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# Evaluation of The dietry Effect of The organic Kelp Powder (Ascophyllum nodosum) on The immune responses, Health benefits and Disease resistance of *Pangasianodon hypophthalmus* fries (Basa fish)

**Rehab, A. Abd-elaziz*; Rasha, Diabb**; Hebatallah, M. Saad***; Mustafa, Shukry****; Hany, M.R. Abdel-Latif***** and Safaa, H. Aboolo******

*Fish Diseases Department, Alexandria Provincial Lab., Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Giza, Egypt, purple-rose20082000@yahoo.com, https://orcid.org/0000--0003-3106-1810

**Immunity Department, Alexandria Provincial Lab, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Giza, Egypt;

***Department of Pathology, Faculty of Veterinary Medicine, Matrouh University, Marsa Matruh 51744, Egypt;

****Department of Physiology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, 33516, Egypt and Department of Biomedical Sciences, College of Veterinary Medicine, King Faisal University, Saudi Arabia,

*****Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University,

******Pathology Department, Damnhour Provincial Lab., Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Giza, Egypt; Safaaboolo.4@gmail.com, https://orcid.org/0000-0003-3141-3994

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**Abstract**

A 60-day feeding trial was conducted on *Pangasianodon hypophthalmus* fries using varying supplementation levels of KP (KP-0 to KP-4). Dietary KP significantly improved growth indices in a dose-dependent manner, with the KP-4 group exhibiting the highest weight gain, specific growth rate, and optimal feed conversion ratio. High survival rates across all groups confirmed the safety of KP as a functional additive. Antioxidant analysis revealed that KP supplementation significantly upregulated superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities while reducing malondialdehyde (MDA) levels, with the KP-3 group providing the most effective physiological priming against post-challenge oxidative stress.

Corresponding author: Safaa H. Aboolo, Pathology Department, Damnhour Provincial Lab., Animal Health Research Institute (AHRI) Dokki, Nadi El-Seid Street, Dokki P.O., Giza 12618, Egypt. Agriculture Research Center (ARC), Giza, Egypt.
Email address: Safaaboolo.4@gmail.com , https://orcid.org/0000-0003-3141-3994

Serum protein profiles showed enhanced total protein and albumin in the KP-3 group, whereas the KP-4 group demonstrated a superior humoral immune response via elevated globulin levels post-infection. Innate immune markers, including lysozyme and myeloperoxidase activities, were significantly activated post-challenge, particularly in KP-supplemented groups. Furthermore, KP exerted a protective effect on renal and hepatic functions, as evidenced by reduced leakage of urea, creatinine, and liver enzymes (ALT, AST, and ALP) following bacterial infection. Histopathological and immunohistochemical examinations of the gills, hepatopancreas, and intestines corroborated these findings, showing that KP supplementation specifically at KP-3 and KP-4 levels mitigated tissue degradation, inflammatory infiltration, and excessive cellular regeneration induced by A. hydrophila. Overall, dietary KP supplementation at levels between KP-3 and KP-4 serves as a potent growth promoter and immunostimulant, enhancing the resilience and structural integrity of P. hypophthalmus against bacterial pathogens

## 1. Introduction

The global aquaculture industry has seen exponential growth, with the *Pangasianodon hypophthalmus*, emerging as a cornerstone species due to its high rate of growth and economic needs **(Khan *et al*. 2021; Tran *et al*. 2025).** However, the rapid expansion of basa fish farming has triggered a sharp rise in disease outbreaks, primarily caused by the ubiquitous Gram-negative bacterium, *Aeromonas hydrophila* **(Chen *et al*. 2022; Ngo *et al*. 2022).** This pathogen is responsible for Motile Aeromonas Septicemia (MAS), a condition characterized by sever economic losses across the aquaculture sector **(Abdel-Latif *et al*. 2020).** Traditionally, fish farmers have relied on chemotherapeutics and antibiotics to mitigate these bacterial threats. However, the indiscriminate use of these agents has triggered the emergence of multidrug-resistant bacterial strains and raised concerns regarding toxic residues in fish flesh and the surrounding environment **(Lulijwa *et al*. 2020).** Consequently, there is an urgent need for sustainable, eco-friendly alternatives such as immunostimulants that can bolster the innate physiological defenses of fish **(Reverter *et al*. 2021).**

