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# EFFICIENCY OF PROTEASE ENZYME AS FEED ADDITIVES ON HEMATOLOGY, BIOCHEMICAL AND HISTOPATHOLOGY IN *CYPRINUS CARPIO* L. CHALLENGED WITH *FLAVOBACTERIUM COLUMNARE*

**Reem N. Talib and Jamal K.A. AL-Faragi**

Department of Pathology, College of Veterinary Medicine, University of Baghdad, Iraq.

## Abstract

This study was point to evaluate the effect of protease enzyme as feed additives on hematology, biochemical response and histopathological changes challenged with *Flavobacterium columnare.* In common carp, *Cyprinus carpio* total of 120 *C. carpio* (initial mass 21.0-22.5 g) were randomly stocked into five treatments in duplicate (10 fish/tank) as follows: T1, T2 and T3 were fed basal diet supplemented with protease at 150 mg/kg, 200 mg/kg, 250 mg/kg while T4 were fed on basal diet plus β-glucan at concentration of 1g/kg as well as the control group was fed basal diet without any addition of protease. Results showed after 60 days of feeding period, that the addition of protease enzyme was significantly ($p<0.05$) affected on hematological parameters, RBC, WBC count and hemoglobin content (g/dl) of T3, T2 recorded significant (Pd" 0.05) increase in comparison to control group. As well as, total protein and albumin levels of T3 revealed a significant increase in comparison to the control group. At the end of experimental period (60 days) six fishes were randomly selected from each treatment group T1, T2, T3, T4 and control (C) which divided into negative and positive control for infection test. At the end of experimental period (60 days) fish were challenged with *Flavobacterium columnare*. Result showed significantly increased ($P<0.05$) in RBC, WBC count in T3 and T2 compared to T4, T1 and control group. For histopathology, various types of destruction were found in gill of fish infected with *Flavobacterium columnare* (positive control group). vasodilation and congestion of central venous, hyperplasia with marked congestion of PL, also multiple MNCs infiltration observed in the space between gill filaments with mild epithelial hyperplasia of SL. Skin of the positive control group showed moderate to severe epithelial sloughing, focal epidermal necrosis. Kidney section of control positive showed various forms of tubular necrosis. All the levels of protease enzyme (T1, T2 and T3) have proven a beneficial and protective effect against *Flavobacterium columnare* infected *C. carpio*.

***Key words:*** Common carp, Protease, Haematological, Biochemical, Histological, *Flavobacterium columnare.*

## Introduction

Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare* is one of the oldest known fish diseases in North America, and has been considered a significant problem in many warm water fish species for decades. *F. columnare* is distributed worldwide in fresh water sources and may infect many different wild and cultured freshwater fish species, such as (but not limited to) carp, channel catfish, goldfish, eel, perch, salmonids and tilapia (Declercq *et al*., 2013). Antibiotics and a variety of synthetic chemicals used for the prevention of diseases and stress cause undesired chemical usages in terms of environment and consumers as well as economic losses. In recent years ,functional diet supplementation has become a topic of interest for improving not only growth rate and feed utilization but also health status of farmed fish (Tiengtam *et al.,* 2015). Feed additives has received great attention in the aquaculture industrial, because the request for protein for human consumption is presently increasing and is mainly provide from aquatic farm animals (Klaenhammer & Kullen, 1999). Enzymes have been used as feed supplements for more than 50 years, but the rapid growth in their use has only been within the last 10 years (Doskovic *et al.,* 2013). Proteolytic enzymes (also termed peptidases, proteases and proteinases) are capable of hydrolyzing peptide bonds in proteins. Proteolytic enzymes can be classified based on their origin: microbial (bacterial,

