive oxygen species (ROS), especially the hydroxyl

radical, via generation of superoxide anions and nitric oxide (Ellis, 1999). Regarding the results of cell mediated immune response the phagocytic percent and phagocytic index (Table 3& Figs 9-12) showed a significant decrease in gp. (2). This explained by Zlotkin *et al.* (2003) who reported that *S. iniae* Serotype II strains able to enter phagocytes and multiply, with subsequently undergoing death through apoptotic processes. Also, these previous parameters revealed a significant decrease in gp. (3). This likely indicated that these antimicrobials had effects on the cellular and adaptive immune response in fish than on the innate humoral immune responses (Caipang *et al.,* 2009). Gp. (5) improved the innate immunity by returning the phagocytic percent and phagocytic index close to the control level. This partially agrees with Tal *et al.* (2007) who suggested that rosemary had a weak affect on the cellular innate immune function of tilapia. Regarding the lysozyme activity, it showed a significant decrease (gp.2). This disagrees with Shoemaker *et al.* (2006) who noted non significant differences for lysozyme value of various treatment groups following the 8-week growth performance trial. On the other hand, lysozyme activity showed a significant increase in gps. (3, 4&5), in comparison with infected group (gp. 2), so this indicate that florfenicol and *R. Officinalis* improved the innate immunity by returning the lysozyme activity towards the normal control level.

Regarding the biochemical studies (Tables 4&5), the serum ALT, AST, ALP, total bilirubin, direct bilirubin and indirect bilirubin showed a significant increase in gp. (2). The serum transferases (ALT and AST) are considered a sensitive indicator to hepatocellular and myocardial damage (Gupta, 1980 and Abo- Hegab *et al.,* 1992); hence the synthesis of these enzymes is mostly of hepatic origin. The liver excretes the breakdown product of hemoglobin, namely bilirubin, so serum bilirubin levels have been used to evaluate the hepatic injury (Gressner *et al.,* 2007). Our results were previously reported by Chen *et al.* (2004) and supported by histopathological results which revealed periportal hydropic degeneration (Fig. 14) and extensive necrosis surrounded with thick zone of leukocytes mostly of polymorphnuclears in the liver (Fig. 15), in comparison with the control (Fig. 13). Fatty change, congested blood vessels, and hemorrhage were detected; with diffuse numerous eosinophilic hyalinized globules (Mallory's bodies) in the degenerated and necrotic hepatic cells. Recent thrombi inside the blood vessels and biliary hyperplasia in the form of bile ductules were also encountered. Hemorrhages and edema in the portal areas and among the hepatic cells were noticed. Diffuse hydropic degeneration and vacuolations of

the hepatic cells were observed besides numerous apoptosis.

Treatment of infected fish with either florfenicol or rosemary (gps. 3& 4) caused a significant decrease in serum ALT activity, in comparison with the infected group (gp. 2). This means that florfenicol and *R. officinalis* decrease liver damage and improved the structural integrity of hepatocyte. This confirmed by histopathological results which were milder in gps. (3 &4) than those described in gp. (2). The liver of gp. (3) showed a diffuse hydropic degeneration and necrosis in the individual hepatocytes. Small areas of coagulative necroses and round cells infiltration in the contagious portal area were seen (Fig. 16). Such portal areas revealed recent thrombi entrapped large numbers of leukocytes and biliary epithelial hyperplasia. Congestion of the hepatic blood vessels and hemorrhages were detected. The liver of gp. (4) revealed mild hydropic degeneration and few interstitial aggregations of lymphocytes. Small areas or individual cell necrosis represented by pyknosis and absence of the nuclei were seen (Fig. 17). Focal hydropic degeneration and fatty change were noticed especially adjacent to the necrotic areas. However, the hepatic function tests were returned to normal control level in gp. (5). Such investigations were supported by the pathologic findings (Fig. 18) and previously obtained by Gutiérrez *et al.* (2010) who demonstrated that *R. officinalis* both prevented and reversed CCl4-induced hepatic damage as evidenced by a reduction in bilirubin levels, suggesting an improvement in biotransformation. *R. officinalis* treatment generated a recovery of both indicator levels suggesting a hepatoprotective effect and preservation of plasma membranes by antioxidative action.

Regarding to proteinogram (Tables 4 &5), gp. (2) showed a significant decrease in serum total protein and globulin levels 2 weeks postinfection, then return to the normal at the end of the experiment (4weeks). The falling in the proteins levels may be attributed to the decrease in circulating immunoglobulin (Evenberg *et al.,*1986) or may be attributed also to the intestinal damage with loss of proteins. However, Shoemaker *et al.* (2006) mentioned that serum protein was significantly ($P<0.05$) elevated in the *S. iniae*-survived tilapia when compared with controls. Gp. (3) showed non significant changes in all serum protein fractions. These results agree with Amer *et al.* (2009). Gps. (4 &5) showed non significant increase in total protein with significant increase in globulin level after 2 weeks then return to normal at the end of the experiment. The serum glucose level showed a significant increase in gp. (2), this may be due to stress resulted from infection. Hyperglycemia was primarily produced by catecholamine and lately by cortisol, that glucocorticoids create peripheral insulin resistance and augmented glucogenolysis (Sumpter, 1997 and Iwama *et al.,* 2006). Gps. (3, 4&5) demonstrated a decrease in the elevated serum glucose of infected group with *S. iniae*. This finding indicates that the *R. officinalis* extract might be produced its hypoglycemic activity by a mechanism independent from insulin secretion, e.g. by the inhibition of endogenous glucose production (Eddouks *et al.,*2003) or by the inhibition of intestinal glucose absorption (Platel and Srinivasan, 1997). In a previous study, it has been suggested that 50% ethanol extract of *R. officinalis*, in part, due to intestinal glucosidase (AGc) inhibitory activity of its active compound might play a role in controlling dietary glucose uptake in the small intestinal track (Koga *et al.,*2006).