The global expansion of intensive aquaculture for P. hypophthalmus has necessitated the development of functional feed additives to mitigate the economic losses caused by opportunistic pathogens **(Abd-elaziz *et al*. 2023; Abdel-Latif *et al*. 2023).** Among these, marine macroalgae, or seaweed, have gained prominence as functional feed additives due to their rich profile of bioactive compounds, including polysaccharides, polyphenols, and essential minerals **(Abdel-Latif *et al*. 2022; Michalak *et al*. 2022).** The brown seaweed Ascophyllum nodosum, commonly known as knotted wrack, has emerged as a potent source of bioactive compounds, including fucoidans, laminarins, and polyphenols, which possess significant biological activities **(Pereira *et al*. 2020).** A. nodosum, a brown seaweed native to the North Atlantic, is particularly noted for its high content of complex carbohydrates like fucoidan and alginates, which possess documented antimicrobial and antioxidant properties **(Abdel-Latif *et al*. 2022).** This macroalga is widely utilized, as a powerful bio-stimulant, for maintaining sustainable agriculture to enhance growth, yield, and resilience of plants against environmental stressors **(Kumari *et al*. 2023).** Beyond its agriculture uses, it is rich in high concentrations of bioactive compounds including polysaccharides (fucoidan, alginic acid), phlorotannins, and essential minerals which enable diverse applications in human, animal, and fish health **(Frazzini *et al*. 2024; Obluchinskaya *et al*. 2024).** In animal nutrition, it is used as a feed additive to increase immune capacity, decrease stress effects and initiate enteric methane emissions in livestock **(Archer 2023; Frazzini *et al*. 2024).** The dietary inclusion of *A. nodosum* in diets of weaned piglets is hypothesized to enhance growth performance by improving intestinal microbiota and enzymatic activity **(Michiels *et al*. 2012).** Furthermore, its antimicrobial and antioxidant properties have sparked interest in the pharmaceutical and cosmetic industries for potential anti-inflammatory, anti-aging, and metabolic health treatments **(Samuels *et al*. 2022).**

In aquaculture, research studies confirmed the

usefulness of Ascophyllum-based diets in several finfish species. For example, dietary supplementation with Ascophyllum meal significantly improved the physiological robusticity, muscle protein composition, and response to air-dipping of red seha bream **(Nakagawa *et al*. 1997).** *A. nodosum* meal was an effective additive for enhancing non-specific cellular immunity in parrot fish with recommended application particularly during periods of low-water temperature stress, when the immune systems of these fish are typically compromised **(Kyoung-Mi *et al*. 2004).** Due to its low digestibility and negative impact on growth at higher concentrations, A. nodosum is not recommended as a primary nutrient source, but rather as a potential additive or prebiotic at or below a 1.0% inclusion level in diets of Nile tilapia fingerlings **(Alves Filho *et al*. 2011).** However, it was found that the inclusion of A. nodosum seaweed meal (20 g/kg diet) in the diet of Nile tilapia fingerlings provides protective health benefits when the fish are challenged with A. hydrophila **(Oliveira *et al*. 2014).**

Intensive rearing conditions often lead to the overproduction of reactive oxygen species, resulting in oxidative stress and cellular damage **(Lushchak 2011).** A. nodosum is rich in phlorotannins, which function as powerful antioxidants by scavenging free radicals and upregulating the activity of endogenous enzymes such as superoxide dismutase and glutathione peroxidase **(Dutot *et al*. 2012).** This enhancement of the antioxidant status ensures the integrity of cellular membranes in *P. hypophthalmus* fries, particularly during the vulnerable early life stages. Further more, the inhance of immune system of fish via A. nodosum provides a prophylactic shield that significantly increases the survival rate of *P. hypophthalmus* fries following a bacterial challenge. This resistance may be lead to the synergistic effect of enhanced mucosal immunity and the direct antibacterial effect of algal metabolites, which inhibit the colonization of A. hydrophila in the host tissues. Despite its proven benefits in other fish species, the specific efficacy of A. nodosum in enhancing the health of *P. hypophthalmus* at the fragile fry stage remains insufficiently explored. This study aims to evaluate how dietary supplementation of this macroalga influences the survival, blood chemistry, and resistance of *P. hypophthalmus* fries during the challenge test with *A. hydrophila*.