fungal and viral), plant, animal and human enzymes can be distinguished (Mótyán *et al.,* 2013). Several studies report that fish viscera can be used as an important source of alkaline proteases from aquatic organisms, especially trypsin, has markedly increased in recent years, since some proteases are stable and active under harsh conditions (high temperature and pH) and in the presence of surfactants or oxidising agents furthermore, the recovery of proteolytic enzymes from fish viscera represents an interesting alternative when the aim is to minimize the economic losses and ecological hazards caused by this waste (Freitas *et al.,* 2012). Digestion is the biodynamic of enzymes. Enzymes play major role in digestion of ingested food of fish (Chakrabarti & Sharma, 2012). Although the array of digestive enzymes in bony ûshes is the same as that in other vertebrates, ûsh digestive enzymes are less well-studied (German *et al.,* 2004). Digestion is a key process in animal metabolism since it determines the availability of nutrients needed for all biological functions. In addition, the study of digestive enzymes is a key tool when studying the nutritional condition and adaptation of the organism to dietary change (Gisbert *et al.,* 2009). In fish, the levels of digestive enzymes may be influenced by the age of the fish, type of feeding, season and/or temperature of acclimatization and so on (Munilla-Morán & Saborido-Rey, 1996). This study was planned to shed light on efficiency of the protease on hematology, biochemical and histopathological changes in *C. carpio* challenged with *Flavobacterium columnare.*

## Materials and Methods

The current study was carried out in the Fish Diseases Laboratory at the College of Veterinary Medicine / University of Baghdad during the period extended from January 2019 until May 2019. A total of 120 of healthy *C. carpio* (weight 21.0-22.5 g) used in this experiment which was obtained from a commercial farm (Al-Musayyib, Babylon). Initially the health status of the experimental fish was inspected, then the fish were immersed in formalin at concentration of 37% (15ml/100L) for 30 min. or until the appearance of the stress on fish. after two weeks of acclimation for the fish before starting the experiment, during this time, they were stocked in two bath with dimension of 150 × 20 ×40 cm. After that, fish were randomly selected and distributed into 12 tanks at rate of 10 fish per tank (two replicates/treatment) were maintained for each of the five treatments (T1, T2, T3, T4 and control (positive and negative). Control was fed on basal diet only, T1, T2 and T3 were fed on basal diet supplemented with protease enzyme at concentration of (150, 200, 250 mg/kg diet respectively), while T4 was fed on basal diet + β-glucan at concentration of 1gm/kg diet. Fish were fed a rate 3% of body weight twice daily for 60 days. Every day tanks were cleaned and water was partially changed. Blood samples were collected to evaluate hematological parameters (RBCs, WBCs count, Hb content, PCV value) biochemical profile (Total Protein, Albumin and Globulin). Also, post challenge test with *Flavobacterium columnare* blood samples were collected to determine the above mentioned tests and histopathological findings in selected organs (gills, skin and kidney) were also studied.

### Preparation of diet

In this study, floating food was obtained from Faradanah Company (Iran) with a diameter of 5 ± 0.4 and was used as a basal diet. Diet was prepared by grinded the basal diet using food grinder and weighed individually feed for each treatment based of body weight. The procedure to prepare feeding experiment was first to dissolve an appropriate dose of protease enzyme into warm water (45°C) and then mix with basal feed (Fox *et al.,* 2006). Then, different concentrations of protease enzyme (150, 200, 250 mg/kg) were added and 1g of β-glucan/kg and mixed well and converted into paste. These pastes were pelletized using food mixer with 1.5 mm diameter and dried at room temperature with using air fun. The control group was fed with basal diet (commercial feed) without adding protease .The food stored in screw plastic container with moisture proof until feeding trial (Sun *et al.,* 2012).

### Isolation of *Flavobacterium columnare*

*Flvobacterum columnare* was isolated previously in College of Veterinary medicine /Pathology Department by Dr.saif wadhah then had been reactivation. Pure stock cultures were kept in semisolid nutrient medium (Hsu-shotts broth) supplemented with 20% (v/v) glycerine at 20°C. Cultures were matured on blood agar or nutrient broth.

### Challenge test

After 60 days of the experimental period, fish were challenged by injection subcutaneous with 0.2ml ($1.7\times10^6$ CFU/ml). Mortality was recorded daily up to 14 days in the five groups T1, T2, T3, T4 and control group. Control groups were divided into two subgroup: negative control (without challenge with *Flavobacterium columnare.)* and positive control (fishes were challenged with *Flavobacterium columnare*).

### Statistical analysis

Statistical analysis was performed using SAS (Statistical Analysis System - version 9.1). One way with

Least significant differences (LSD) post hoc test was performed to assess significant difference among means. $P < 0.05$ was considered statistically significant. All results were presented as means ± standard error (SE).