Concerning the kidney function tests, a significant increase in the serum creatinine and urea levels was recorded in gp. (2). The increase in serum creatinine level may be indicated the kidney disease because it excreted mainly through the kidney (Stoskopf, 1993). This confirmed by histopathological lesions in the kidneys comparatively with the normal structure of control (Fig. 19). Hypercellularity of glomerular tufts and mild vacuolation in the tubular epithelium were noticed (Fig. 20), in addition to, coagulative necrosis in tubular epithelium and depletion of the hemopoietic and melanomacrophage centers (Fig. 21). Many hemopoietic cells were necrotic, represented by pyknosis, karyorhexis and cellular fragmentation and hemorrhage were seen within the necrotic lesions. Almost all glomeruli were collapsed with shrunken glomerular tufts and the adjacent tubules were necrotic. The serum creatinine and urea showed non significant increase in gps. (3, 4&5). Our results agree with Amer *et al.* (2009) and confirmed the improvement effect of both types of treatments.

Several types of antioxidative compounds are found in all fish species to protect their lipids against damage caused by reactive oxygen species. The antioxidant systems in living organisms may be represented by enzymes, such as superoxide dismutase, catalase and peroxidases, which remove reactive oxygen species (Bragadóttir *et al.,* 2001). Catalase is a hemoprotein, which catalyzes the reduction of hydrogen peroxides (Punitha *et al.,*2005) and known to be involved in detoxification of H_2O_2 concentrations (Manonmani *et al.,*2005). Fish infected with *S. iniae* showed a significant decrease in the hepatic catalase levels. However, treatment of infected fish with florfenicol resulted in slight elevation in the catalase levels. Fish could develop an adaptive response based on the antioxidant activities which are able to neutralize the oxidative stress seen in fish in various circumstances. Further, the obtained adaptive

response after antibiotic treatment could also be explained by the possible reduction in bacterial load with consequent reduction in antigen stimulation, which was indirectly responsible for maintaining a well-balanced antioxidant status (Ahmad *et al.,* 2000). Treatment of infected fish with *R. officinalis* improved the antioxidant activity by slight elevation to catalase levels. These results previously obtained by Gutiérrez *et al.* (2010) who suggested *that R. officinalis* acts as an antioxidant or as a free radical scavenger and able to stabilize membrane structures, thus preserving the cellular integrity and restraining the severity of CCl4-induced injury. On the other hand, *R. officinalis* accelerated recuperation of glycogen stores and stimulated the activity of GST, a phase II enzyme, in the liver. Based on these results, it is reasonable to suggest that *R. officinalis* might exert other mechanism different from its antioxidant action or free radical scavenger activity. Several reports indicate that the compounds responsible for antioxidative activity of *R. officinalis* are mainly phenolic diterpenes such as carnosoic acid, carnosol, rosmanol (Hras *et al.,*2000), and other phenolic acids, such as rosmarinic and caffeic acids (Carvalho *et al.,*2005 and Perez *et al.,*2007). It is possible that the *R. officinalis* extract due to its presence of several

bioactive antioxidant principles and their synergistic properties may be caused an improving effect in antioxidant status. The hepatic malondialdehyde (MDA) level showed a significant increase in fish infected with *S. iniae*, but treatment of infected fish with florfenicol or *R. officinalis* resulted in slight decrease the levels of hepatic MDA. This agrees with Botsoglou *et al.* (2009) who said that the levels of MDA in serum, liver, kidney and heart tissues of rat feeding oregano, rosemary, or both before CCl4 treatment resulted in significant decrease that did not differ from those of the control group. The decline was lowest in the oregano group, and highest in group given both herbs. The major implication from this assay is that the herb supplements were an effective direct quencher of free radicals.

4. Conclusions

The development of drug resistance in bacteria and the accumulation of chemicals in the environment and in the fish have led to strict regulations that limit the use of antibiotics and other chemicals in fish aquaculture. The results presented here show the potential of *R. officinalis* for controlling *S. iniae* in tilapia. Such treatment could be used in organic fish culture.

Table 1: Erythrogram (mean values ± SE) in different groups of Nile tilapia. Gp.(1): Normal control, Gp.(2): Infection with *S. iniae,* Gp.(3): *S. iniae* and florfenicol, Gp.(4): *S. iniae* and *R. officinalis* and Gp.(5): *R. officinalis* then *S. iniae* then *R. officinalis* after 2&4 weeks of treatment.

Parameters / Gps.	RBCs x10^6/µl		Hb g%		PCV %		MCV fl		MCH pg		MCHC %	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Gp.(1)	1.36 [a] ±0.09	1.41 [a] ± 0.09	8.75 [a] ± 0.26	7.8 [a] ± 0.24	29.00 [a] ± 1.0	27.00 [a] ±0.71	216.25 [bc] ± 5.10	192.98 ±8.15	65.00 [bc] ± 3.00	55.71 [b] ±2.22	30.25 [bc] ± 0.85	28.75 [c] ±0.49
Gp.(2)	0.83 [cd] ±0.18	0.87 [c] ±0.13	6.45 [b] ± 0.32	5.95 [b] ±0.34	18.25 [bc] ± 1.11	13.75 [d] ±1.49	238.50 [ab] ± 27.89	163.06 ±12.16	85.00 [b] ± 1.05	72.17 [ab] ±8.04	35.25 [ab] ±0.85	44.13 [a] ±2.83
Gp.(3)	0.99 [bc] ±0.03	0.73 [c] ±0.08	7.00 [b] ± 0.33	6.05 [b] ±0.13	16.50 [c] ± 0.96	14.75 [c] ±1.03	166.25 [bc] ± 4.80	206.40 ±13.33	70.50 [bc] ± 1.94	85.88 [a] ±8.00	42.50 [a] ± 1.50	41.40 [a] ±2.27
Gp.(4)	0.64 [d] ± 0.05	0.81 [c] ±0.05	6.80 [b] ± 0.08	5.65 [b] ±0.28	17.50 [bc] ± 1.85	14.50 [c d] ±1.04	274.50 [a] ± 16.50	178.69 ±1.83	108.75 [a] ± 8.25	69.95 [b] ±1.28	40.00 [a] ± 3.34	39.20 [ab] ±1.10
Gp.(5)	1.07 [bc] ± 0.09	1.18 [ab] ±0.13	8.10 [ab] ± 0.31	8.00 [a] ±0.37	22.50 [b] ± 0.87	19.75 [b] ±1.65	214.12 [bc] ± 11.63	163.60 ±19.16	77.83 [bc] ± 8.21	69.33 [b] ±5.14	36.15 [ab] ± 2.00	41.13 [a] ±3.12
F test	**	**	*	**	**	**	**	N.S	**	*	**	**