## 2. Materials and Methods

### 2.1. Kelp powder (KP) and experimental diets formulation

To prepare the experimental diets, organic macroalga Canadian kelp powder (Ascophyllum nodosum; Organic Kelp Powder, XPRS Nutra, Canada) was incorporated at five distinct concentrations: 0 (control), 1.0, 2.0, 3.0, and 4.0 g/kg, designated as KP-0 through KP-4, respectively. All formulations were designed to be isocaloric and isonitrogenous, adhering to the nutritional standards (NRC 1993). The basal mixture (Table S1 – Supplementary material) (comprising fish meal, soybean meal, corn gluten, ground corn, rice bran, fish oil, starch, and essential supplements such as di-calcium phosphate, vitamins, minerals, and antioxidants) was finely ground and homogenized before KP supplementation. After ensuring a uniform distribution of ingredients, the resulting dough was processed into 2-mm pellets using a laboratory-scale pelletizer. These pellets were subsequently air-dried and maintained in sealed containers at 4 °C throughout the duration of the study to preserve nutritional integrity.

### 2.2. Experimental fish and culture conditions

To ensure the experimental design was robust and followed standard aquaculture protocols, healthy striped catfish, *P. hypophthalmus* fries were acquired from an indoor facility in Cairo–Alexandria Desert Road, Alexandria, Egypt, and subjected to a two-week acclimation period in 500-L aerated fiberglass units. Following this adaptation phase, 150 individuals (mean weight 6.35 ± 0.1 g) were allocated into five distinct dietary groups, each consisting of three replicates (10 fish per 100-L glass aquarium) (100 cm × 90 cm × 70 cm). Throughout the 60-day study, the fish were fed their experimental rations twice per day at 08:00 and 17:00 at a fixed rate of 3% of their biomass, with quantities recalculated every 14 days to account for growth. Environmental stability was prioritized through daily siphoning of organic waste and a

33% water exchange, keeping dissolved oxygen at (6.5 ± 0.50 mg $L^{-1}$), pH between 7.60–7.65, and un-ionized ammonia within the safe threshold of (0.02–0.20 mg $L^{-1}$). A consistent 12-hour natural light cycle was maintained, while thermal conditions remained steady at 27.5–29.0 °C, ensuring all parameters stayed within the ideal physiological range for this species.

### 2.3. Growth performance and feed utilization

Following the 60-day experimental period, a 24-hr fasting period was observed before the fish were sedated using clove oil (20 mg/L) to facilitate accurate counting and weighing. Growth and feed efficiency were assessed through several metrics: -

Weight gain (WG) was defined as the increment between initial (W1) and final (W2) weights, while the specific growth rate (SGR) was derived using the logarithmic growth equation: -

$$SGR\ (\%/day) = [(\ln W_2 - \ln W_1) / 60] \times 100.$$

Individual feed intake (FI) was calculated by partitioning total consumption among the fish population, and the feed conversion ratio (FCR) was established by relating cumulative feed intake to total biomass gain. Finally, the survival rate (SR) was expressed as the percentage of the initial stock remaining at the conclusion of the trial.

### 2.4. Bacterial challenge test

To prepare the challenge inoculum, A. hydrophila was incubated overnight in TSB (tryptic soy broth, Merck, Germany) at 31 °C, then harvested by centrifugation (5000 × g for 15 minutes at 25 °C) as per the methodology of **Abdel-Latif and Khafaga (2020) and El-Son *et al*. (2021).** The resulting pellets were washed three times in sterile PBS and adjusted to an optical density equivalent to a 0.5 McFarland standard (wavelength = 600 nm), corresponding to $1 \times 10^8$ CFU/mL. Following the 60-day nutritional trial, fish from all experimental groups were subjected to an A. hydrophila challenge while maintained on their respective test diets. Each fish received an intraperitoneal injection of 0.1 mL of PBS containing a predetermined LD50 dose ($1 \times 10^6$ CFU/mL) of live cells, adhering to the protocols established by **Abdel-Latif *et al*. (2023).** During the subsequent 14-day post-challenge observation period, the fish were monitored daily for the onset of clinical symptoms and cumulative mortality.

### 2.5. Blood and tissue sampling

**Twice during the study:** initially on day 60 (**pre-challenge phase**) and subsequently 14 days following the challenge (**post-challenge phase**), blood samples was collected from the caudal vein of the fish. To obtain the serum, blood samples centrifugated at 3000 × g for 15 minutes, after which the resulting supernatant was preserved at -80°C for future immunological and biochemical evaluation. Furthermore, specimen collections of the intestine, hepatopancreas, and gills were performed; these tissues submerged in a 10% neutral buffered formalin solution to prepare them for subsequent immunohistochemical and histological examinations.