## Results

### Hematological parameters

Results of hematological parameters pre and post-challenge with *Flavobacterium columnare* are presented in table 1 & 2. The results revealed a significant increases ($P < 0.05$) in RBCs count of *C. carpio* at 60 days (pre challenge with *Flavobacterium columnare*) in treatments T1, T2, T3 and T4 (3.68, 4.12, 4.75 and 4.02 cell$\times 10^6$/ $mm^3$ respectively) compared with control group(2.65 cell$\times 10^6$/$mm^3$) table 1. Also, similar results were revealed in RBCs count at 74 days (post challenge with *Flavobacterium columnare*) with slight decreases in values compared with pre challenge values, table 2. In table 1, there were a significant increases ($P < 0.05$) in haemoglobin concentration and packed cell volume at 60 days (pre challenge) in treatments T1,T2, T3 and T4 that were (8.34, 8.43, 8.67and 8.46 g/dl respectively) and (28.50, 28.83, 29.00 and 28.16 % respectively) compared with control group (6.87 g/dl and 25.00%), the highest values were recorded in T3 from all other treatments. Also, similar results were revealed in Hb concentration and PCV% at 74 days (post challenge) with slight decreases in values compared with pre challenge values, table 2. In table 1, the results revealed a significant increases ($P < 0.05$) in WBCs count of *C. carpio* post treatment at 60 days (pre challenge) in all treatments compared with control treatment, and the highest mean values were recorded in T3 (18.34 $\times 10^3$ cell/$mm^3$) in comparison with other treatments. While, in table 2 WBCs count increased significantly ($P < 0.05$) at 74 days (post challenge) in treatments T1, T2, T3 and T4 and recorded highest mean values in T3and T2 (21.26, 20.56 x$10^3$ cell/ $mm^3$respectively), but it was no significant difference ($P > 0.05$) between them.

**Table 1:** Blood parameters of *C. carpio* at 60 days (pre challenge).

| Treatment | RBCs count $\times 10^6$ | WBCs count $\times 10^3$ | PCV % | Hb (gm/dl) |
|---|---|---|---|---|
| Control | 2.65 ± 0.16 c | 11.98 ± 0.04 d | 25.00 ± 1.18 b | 6.87 ± 0.26 b |
| T1 | 3.68 ± 0.14 b | 13.70 ± 0.74 cd | 28.50 ± 1.35 a | 8.34 ± 0.39 a |
| T2 | 4.12 ± 0.26 b | 16.79 ± 1.09 ab | 28.83 ± 1.20 a | 8.43 ± 0.23 a |
| T3 | 4.75 ± 0.18 a | 18.34 ± 0.42 a | 29.00 ± 1.47 a | 8.67 ± 0.44 a |
| T4 | 4.02 ± 0.06 b | 15.45 ± 0.87 bc | 28.16 ± 1.22 a | 8.46 ± 0.35 a |
| LSD value | 0.509 * | 2.144 * | 3.071 * | 1.167 * |

Means having with the different letters in same column differed significantly. *($P \leq 0.05$).

**Table 2:** Blood parameters of *C. carpio* at 74 days (14 days post challenge).

| Treatment | RBCs count $\times 10^6$ | WBCs count $\times 10^3$ | PCV % | Hb (gm/dl) |
|---|---|---|---|---|
| Control | 2.57 ± 0.15 c | 12.37 ± 0.28 c | 24.83 ± 0.79 b | 6.62 ± 0.22 b |
| T1 | 3.68 ± 0.12 b | 15.62 ± 0.53 b | 27.66 ± 1.03 ab | 8.15 ± 0.34 a |
| T2 | 4.05 ± 0.28 b | 20.56 ± 1.17 a | 27.50 ± 1.14 ab | 8.22 ± 0.39 a |
| T3 | 4.72 ± 0.03 a | 21.26 ± 0.60 a | 28.83 ± 1.27 a | 8.42 ± 0.50 a |
| T4 | 4.01 ± 0.05 b | 17.11 ± 0.37 b | 27.66 ± 1.14 ab | 8.31 ± 0.35 a |
| LSD value | 0.463 * | 1.956 * | 3.261 * | 1.269 * |

Means having with the different letters in same column differed significantly. *($P \leq 0.05$).