Means within the same column having different superscript letters are significantly different.
*: Significant at 0.05 probability   **: Highly significant at 0.01 probability   N.S: Non Significant
RBCs: Red Blood Cells   Hb: Hemoglobin   PCV: Packed Cell Volume
MCV: Mean Corpuscular Volume   MCH: Mean Corpuscular Hemoglobin
MCHC: Mean Corpuscular Hemoglobin Concentration

Table 2: Leukogram (mean values ± SE) in different groups of Nile tilapia. Gp.(1): Normal control, Gp.(2): Infection with _S. iniae_, Gp.(3): _S. iniae_ and florfenicol, Gp.(4): _S. iniae_ and _R. officinalis_ and Gp.(5): _R. officinalis_ then _S. iniae_ then _R. officinalis_ after 2&4 weeks of treatment.

Parameters / Gps.	T.L.C $\times 10^3/\mu l$		Neutrophil $\times 10^3/\mu l$		Basophil $\times 10^3/\mu l$	Lymphocyte $\times 10^3/\mu l$		Monocyte $\times 10^3/\mu l$	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Gp.(1)	51.31 [c] ±2.30	48.33 [ab] ±1.69	14.19 [d] ±1.17	14.58 [b] ±1.90	0.12 [bc] ± 0.12	32.78 [ab] ±2.26	31.99 [ab] ± 2.23	4.23 [ab] ± 0.85	1.75 [ab] ± 0.43
Gp.(2)	67.88 [b] ±2.29	50.17 [a] ±2.59	29.21 [c] ± 0.97	9.41 [c] ±0.98	0.66 [ab] ± 0.23	36.78 [a] ±1.55	39.33 [a] ± 3.13	1.82 [bc] ± 0.26	1.43 [abc] ± 0.54
Gp.(3)	83.00 [a] ±1.91	32.50 [d] ±1.61	42.62 [a] ± 1.82	5.82 [c] ± 0.07	0.73 [a] ± 0.25	36.36 [a] ±1.14	26.06 [bc] ± 1.63	3.29 [bc] ± 1.24	0.61 [c] ± 0.08
Gp.(4)	59.00 [c] ±5.00	44.83 [bc] ±1.48	34.56 [b] ± 0.72	10.17 [c] ±1.09	0 [c]	23.44 [c] ± 4.00	33.94 [a] ± 2.44	1.02 [c] ± 0.30	1.21 [bc] ± 0.48
Gp.(5)	58.00 [c] ±3.06	40.17 [c] ±0.60	40.01 [a] ± 0.96	8.92 [c] ± 0.35	0.64 [ab] ± 0.03	13.71 [d] ± 2.58	32.70 [ab] ± 3.19	5.98 [a] ± 1.34	2.48 [a] ± 0.23
F test	**	**	**	**	**	*	**	**	*

Means within the same column having different superscript letters are significantly different.
*: Significant at 0.05 probability   **: Highly significant at 0.01 probability   N.S: Non Significant
T.L.C: Total Leukocytic Count

Table 3: Phagocytic percent, Phagocytic index and lysozyme activity (mean values ± SE) in different groups of Nile tilapia. Gp.(1): Normal control, Gp.(2): Infection with _S. iniae_, Gp.(3): _S. iniae_ and florfenicol, Gp.(4): _S. iniae_ and _R. officinalis_ and Gp.(5): _R. officinalis_ then _S. iniae_ then _R. officinalis_ after 2&4 weeks of treatment.

Parameters / Gps.	Phagocytic percent		Phagocytic index		Lysozyme µmol/ml	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Gp.(1)	81.67 [a] ± 3.76	82.00 [a] ±1.53	0.88 [ab] ±0.04	0.91 [ab] ± 0.02	251.47 [ab] ± 7.37	253.47 [b] ± 5.37
Gp.(2)	50.67 [d] ± 1.76	54.00 [c] ± 2.08	0.58 [d] ±0.02	0.58 [d] ± 0.02	211.07 [c] ± 6.38	213.17 [c] ± 6.35
Gp.(3)	64.33 [bc] ± 2.96	68.00 [b] ± 3.51	0.69 [cd] ±0.02	0.73 [c] ± 0.02	251.47 [ab] ± 7.37	250.47 [b] ± 7.37
Gp.(4)	58.67 [cd] ± 2.33	60.67 [bc] ± 2.33	0.66 [cd] ±0.03	0.65 [d] ± 0.02	229.40 [bc] ± 14.70	249.40 [b] ±14.70
Gp.(5)	73.67 [ab] ± 6.06	79.33 [a] ± 4.91	0.81 [ab] ±0.06	0.83 [b] ± 0.05	266.20 [ab] ± 12.76	267.20 [b] ± 12.66
F test	**	**	**	**	**	**

Means within the same column having different superscript letters are significantly different.
*: Significant at 0.05 probability   **: Highly significant at 0.01 probability   N.S: Non Significant

Table 4: some liver function tests (mean values ± SE) in different groups of Nile tilapia Gp.(1): Normal control, Gp.(2): Infection with _S. iniae_, Gp.(3): _S. iniae_ and florfenicol, Gp.(4): _S. iniae_ and _R. officinalis_ and Gp.(5): _R. officinalis_ then _S. iniae_ then _R. officinalis_ after 2 weeks of treatment.