### 2.6. Evaluation of systemic redox status and lipid peroxidation

To assess systemic redox balance and lipid peroxidation, liver tissue was processed by homogenization in chilled 0.86% saline (1:10 ratio) followed by a 10-minute centrifugation at 13,600 × g and 4 °C. The isolated supernatant was used to measure the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as the concentration of malondialdehyde (MDA) via the thiobarbituric acid reactive substances (TBARS) method. These biochemical assays were conducted using specialized colorimetric kits from MyBioSource Inc. (San Diego, California, USA), adhering to the established methodologies of **Marklund and Marklund (1974) for SOD, Aebi (1984) for CAT, Paglia and Valentine (1967)** for GPx, and **Ohkawa *et al*. (1979)** for MDA.

### 2.7. Assessment of serum protein fractions

To determine the serum protein profile, total protein (TP) and albumin (ALB) levels were quantified using commercially available diagnostic kits (Bio-Diagnostic, Egypt), with globulin (GLB) concentrations subsequently derived by subtracting albumin from the total

protein value. The albumin-to-globulin (A/G) ratio was then calculated for each sample. All biochemical assays were conducted in accordance with the manufacturer's standardized protocols to ensure precision in the fractionation of these serum components.

**2.8. Liver and kidney functional indices**

To assess organ health, biochemical markers of hepatic and renal function were analyzed. Liver integrity was evaluated by measuring the enzymatic activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Kidney performance was determined through the quantification of nitrogenous waste products, specifically urea, creatinine, and uric acid levels. Commercially purchased diagnostic kits (Bio-Diagnostic, Egypt) were used for the aforementioned parameters.

**2.9. Systemic innate immune parameters**

To assess systemic innate immunity, several key biochemical parameters were evaluated. Serum lysozyme (LYZ) activity was quantified using the turbidimetric technique as described by **Ellis (1990),** while myeloperoxidase (MPO) levels were measured following the methodology of **Kumari and Sahoo (2006).** Additionally, serum bactericidal activity (BA) against A. hydrophila was determined according to the protocol established by **Ji *et al*. (2011),** with all assays performed in accordance with these standardized procedures to ensure consistency and accuracy. MPO activity was expressed as U/mg serum at OD 540 nm.

**2.10. Histopathological assessment**

Six fish per group were sacrificed then gills, livers, and intestines were carefully dissected and fixed in 10% neutral buffered formalin. After that fixed samples were processed through the paraffin embedding technique, then cut into slices with 5 µm thickness and stained with H & E stain **(Suvarna *et al*. 2018).** Briefly, scoring parameters such as inflammatory infiltrates, lamellar epithelial hyperplasia, necrosis of epithelium, and telangiectasis were used for gills. In the case of the liver, scoring was conducted for vascular congestion, vacuolar degeneration, inflammatory infiltrates and hepatic and acinar necrosis. While scoring parameters such as vascular congestion, villar necrosis and inflammatory infiltrates were used for the intestinal score. The scoring criteria were classified as a score of 0 = Normal, 1–25% = mild (1), 25–50% = moderate (2), 50–100% = severe (3) **(Khafaga *et al*. 2020).**

**2.11. Immunohistochemical protein assay**

Multiple slices with 4 µm thickness were obtained from paraffin blocks after rehydration in a decreasing grade of ethyl alcohol. Antigen retrieval was conducted using citrate-buffered saline (pH 6.0), and endogenous peroxidase activity was quenched with 0.3% $H_2O_2$ in phosphate-buffered saline (PBS). Subsequently, to inhibit the non-specific binding of immunological reagents, samples were treated for one hour with 10% (v/v) normal goat serum, after which tissue slices underwent an overnight incubation at 4 °C with proliferative cell nuclear antigen antibody (PCNA, Catalog # (F-2): sc-25280, 1:50 dilution, Santa Cruz). Subsequently, slices were rinsed with phosphate buffer saline and treated with biotin-conjugated goat anti-rabbit IgG antiserum for 60 minutes. Subsequently, the slices were rinsed with phosphate buffer saline and treated with a streptavidin-peroxidase conjugate for 30 minutes. Then, a 3,3′-diaminobenzidine tetrahydrochloride (DAB)- $H_2O_2$ solution was applied for 3 minutes. Ultimately, the sections were counterstained by Mayer's hematoxylin solution **(Dabbs 2021).**

**2.12. Statistical Analysis**

Data was expressed as means ± SE. Prior to testing, the assumptions of normality and homogeneity of variances were assessed to confirm the appropriateness of parametric procedures. A two-way ANOVA was executed using IBM SPSS Statistics (v.26, Armonk, NY) to evaluate the primary effects of KP, bacterial challenge, and their potential interaction. In instances where significant differences were detected ($p < 0.05$), mean separation was performed using Tukey's Honestly Significant Difference (HSD) post-hoc test.