### Biochemical profile (total protein, albumin, globulin and AG ratio).

Total protein level significantly increased ($P < 0.05$) at 60 days (pre challenge) in all treatments compared with control group table 3, the maximum levels were recorded in T3, T2 and T4 (8.09, 7.65 and 7.11 g/dl) respectively. No significant difference ($p > 0.05$) between T2, T4; T1 and control. Also, there were significant increases ($P < 0.05$) at 74 days (post challenge) in all treatments compared with control group table 4, the maximum level was recorded in T3 (9.04g/dl) followed by T2, T4 and T1 (8.22, 8.04 and 8.04 g/dl) respectively. However, no significant difference ($p > 0.05$) between them.

Albumin content in pre table 3 challenge revealed significant increase in all treatment compared with control group. The maximum levels were recorded in T3 followed by T2, T4 and T1 (5.60, 5.24, 5.12 g/dl) respectively. At 74 days (14 days post challenge) significant increase ($p < 0.05$) among all groups at albumin level table 4, highest values was recorded by T3 (6.11g/dl). Between T2, T1 and T4 no significant difference ($p > 0.05$) (5.73, 5.57 and 5.51g/dl) compared with control group (5.21g/ dl). Whereas globulin content increased significantly ($P < 0.05$) in T3, T2, T4 and T1 (2.58, 2.37, 2.28 and 2.17 g/dl respectively) compared with control treatment (1.83g/dl) at 60 days (pre challenge) table 3. Also, globulin increased in T3, T2, T4 and T1 ( 3.64, 3.18, 2.89 and 2.75 g/dl respectively) compared with control group (2.48g/ dl) at 74days (post challenge table 4).

Albumin/globulin ratio in table 3 decreased without significant difference ($P > 0.05$) in treated groups (T1, T2, T3, T4 and control group at 60 days (pre challenge), the best ratio recorded in T3and T2 from all other treatments. A/G ratio in table 4 decreased significantly ($P < 0.05$) in treated

groups (T1, T2, T3, T4) compared with control treatment at 74 days (post challenge), the best ratio recorded in T3 and T2 but not differ significantly (p>0.05) between T1, T2 and T4.

**Histopathological**

**Gills**

Gill sections of *c.carpio* of negative control showed normal structural details of primary lamella and secondary lamella with central vein. Fig. 1A while the most morphological lesions that in positive control group which showed vasodilation and congestion of central venous sinus that contain numbers inflammatory cells Fig. 1B. Gill sections in T1 was similar to positive control, extensive telangiectasis and hyperemia of SL accompanied with moderate loss of lamellar epithelium result in marked atrophy of SL Fig. 1C. Gill sections in T2 showed moderate lamellar epithelial hyperplasia with marked congestion of PL, while extensive proliferative changes of lamellar epithelium result in lamellar hyperplasia with moderate elongation and circulating of SL Fig. 1D. Gill sections in T3 showed various degrees of venous dilation with different inflammatory cells associated with normal lamellar epithelium Fig. 1E. Gill sections in T4 showed moderate proliferative changes were recognized in the gill tissue associated with moderate hyperplasia of lamellar epithelium with evidence of focal cellular proliferation observed at the tip of SL Fig. 1F.

**Skin**

Skin sections of *c. carpio* of negative control showed epidermis, scales, dermis and muscles Fig. 2A, while in control positive showed moderate to severe epithelial sloughing were recognized in many portions of epidermal tissue with cytoplasmic vaculation of some epidermal cells that exhibit few number of alarm cells Fig. 2B, Skin sections in T1 showed focal epidermal necrosis with various sized vacuoles of epidermal squamous epithelium associated with irregular appearance of survival alarm cells Fig. 2C, Skin sections in T2 showed moderate destructive lesions recognized in both dermal and epidermal layers with evidence of superficial desquamation of squamous epithelial and dermal edema associated with perivascular MNCs aggregation Fig. 2D, Skin sections in T3 showed the specific cutaneous lesions observing in the hypo dermal tissue in which the interstitial tissue heavily infiltrated by MNCs with multiple melanin deposition Fig. 2E, Skin sections in T4 showed moderate epidermal tissue hyperplasia with mild desquamation of squamous epithelial cell accompanied with focal vacuolation of basal layer and increase number of both alarm and mucous cells Fig. 2F.