Parameters / Gps.	ALT U/l	AST U/l	ALP U/l	Total bilirubin mg/dl	Direct bilirubin mg/dl	Indirect bilirubin mg/dl	Total protein g/dl	Albumin g/dl	Globulin g/dl	Glucose mg/dl
Gp.(1)	43.05 [c] ± 3.05	239.75 [c] ±4.25	25.60 [b] ±0.60	0.40 [e] ± 0.04	0.15 [b] ± 0.03	0.25 [b] ± 0.005	4.43 [a] ± 0.30	1.23 ±0.15	3.20 [ab] ± 0.15	85 [d] ± 2.11
Gp.(2)	62.67 [a] ± 1.33	297.10 [a] ±0.90	81.17 [a] ±9.34	0.94 [a] ± 0.05	0.37 [a] ± 0.09	0.57 [a] ± 0.12	3.25 [b] ± 0.25	1.20 ± 0.12	2.30 [c] ± 0.18	123.95 [a] ± 1.43
Gp.(3)	51.93 [b] ±2.91	256 [b] ± 5.51	74.60 [a] ±9.70	0.66 [bc] ± 0.05	0.26 [ab] ± 0.03	0.40 [ab] ± 0.06	4.25 [a] ± 0.14	1.00 ± 0.00	3.25 [ab] ± 0.14	95.45 [bc] ± 2.37
Gp.(4)	37.37 [cd] ±1.77	234.80 [c] ± 4.31	43.80 [b] ±2.46	0.75 [b] ± 0.04	0.34 [a] ± 0.02	0.41 [ab] ± 0.02	4.25 [a] ± 0.25	1.13 ± 0.08	3.13 [ab] ± 0.24	115.80 [a] ±1.90
Gp.(5)	43.83 [c] ± 2.00	232.07 [c] ± 4.49	33.17 [b] ±2.47	0.52 [de] ± 0.03	0.22 [ab] ± 0.02	0.30 [b] ± 0.04	4.75 [a] ± 0.25	1.18 ± 0.10	3.58 [a] ± 0.21	90.10 [cd] ± 3.34
F test	**	**	**	**	*	*	*	N.S	**	**

Means within the same column having different superscript letters are significantly different.
*: Significant at 0.05 probability   **: Highly significant at 0.01 probability   N.S: Non Significant
ALT: Alanine Aminotransferase   AST: Aspartate Aminotransferase
ALP: Alkaline Phosphatase

Table 5: Some liver function tests (mean values ± SE) in different groups of Nile tilapia. Gp.(1): Normal control, Gp.(2): Infection with *S. iniae*, Gp.(3): *S. iniae* and florfenicol, Gp.(4): *S. iniae* and *R. officinalis* and Gp.(5): *R. officinalis* then *S. iniae* then *R. officinalis* after 4 weeks of treatment.

Parameters / Gps.	ALT U/l	AST U/l	ALP U/l	Total bilirubin mg%	Direct bilirubin mg%	Indirect bilirubin mg%	Total protein g/dl	Albumin g/dl	Globulin g/dl	Glucose mg/dl
Gp.(1)	36.83^{abc} ± 3.66	207.50^b ± 5.00	37.33^c ± 1.45	0.37^c ± 0.01	0.18^c ± 0.01	0.19^c ± 0.007	4.67 ± 0.33	1.00 ± 0.00	3.67 ± 0.33	75.93^d ± 0.82
Gp.(2)	39.17^{ab} ± 3.03	259.53^a ± 6.30	91^a ± 2.08	0.64^a ± 0.04	0.31^a ± 0.02	0.33^a ± 0.03	4.67 ±0.33	1.33 ± 0.33	3.33 ± 0.67	156.80^a ± 3.44
Gp.(3)	33^{bc} ± 1.52	208.23^b ± 5.62	62.67^b ± 2.19	0.50^b ± 0.03	0.26^{abc} ±0.03	0.24^{bc} ± 0.03	3.00 ± 0.00	1.00 ± 0.00	2.00 ± 0.00	113.90^b ± 3.16
Gp.(4)	33.50^{bc} ± 1.60	200.33^b ± 2.96	39^c ± 2.89	0.53^b ± 0.03	0.26^{abc} ±0.05	0.28^{ab} ± 0.03	3.33 ± 0.33	1.00 ± 0.00	2.33 ± 0.33	69.37^d ± 4.99
Gp.(5)	42.07^a ± 1.67	108.43^c ± 0.81	39.67^c ±4.10	0.36^c ± 0.02	0.19^{bc} ± 0.02	0.17^c ± 0.03	4.33 ± 0.33	1.00 ± 0.00	3.33 ± 0.33	66.60^d ± 0.85
F test	**	**	**	**	**	**	N.S	N.S	N.S	**

Means with the same column having different superscript letters are significantly different.
*: Significant at 0.05 probability.   **: Highly significant at 0.01 probability.   N.S: Non Significant
ALT   alanine aminotransferase   AST   aspartate aminotransferase   ALP   alkaline phosphatise

Table 6: Some kidney function tests and Antioxidant enzymes in the liver tissue (mean values ± SE) in different groups of Nile tilapia. Gp.(1): Normal control, Gp.(2): Infection with *S. iniae*, Gp.(3): *S. iniae* and florfenicol, Gp.(4): *S. iniae* and *R. officinalis* and Gp.(5): *R. officinalis* then *S. iniae* then *R. officinalis* after 2 &4 weeks of treatment.