## 3. Results

### 3.1. Growth performance, feed efficiency and survival

Dietary KP supplementation significantly influenced the growth parameters and feed efficiency of the treated fish over the 60-day feeding period. A clear dose-dependent relationship was observed, with performance metrics peaking in the KP-4 treatment group ($P < 0.05$). As detailed in Table 1, the KP-4 group demonstrated superior outcomes across all primary growth indices, including final weights, WG and WG% and reached maximum values in the KP-4 inclusion group. The SGR values were significantly higher in KP-4 compared to the control and lower-inclusion groups. The FI increased proportionally with KP levels, suggesting high palatability of the supplemented test diets for this fish species. Furthermore, feed utilization was optimized in the KP-4 group, which exhibited the most favorable FCR among all treatments ($P < 0.05$). Notably, no mortality was recorded across any treatment groups with non-significant variations after the end of the 60-day feeding trial ($P > 0.05$), indicating that KP is a safe and well-tolerated functional ingredient for this fish species.

### 3.2. Oxidative stress biomarkers

The results presented in Table 2 demonstrated that both A. hydrophila infection and dietary KP supplementation significantly ($P < 0.05$) influenced the oxidative stress profile of the experimental fish. In the pre-challenge phase, it was found that following the 60-day feeding trial, dietary KP inclusion significantly enhanced the activity of enzymatic antioxidants. The levels of SOD, CAT, and GPx enzymes exhibited a dose-dependent increase, with the KP-4 group displaying the most robust antioxidant capacity. Conversely, MDA levels were significantly attenuated by KP supplementation as the lowest MDA concentrations were observed in the KP-4 group, suggesting a protective effect against oxidative damage. In the post-challenge phase, the stimulatory effect of KP on the antioxidant defense system persisted following the bacterial infection. While the general trend of enhanced enzyme activity remained consistent with the pre-challenge observations, the peak antioxidant response shifted as the KP-3 group recorded the highest activities for SOD, CAT, and GPx post-infection. This suggests that while higher inclusion levels (KP-4) provide optimal baseline protection, the KP-3 formulation may offer the most effective physiological priming for oxidative stress responses during an active pathogen challenge.

### 3.3. Blood protein profile

The serum protein profile of *P. hypophthalmus* fries, including TP, ALB, GLB, and the A/G ratio, exhibited significant variations in response to dietary KP supplementation both pre- and post-bacterial challenge (Table 3). Prior to the bacterial challenge, fish fed the KP-3 diet displayed the highest concentrations of TP and ALB among all experimental groups. Interestingly, GLB levels remained statistically uniform across all treatments during this period, suggesting that the initial physiological response to KP supplementation primarily influenced the ALB fraction. Following the bacterial infection, a distinct shift in the protein profile was observed, whereas the KP-3 group sustained the highest levels of TP and ALB. While ALB values dominated the pre-infection phase, the post-infection period presented a significant elevation in GLO values within the KP-4 group compared to all other treatments. The increase in TP and ALB in the KP-3 group suggests enhanced nutritional status and liver function, while the significant rise in GLO in the KP-4 group post-infection likely indicates a more robust humoral immune response to the pathogen.

### 3.4. Serum immune responses

The effects of dietary KP powder supplementation on the systemic immune status of *P. hypophthalmus* fries (both pre- and post-challenge) are summarized in Table 4. Prior to the bacterial challenge, it was noticed that serum LYZ and MPO activities showed no significant deviations across the experimental groups ($P > 0.05$), remaining relatively constant regardless of the KP inclusion levels. However, serum bactericidal activity was significantly influenced by the diet, with the KP-3 group exhibiting the most robust response relative to the control and other treatment groups. Following the bacterial infection, a systemic

activation of the innate immune system was observed, as both serum LYZ and MPO activities significantly increased across all groups. Notably, the KP-supplemented groups reached the highest recorded values for these parameters, suggesting that KP powder may enhance the fish's capacity for rapid immune response during a pathogen challenge.