**Kidney**

Kidney sections of *C. carpio* of negative control showed normal tubules, interstitial lymphoidal tissues, melanomacrophage with evidence of thyroid follicles Fig. 3A. While the section of control positive marked cellular infiltration with fibrinous exudate noticed in capsular region that filled with various inflammatory cells Fig. 3B. Kidney sections in T1 showed hydropic swelling of most tubules with focal tubular necrosis accompanied with inflammatory edema between affected tubules Fig. 3C, Kidney sections in T2 showed epithelial cellular swelling associated with focal MM aggregation with no clear changes of glomerular tissue Fig. 3D, Kidney sections in T3 showed many of renal tubules appeared with normal structure with prominence of haemopoietic tissue with presence of eosinophilc odematous substance accompanied with scattered melanomacrophage Fig. 3 E, Kidney section in T4 showed moderate fibroplasia of renal interstitial tissue associated with moderate cellular infiltration Fig. 3F.

**Table 3:** Biochemical parameters of *C. carpio* at 60 days (pre challenge).

| Treatment | Total protein (g/dl) | Albumin (g/dl) | Globulin (g/dl) | Albumin/ Globulin |
|---|---|---|---|---|
| Control | 6.91 ± 0.35 b | 4.63 ± 0.15 b | 1.83 ± 0.04 b | 2.67 ± 0.07 a |
| T1 | 7.00 ± 0.41 b | 4.87 ± 0.22 ab | 2.17 ± 0.07 ab | 2.41 ± 0.07 a |
| T2 | 7.65 ± 0.48 ab | 5.24 ± 0.19 ab | 2.37 ± 0.07 a | 2.21 ± 0.04 a |
| T3 | 8.09 ± 0.53 a | 5.60 ± 0.26 a | 2.58 ± 0.05 a | 2.18 ± 0.03 a |
| T4 | 7.11 ± 0.39 ab | 5.12 ± 0.20 ab | 2.28 ± 0.02 ab | 2.29 ± 0.05 a |
| LSD value | 1.082 * | 0.892 * | 0.503 * | 0.549 NS |

Means having with the different letters in same column differed significantly.* (P≤0.05).

**Table 4:** Biochemical parameters of *C. carpio* at 74 days (14 days post challenge).

| Treatment | Total protein (g/dl) | Albumin (g/dl) | Globulin (g/dl) | Albumin/ Globulin |
|---|---|---|---|---|
| Control | 7.23 ± 0.38 b | 5.21 ± 0.15 b | 2.48 ± 0.05 b | 2.16 ± 0.07 a |
| T1 | 8.04 ± 0.52 ab | 5.57 ± 0.24 ab | 2.75 ± 0.11 b | 2.04 ± 0.03 ab |
| T2 | 8.22 ± 0.57 ab | 5.73 ± 0.19 ab | 3.18 ± 0.14 ab | 1.83 ± 0.03 ab |
| T3 | 9.04 ± 0.48 a | 6.11 ± 0.32 a | 3.64 ± 0.09 a | 1.68 ± 0.04 b |
| T4 | 8.04 ± 0.55 ab | 5.51 ± 0.24 ab | 2.89 ± 0.08 ab | 1.93 ± 0.05 ab |
| LSD value | 1.144 * | 0.883 * | 0.795 * | 0.335 * |

Means having with the different letters in same column differed significantly.* (P≤0.05).