Parameters / Gps.	Creatinine mg/dl		Urea g/dl		MDA nmol/ml		Catalase U/g	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Gp.(1)	0.75^{bc} ± 0.05	7.77^{bc} ± 0.27	0.63^{bc} ±0.07	6.27^{bc} ± 0.69	59.64^c ± 2.41	60.64^c ± 1.41	21.46^a ± 0.40	22.36^b ± 0.30
Gp.(2)	1.11^a ±0.13	1.06^a ± 0.22	10.30^a ±0.73	9.77^a ± 0.87	89.57^a ± 5.58	88.77^a ± 4.58	12.81^d ± 1.07	13.81^e ± 1.17
Gp.(3)	0.91^{ab} ±0.20	0.89^{ab} ± 0.12	8.43^{ab} ± 0.78	9.20^a ±1.48	79.98^{ab} ±63.80	78.98^{ab} ± 5.38	19.06^b ± 0.19	16.68^d ± 1.07
Gp.(4)	0.87^{ab} ±0.098	0.75^{ab} ±0.03	8.0^{bc} ± 0.91	8.13^{ab} ± 0.83	72.46^b ± 1.71	71.46^b ± 1.71	16.68^c ± 1.17	19.66^c ± 0.18
Gp.(5)	0.58^{bc} ±0.03	0.45^c ± 0.08	5.98^c ± 0.36	4.70^c ± 0.50	61.63^c ± 1.88	60.62^c ± 1.78	22.29^a ± 0.61	22.29^b ± 0.62
F test	**	***	**	***	**	**	**	**

Means with the same column having different superscript letters are significantly different.
*: Significant at 0.05 probability.   **: Highly significant at 0.01 probability.   N.S: Non Significant
MDA: Malondialdhyde

Fig. (1): Nile tilapia experimentally infected with *S. iniae* showed erythema on tail, pectoral and pelvic fins (arrow).

Fig. (2): Nile tilapia experimentally infected with *S. iniae* showed ascitis(arrow).
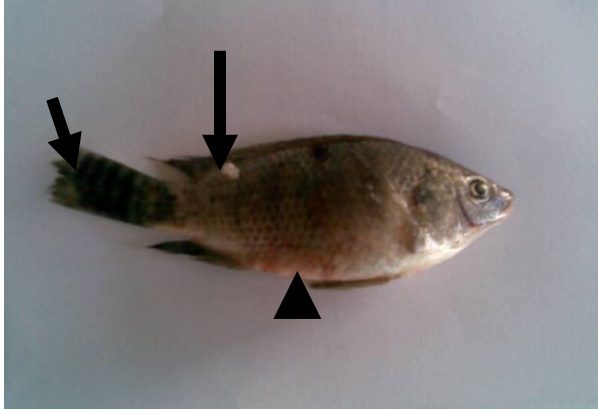
Fig. (3): Nile tilapia experimentally infected with *S. iniae*, showed slight ulcers with erythema.

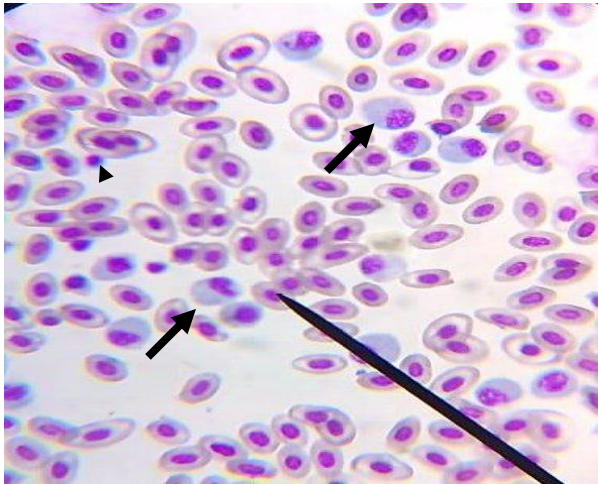**Fig. (4): Nile tilapia experimentally infected with *S. iniae*, showed exophthalmia.**

Fig. (5): blood film showed neutrophil (arrow) and lymphocyte (arrow- head) in *Nile tilapia* experimentally infected with *S. iniae* (gp. 2).
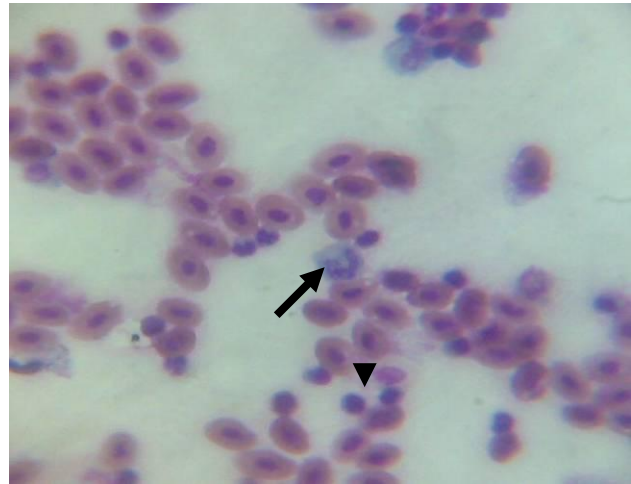

Fig. (6): blood film showing neutrophil (arrow) and lymphocyte (arrow- head) in infected fish and treated with florfenicol (gp. 3).
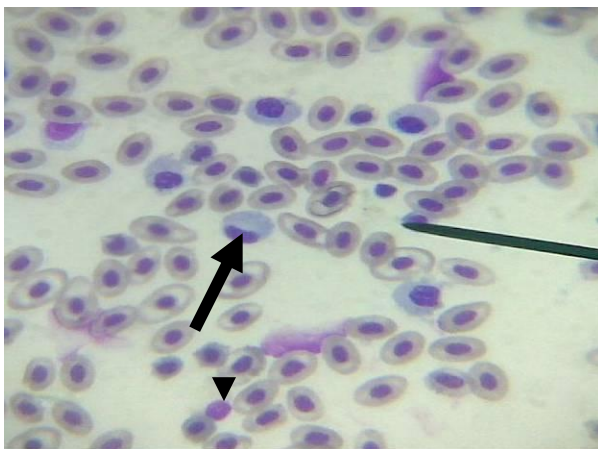