### 3.5. Serum biochemical parameters

The impact of dietary KP powder on the serum biochemical profile of *P. hypophthalmus* fries both under basal conditions and following bacterial challenge is summarized in Table 5. The data indicates that both KP supplementation and bacterial infection significantly ($P < 0.05$) modulated the physiological status of the fish. Prior to the bacterial challenge, a dose-dependent reduction in kidney function markers (urea, creatinine, and uric acid) was observed in correlation with increasing KP inclusion levels. Following bacterial infection, these indices increased across all experimental groups; however, fish receiving the highest supplementation (KP-4) maintained significantly ($P < 0.05$) lower levels compared to other groups, suggesting a protective effect against infection-induced renal stress. Dietary KP supplementation appeared to enhance liver health, as evidenced by the significant decrease in ALT, AST, and ALP activities in the KP-treated groups relative to the control under pre-infection conditions. While the bacterial challenge triggered a systemic increase in these liver enzymes across all groups, the KP-2 group exhibited the highest degree of hepatoprotection, recording the lowest levels of enzymatic leakage post-infection. These results suggest that KP supplementation may mitigate tissue damage and improve the resilience of P. hypophthalmus fries against bacterial pathogens.

### 3.6. Histopathological analysis

The histopathological examination of the gills from KP-treated groups before infection and KP-0 after infection showed normal lamellar histoarchitecture, including mucous cells, chloride cells, and primary and secondary lamellae (Fig. 1A-E & A1, Table 6). However, the KP-1 treated group after infection showed marked vascular congestion, hyperplasia, and lymphocytic infiltration with moderate telangiectasia (Fig. 1B1 & B2, Table 6). Furthermore, KP-2, KP-3 and KP-4 treated groups after infection showed less degenerative changes in a dose-dependent manner (Fig. 1C1-E1, Table 6).

The histopathological examination of the hepatopancreas from the KP-treated groups before infection and KP-0 after infection demonstrated typical histoarchitecture of hepatic lobules and hepatic cords (Fig. 2A-E &A1, Table 6). The liver of *P. hypophthalmus* had polygonal hepatocytes with central to subcentral rounded nuclei and uniform cytoplasm with multiple vacuoles indicating glycogen accumulation. Furthermore, the hepatic vasculature consisted of central and portal veins, along with hepatic sinusoids showing a normal vascular wall structure and blood capacity. Moreover, the exocrine pancreatic acini showed normal acinar cells with a basophilic component containing a nucleus and eosinophilic cytoplasm distributed around the portal vein branches in the portal area. The KP-1 treated group after infection showed marked vascular congestion, hepatocellular vacuolation, and necrosis of pancreatic acinar cells with mononuclear inflammatory infiltrates (Fig. 2B1-3, Table 6). The KP-2 treated group showed the same lesion that was noiced in KP1 but less in distribution and severity (Fig. 2C1, Table 6). Furthermore, KP-3 and KP-4 treated groups after challenge with A. hydrophila showed typical histologic structure just limited to hepatocellular vacuolar degeneration (Fig. 2 D1&E1, Table 6).

The histopathologic observation of intestinal tissues in the KP-treated groups before infection and KP-0 after infection exhibited typical histoarchitecture of intestinal villi, crypts, and the internal and external tunica muscularis (Fig. 3A-E&A1, Table 6). Moreover, the villus was lined by simple columnar epithelium with goblet cells. Conversely, the KP1-treated group after infection showed necrosis of enterocytes with marked mononuclear inflammatory infiltrates (Fig. 3B1, Table 6). These changes decreased in a dose-dependent manner in KP-2, KP3 and KP-4 supplemented groups after A. hydrophila challenge (Fig. 3 C1-E1, Table 6).