## Discussion

**Hematological parameters**

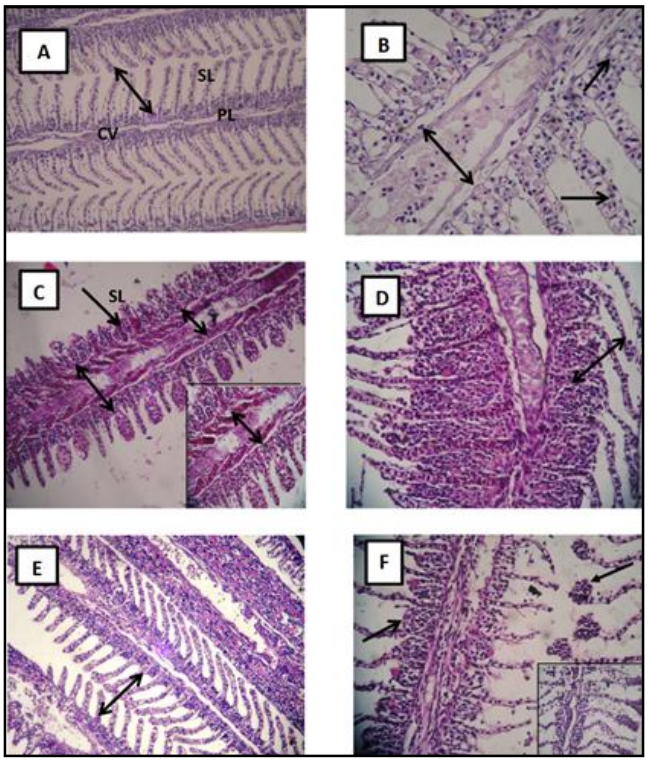


**Fig. 1:** microscopic section of gill of *C. carpio* after treatment of dietary protease and challenge with *F. columnare* (A): control negative healthy fish showing normal gill histology (PL, SL, CV) (H & E stain x20); (B) control positive showing marked vasodilation of C.V.S contain inflammatory cell ↔ with mild lamellar epithelial vacuolation of SL → (H & E stain x40); microscopic section of gill of *C. carpio* after treatment of dietary protease and challenge with *F. columnare* (C): T1 showing extensive lamellar telengiectasis and congestion ↔ with marked atrophy of adjacent SL → (H&E stain x10); (D) T2 showing intensive lamellar epithelial hyperplasia with moderate elongation of SL ↔ (H& E stain x40); (E): T3 showing moderate venous dilation of PL with normal lamellar epithelium of SL ↔ (H&E stain x20); (F) T4 showing mild L.E.H → with focal cellular aggregation at the tip of SL (H & E stain x40).

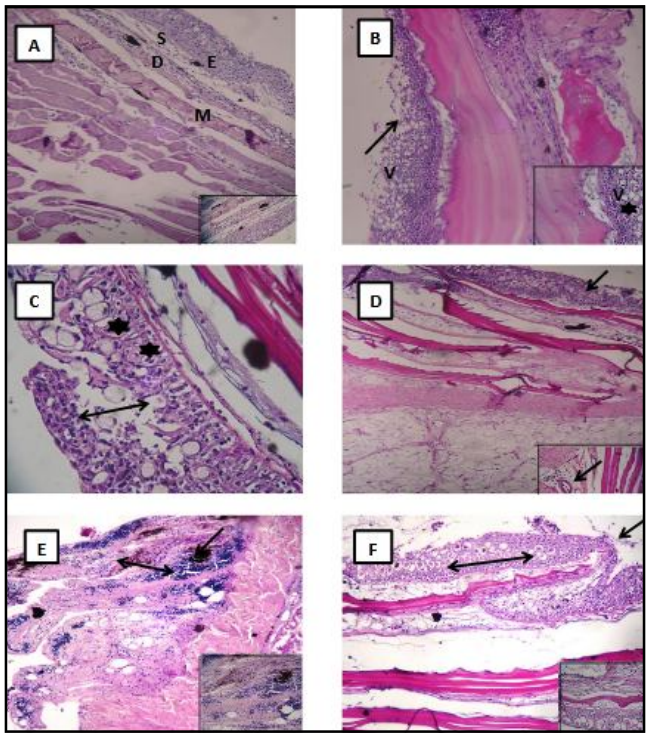


**Fig. 2:** Microscopic section of skin of *C. carpio* after treatment of dietary protease and challenge with *F. columnare* (A) control negative showing normal skin histology (epidermis, scales, dermis and muscle) (H & E stain x40); (B) control positive showing moderate epidermal sloughing and cytoplasmic vaculation (V) → with few alarm cells *(H & E stain x10); (C) T1 showing focal epidermal ↔ necrosis with irregularity of survival alarm cells *(H & E stain x40); (D) T2 showing mild epidermal desquamation → with dermal edema and perivascular MNCS aggregation → (H & E stain x40); (E) T3 showing hypodermal multifocal MNCS infiltration with multiple melanin deposite ↔ (H & E stain x20); (F) T4 showing moderate epidermal hyperplasia → with increased number of alarm cells and mucous cells ↔ (H & E stain x20).