Fig. (7): blood film showing lymphocyte (arrow) and neutrophil (arrow- head) in infected fish and treated with *R. officinalis* (gp. 4).
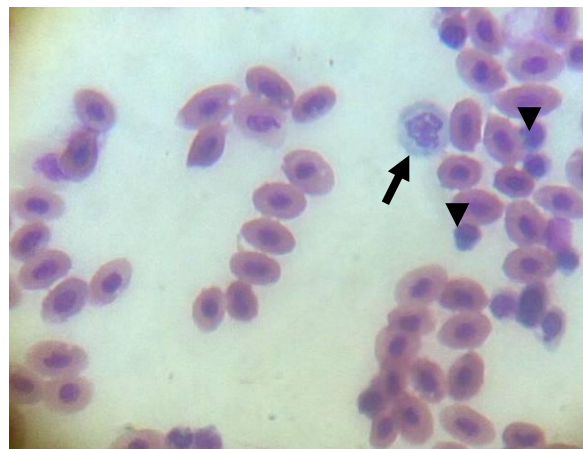
Fig. (8): blood film showing lymphocyte (arrow-head) and monocyte (arrow) in treated fish with *R. officinalis* before snd after infection with *S. iniae* (gp. 5).
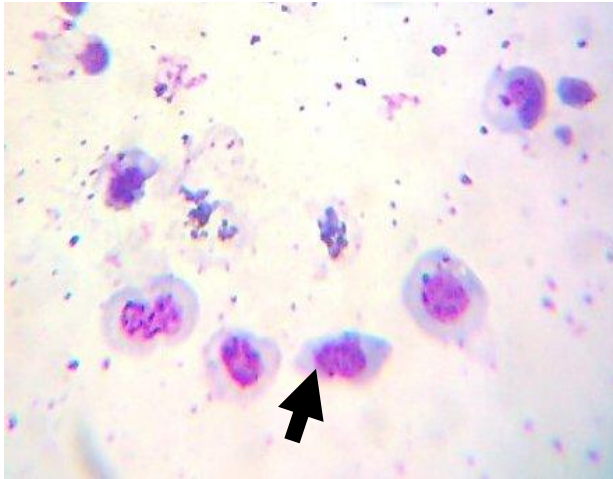
Fig. (9): showing phagocytic cell (arrow), in film from infected fish with *S. iniae* (gp. 2).
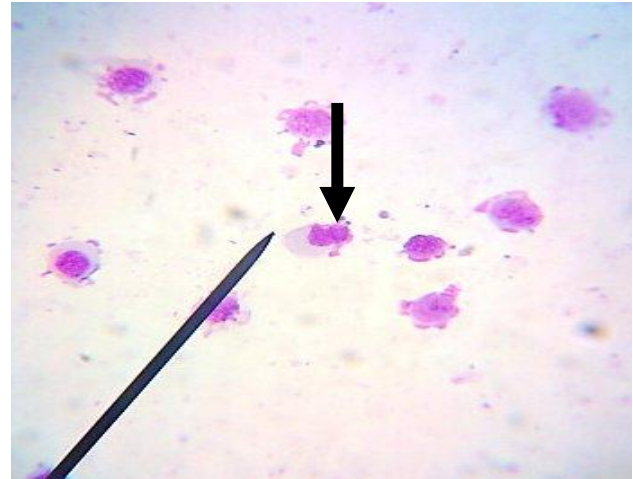
Fig. (10): showing phagocytic cell (arrow) in infected fish and treated with florfenicol (gp. 3).
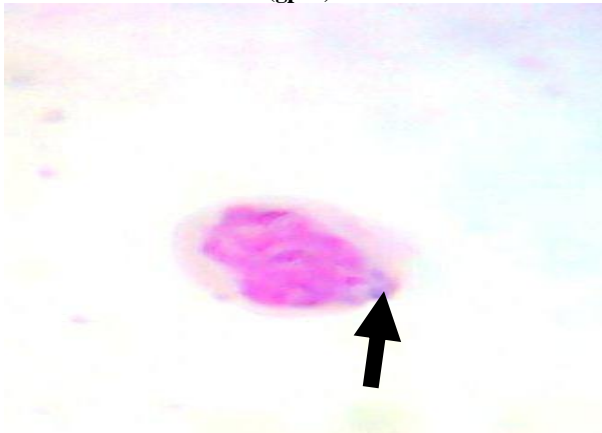
Fig. (11): showing phagocytic cell (arrow), in film from infected fish that treated with *R. officinalis* (gp. 4).
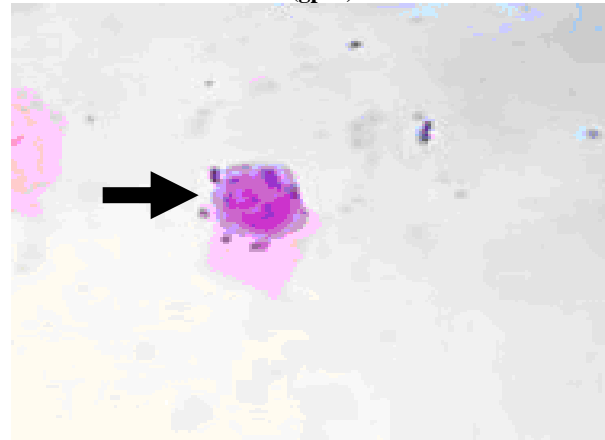
Fig. (12): showing phagocytic cell (arrow) in fish treated with *R. officinalis* before and after infection with S. iniae (gp. 5).
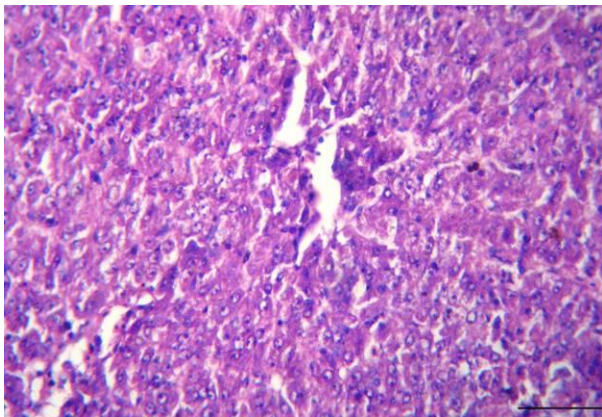
Fig. (13): Liver of fish of control (gp. 1) showing normal hepatocyte and sinusoidal architecture, HE (Bar = 100 μm).
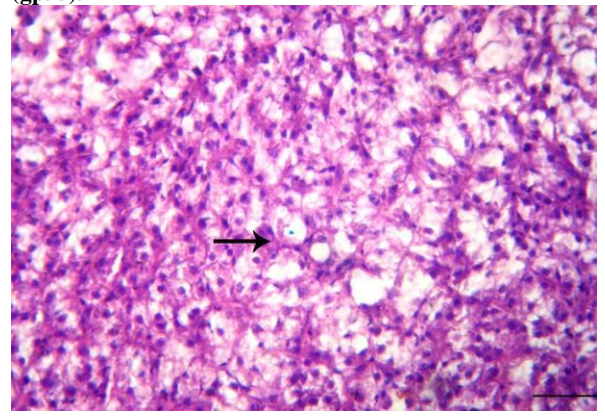
Fig. (14): Liver of fish infected with *S. Iniae* (gp.2) showing periportal hydropic degeneration (arrow), HE (Bar = 100 μm).
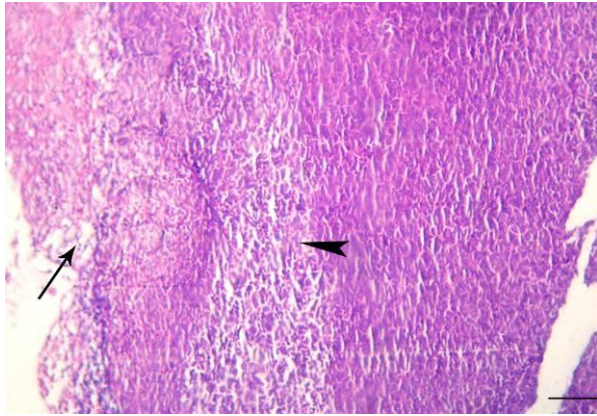
Fig. (15): Liver of fish infected with *S. Iniae* (gp. 2) showing extensive necrosis (arrow) surrounded with thick zone of leukocytes mostly of polymorphnuclears (arrowhead), HE (Bar = 100 µm).
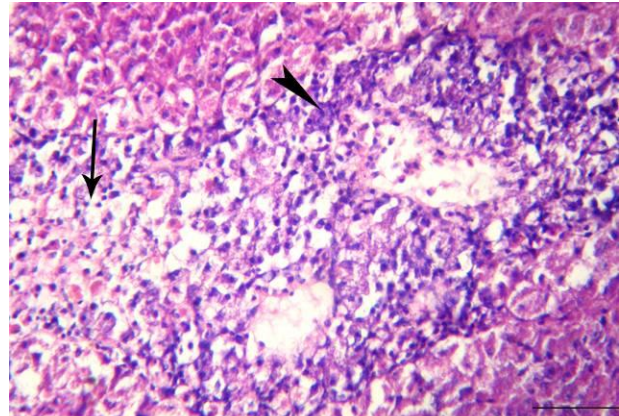
Fig. (16): Liver of fish infected with *S. iniae* and treated with florfenicol (gp.3) showing small necrotic area (arrow) and round cells infiltration in the contagious portal area (arrowhead), HE (Bar = 100 µm).
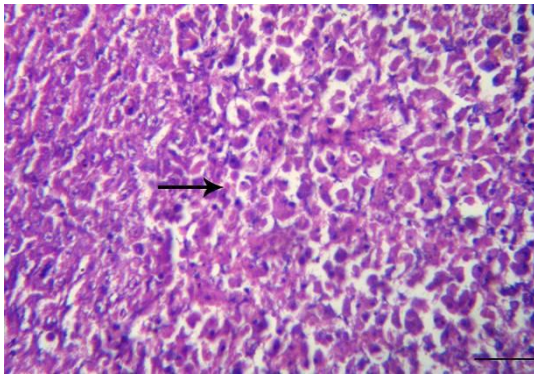