### 3.7. Immunohistochemical results

Gills of *P. hypophthalmus* showed variable degrees (mild, moderate and marked ) of specific granular brown immunoperoxidase reaction against PCNA within the epithelial cells lining lamellae and within pillar cells befor infection with A. hydrophila and in KP-0 group after infection (Fig. 4A-E & A1) . After infection with A. hydrophila , the KP1 group showed moderate to severe PCNA nuclear immunoreactivity (Fig. 4 B1) compared to other groups and aminimal PCNA nuclear immunoreactivity appeared in KP4 group (Fig. 4 B1 - E1). Hepatopancrease of P. hypophthalmus showed comparative immunohistochemical reaction against PCNA antigen , in after infection with *A. hydrophila* groups noticed mild to moderate specific brown immunoperoxidase reaction against PCNA antigen within the exocrine pancreatic tissues and hepatic paranchyma(Fig. 5B1-E1). .In befor infection with A. hydrophila groups and KP-1 treated group after infection there was no detected reaction (Fig. 5A-E & A1). Intestine of *P. hypophthalmus* showed comparative immunohistochemistry reaction against PCNA protein within the cytoplasm of epithelial lining the intestinal villi as a golden brown stain which indicated the presence of PCNA after infection with A. hydrophila showed moderate specific brown immunoperoxidase reaction (Fig. 6 C1) while KP1 group showed sever marked immunoperoxidase reaction (Fig. 6 B1). While The immunohistochemical expression of the PCNA in the intestinal tissues in KP-supplemented groups before the challenge and KP-0 after infection showed mild brown nuclear immunoreactivity (Fig. 6A-E & Fig. 6A1). Furthermore, KP-3 and KP-4 treated groups after infection with A. hydrophila showed mild and week PCNA nuclear immunoreactivity, respectively (Fig. 6 D1 & Fig. 6 E1).

## 4. Discussion

The significant improvement in growth parameters (final weight, WG, and SGR) and feed efficiency (FCR) in the KP-4 group suggests that Kelp powder (KP) could enhance nutrient assimilation. The dose-dependent increase in Feed Intake (FI) indicates that KP improves the palatability of the diet, a phenomenon often attributed to the presence of attractive amino acids and polysaccharides in marine algae. The optimized FCR in the KP-4 group aligns with previous studies on seaweed supplementation in aquaculture, where brown algae have been shown to improve intestinal enzyme activity and nutrient digestibility. Extensive literature supports the inclusion of seaweed in aquatic diets as a functional ingredient to bolster productivity. Specifically, studies on Nile tilapia, European seabass, and white-spotted snapper juveniles have confirmed that macroalgae supplementation significantly optimizes weight gain, feed utilization, and overall survival rates while refining carcass quality **(Ashour *et al*. 2020; Khalafalla and El-Hais 2015; Kubai *et al*. 2023; Wassef *et al*. 2013; Zhu *et al*. 2016).** Several synergistic pathways underpin the enhanced growth observed in this study. The diverse profile of bioactive constituents within *A. nodosum* include phlorotannins, essential fatty acids, and structural polysaccharides like alginates and fucoidans collectively optimize digestive function by stimulating enzyme production and refining nutrient **uptake (Gisbert *et al*. 2023; Hamed *et al*. 2018; Martins *et al*. 2022).** These compounds appear to bolster anabolic efficiency by upregulating mitochondrial metabolism and protein biosynthesis **(Wang *et al*. 2023).** This metabolic advantage is likely complemented by a superior oxidative status, which conserves metabolic energy by mitigating the physiological demands of cellular repair, thus channeling more dietary resources toward biomass accumulation **(Begum *et al*. 2021; Kamunde *et al*. 2019; Siddik *et al*. 2023).** Such health-promoting benefits are further evidenced by the steady improvement in survival rates, indicating that KP supplementation strengthens physiological resilience and minimizes the impact of subclinical stressors.

Dietary KP markedly bolsters the antioxidant profile of *P. hypophthalmus* by stimulating endogenous defense mechanisms. The observed dose-responsive elevation in SOD, CAT, and GPx activities alongside a concomitant decrease in MDA concentrations underscores the potent radical-neutralizing capacity of KP. These antioxidant benefits are largely attributed to bioactive phlorotannins and fucoidans, which serve as cellular antioxidant system **(Begum *et al*. 2021; Gisbert *et al*. 2023).** At a mechanistic level, these polyphenolic and pol-

ysaccharide compounds protect against oxidative stress by directly scavenging reactive oxygen species (ROS) and triggering the Nrf2/ Keap1 signaling axis, thereby up regulating the transcription of vital antioxidant enzymes **(Marmelo *et al*. 2024; Michalak *et al*. 2022).** The increased SOD levels facilitate the conversion of superoxide radicals into hydrogen peroxide, which is subsequently detoxified into water by enhanced CAT and GPx activity, preventing cellular damage. The reduction in MDA further illustrates preserved membrane integrity through inhibited lipid peroxidation. Notably, while KP-4 offered superior protection, the KP-3 group exhibited the most robust antioxidant surge following infection, suggesting that moderate-to-high seaweed inclusion primes the physiological machinery for rapid mobilization against pathogen-induced oxidative bursts. These results align with broader evidence that macroalgal derivatives enhance respiratory burst and phagocytic functions in several finfish species like *Epinephelus coioides* **(Cheng *et al*. 2007)** and *Dicentrarchus labrax*, where *Gracilaria* inclusion boosts GPx **(Martínez-Páramo *et al*. 2013; Peñalver *et al*. 2020).** Similar improvements in redox homeostasis and mitochondrial enzyme activity have been documented in Atlantic salmon fed *Laminaria* **(Kamunde *et al*. 2019),** and rainbow trout **(Sotoudeh and Mardani 2018),** confirming the universal efficacy of KP in mitigating oxidative stress across several fish species.