Hematological parameters have been recognized as valuable tools for monitoring the health condition of fish. These parameters influenced by many factors such as fish species, age, the cycle of sexual maturity and health condition (Blaxhall, 1972); (Hrubec *et al.,* 2001). Quality of water, temperature, food availability and physiological status of fish either or indirectly influence on blood constituents of fish (Punniyamurthy & Iqbal, 1997).

The results of this study revealed a significant increase in RBCs count, WBCs count, differential WBCs count, Hb content and PCV% in all protease treatments and β-glucan treatment compared with control group. significant improvement in RBCs count, Hb content, PCV% in this study accordance with the study on tilapia (*Oreochromis niloticus*) were fed with practical diet supplemented with 200 mg/kg protease in comparison with control group (Adeoye *et al.,* 2016). The increased red blood cell could infer better immune response (Zimring & Hudson, 2016). As there is no clear understanding of established interaction between exogenous enzymes and fish hematological status, further study is required to establish the mode of action between exogenous enzyme and hematological parameters. Also (ali Zamini *et al.,* 2014) showed improvement in RBCs count, Hb, PCV%, WBCs count in protease supplemented diet groups and this applied as a measure of general immune response. Increase in number of WBCs in diseased fish could be serving as a protective barrier towards any infection (Talpur *et al.,* 2013). Moreover, WBCs are one of the

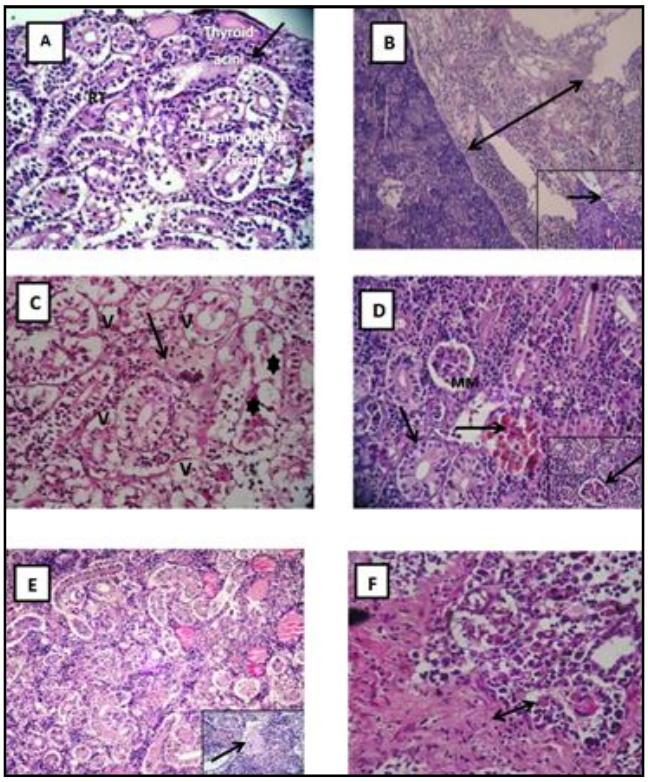


**Fig. 3:** Microscopic section of kidney of *C. carpio* after treatment of dietary protease and challenge with *F. columnare* (A) control negative showing normal histology of kidney renal tubules Rt, hemopoietic tissue and thyroid acini (H & E stain x40); (B) control positive showing marked cellular infiltration in renal capsular tissue with fibrinous exudate ↔ (H & E stain x10); (C) T1 showing focal tubular necrosis *with interstitial inflammatory edema →; (D) T2 showing moderate cellular swelling of renal tubules, moderate congestion of glomerular tissue → with MM hyperplasia (H & E stainX40); (E) T3 showing eosinophilic edematory fluid with scattered MM presence → (H & E stain x40); (F) T4 showing moderate fibroplasia of interstitial tissue with moderate cellular infiltration (H & E stain x40).

most affecting factors in immunity of fish and WBCs counts has been used as marker of health of aquatic animals (Duncan & Klesius, 1996).