Fig. (17): Liver of fish infected with *S. iniae* and treated with *R. officinalis* (gp. 4) showing small focal area of coagulative necrosis presented by pyknosis and absence of the nuclei (arrow), HE (Bar = 100 µm)
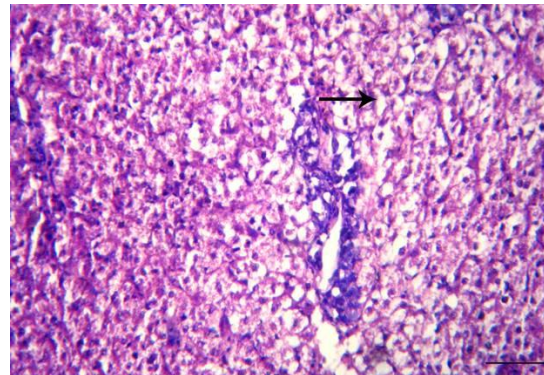
Fig. (18): Liver of fish administered *R. officinalis* before and after infection with *S. iniae* (gp. 5) showing focal hydropic degeneration (arrow), HE (Bar = 100 µm).
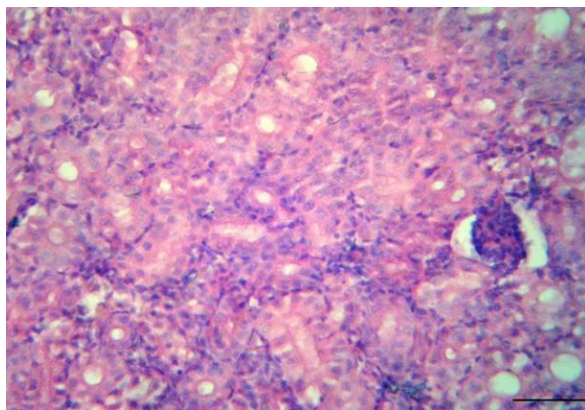

Fig. (19): Kidney of fish of control (gp. 1) showing normal glomerular and tubular structure, HE (Bar = 100 µm).
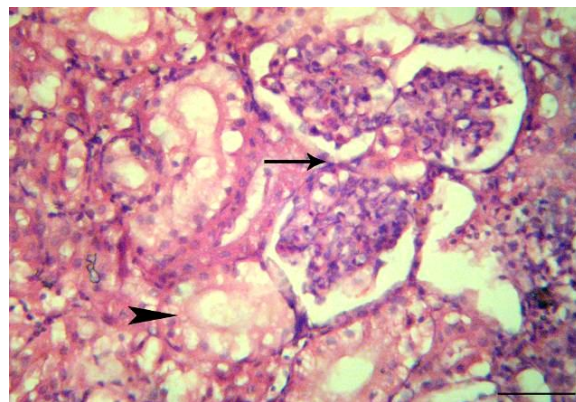

Fig. (20): Kidney of fish infected with *S. iniae* (gp. 2) showing hypercellularity of glomerular tufts (arrow) and mild vacuolation in the tubular epithelium (arrowhead), HE (Bar = 100 µm).
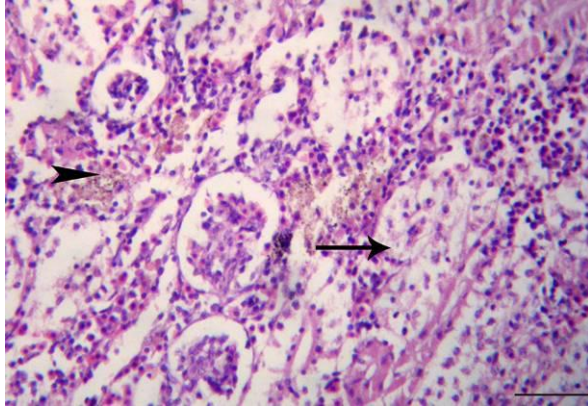
Fig. (21): Kidney of fish infected with *S. iniae* (gp. 2) showing coagulative necrosis in tubular epithelium (arrow) and depletion of the hemopoietic and melanomacrophage centers (arrowhead), HE (Bar = 100 μm).
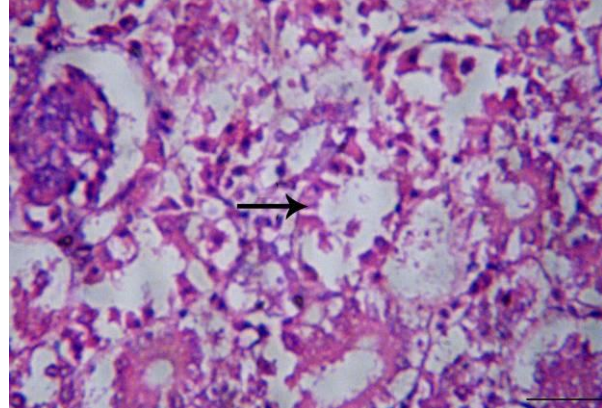

Fig. (22): Kidney of fish infected with *S. iniae* and treated with florfenicol (gp. 3) showing coagulative necrosis in the tubular epithelium (arrow), HE (Bar = 100 μm).
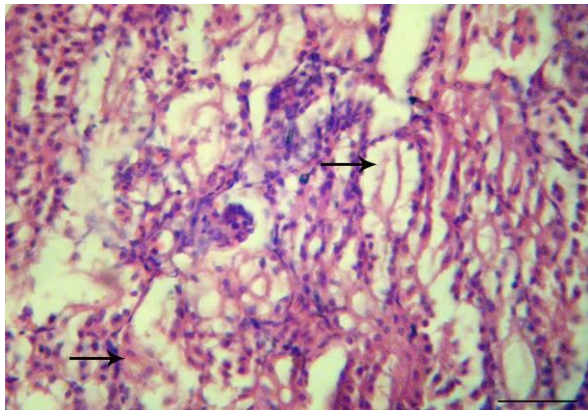

Fig. (23): Kidney of fish infected with *S. iniae* and treated with *R. officinalis* (gp. 4) showing vacuolation of the tubular epithelium (arrows), HE (Bar = 100 μm).
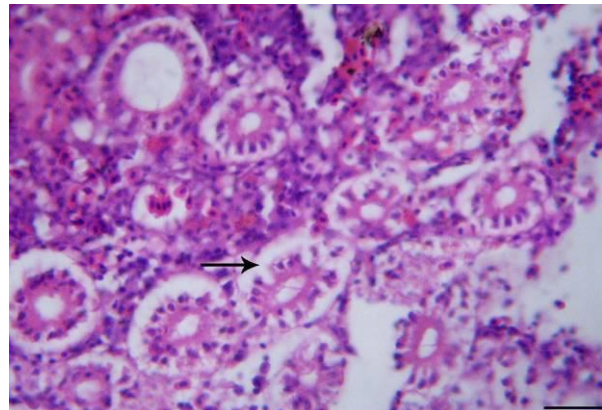

Fig. (24): Kidney of fish administered *R. officinalis* before and after infection with *S. iniae* (gp. 5) showing edema in the interstitial tissue and hydropic degeneration in the tubular epithelium (arrow), HE (Bar = 100 μm).

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