**Biochemical Parameters**

The concentration of total protein in blood serum is used as a basic index for the health status of fish (Kumar *et al.*, 2010), increasing in the serum protein Albumin, Globulin levels are thought to be associated with a stronger innate response in fish (Kaleeswaran *et al.,* 2012).

In the present study, there were significant increases in serum total protein, albumin and globulin in all treatments compared with control group. This agree with (W. Liu *et al.*, 2018) who used protease supplement in diet in juvenile Gibel carp (*Carassius auratus gibelio*), because protease enzymes always act as catalysts and small quantities compared to their substrate are required to considerably increase the rate of chemical reaction, in addition to catalytic properties, enzymes exhibit the physico-chemical behavior of proteins: their solubility, electrophoretic properties, electrolytic behaviors and chemical reactivity (Agarwal, 2006). Unlike other feed additives supplements (such as antibiotic growth promoters) which may have adverse impact on human health and the environment, exogenous digestive enzymes are perceived to be harmless, environment friendly and natural (N. Liu *et al.,* 2010). The exogenous digestive enzymes have the potential to reduce environmental pollution arising from aquaculture operations (Kumar *et al.,* 2011). On other hand, results of serum parameters at (Kamel *et al.,* 2015) who used protease supplement in Broiler chicken diet showed no significant effect, this may be due to different immune stimulants that used and the change in their feeding form.

**Histopathological Study**

In the present study massive tissue damage in gill, skin and kidney a of common carp at 14 days post experimental infection with *F. columnare* particulary in positive control and T1 compared with very mild effects observed in the infected fish in other treated group during the same period. Most histopathlogical studies of columnaris disease focused on gill and skin tissues, while in internal organs specially kidney are restricted. In this study, gill necrosis and massive destruction in primary and secondary lamellae, and lamellar epithelial invaded by numerous elongated thin filamentous bacteria were observed in the experimentally infected fish, which would have compromised respiration, that observed as behavioural changes in *C. carpio*. The gill necrosis and bacterial presence observed in *C. carpio* was similar to that described by (Decostere *et al.,* 1999) in black molly. (Tripathi *et al.,* 2005) showed, that following attachment of *F. columnare* to the gill, initial microscopic lesions include branchial epithelial cell and goblet cell hyperplasia. These lesions rapidly progress to severe neutrophilic inflammation and gill necrosis in koi carp, *C. carpio*. In acute bacterial infection, hypoxia and death may result from extensive damage to the gills. In advanced stage of the disease extensive loss of bronchial structures (disappearance of the normal structure of primary and secondary filaments) is visible, in which the filaments and the lamellae have fused and the gill epithelium is destroyed. In skin sections, Similar histopathological observations were found in the experimentally challenged striped catfish, *Pangasiandon hypophthalmus* with *F. columnare* by (Tien *et al.,* 2012). They observed: loss of

structure in the skin showing flexible bacteria intermixed with collagen; necrosis of muscle areas, together with high leukocyte influx associated with the presence of *F. columnare*.(Tripathi *et al.*, 2005) reported the presence of the bacteria on the skin is typical of columnaris infections, resulting in skin and muscle injury with inflammatory cell influx in koi carp *Cyprinus carpio*.

In this study, renal section of control positive showed massive areas of haemorrhage and congestion seen in renal tissue mixed with bacterial filaments accompanied with extensive tubular degeneration, tubular rupture and necrosis, also massive destruction in tubular and glomerular structure. The kidney plays a role in the formation of various white blood cells group such as the monocytes and granulocytes (neutrophils, basophils and eosinophils). High intensity attacks from pathogenic bacteria causes the kidney to work in overdrive, causing cell damage. In addition, the bacteria which succeed in attacking the kidney will secrete exotoxins which have the ability to cause haemorrhages in the epithelial lining of the tubules (Widanarni & Tanbiyaskur, 2015).

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