The shift in the blood protein profile post-infection specifically the rise in GLB in the KP -4 group indicates a robust humoral immune response. Globulins are the primary carriers of immunoglobulins in fish; thus, their elevation suggests enhanced antibody production. Improved immune states and growth parameters suggesting that KP had a nutritional and energy requirmentsthat stimulate immune response in case of infection. Serum ALB and TP levels were increased in all KP-supplemented groups, in which stimulate hepatic function and metabolic status **(Akbary *et al*. 2018; Manikandan *et al*. 2022).** ALB, a liver-synthesized protein, serves as an important antioxidant against effects of oxidative stress, while TP elevation reflect acute-phase protein synthesis that important for immune competence **(Akbary *et al*. 2018; Ashour *et al*. 2020).** Also, dietary boron supplementation showed an improve in growth performance, survival rate, plasma TP, and non-specific immune responses in Nile tilapia, leading to increase resistance to A. hydrophila infection **(Ardó *et al*. 2008).**

The pre-challenge results showed that while LYZ and MPO were unaffected, bactericidal activity was significantly higher in the KP-3 group. This implies that KP enhances specific innate pathways before others. However, the systemic surge in LYZ and MPO across all KP groups post-infection confirms that KP supplementation accelerates the activation of neutrophils and macrophages, which are critical for the early containment of A. hydrophila. The immunological changes enhance the bioactive role of A. nodosum in innate immunity in aquatic species such as *O. niloticus* **(Negm *et al*. 2021; Sheikhzadeh *et al*. 2022; Thépot *et al*. 2021).** LYZ, a key antibacterial enzyme that hydrolyses peptidoglycan bonds in bacterial cell wall degradation by lysozyme (LYZ) serves as an initial barrier against host infection, whereas elevated bactericidal activity (BA) indicates a wider strengthening of systemic immune defense **(Ferraboschi *et al*. 2021; Song *et al*. 2021).** These outcomes align with previous studies demonstrating that marine algal polysaccharides and phenolics boost LYZ performance and disease immunity across diverse teleost species **(Peixoto *et al*. 2016; Siddik *et al*. 2024).** The immunomodulatory capacity of Kappaphycus (KP) stems primarily from its sulfated polysaccharides—notably fucoidans—which function as pathogen-associated molecular pattern (PAMP) mimetics. These molecules bind pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) expressed on macrophages and neutrophils **(Hwang *et al*. 2022).** This receptor engagement triggers signaling pathways that stimulate phagocytosis, cytokine secretion, and antimicrobial peptide synthesis, ultimately fortifying both innate and adaptive immunity **(Siddik *et al*. 2023; Vidhya Hindu *et al*. 2019).** The simultaneous rise in BA and LYZ levels at elevated dietary inclusions suggests a dose-dependent threshold, wherein adequate bioactives are required to fully activate PRRs and downstream signaling cascades **(Yangthong *et al*. 2016; Yengkhom *et al*.**

**2018).** Mechanistically, LYZ cleaves bacterial peptidoglycan as a primary defensive step **(Saurabh and Sahoo 2008),** and its upregulation mirrors observations in fish supplemented with algal polysaccharides or polyphenols **(Marmelo *et al*. 2024).** Similarly, **Yengkhom *et al*. (2019)** observed that Caulerpa scalpelliformis extracts up-regulated LYZ transcript levels and myeloperoxidase (MPO) activity in *Oreochromis niloticus*. Serum biochemical markers, including ALT, AST, and ALP, serve as critical indicators of hepatocellular integrity, while urea and creatinine levels are gold standards for assessing glomerular filtration and renal health. The observed reduction in these enzymes following infection in fish fed KP-based diets suggests that bioactive compounds within brown macroalgae, such as fucoidans, phlorotannins, and laminarin, provide significant organoprotection **(Michalak *et al*. 2022).** Specifically, these seaweeds have been shown to lower AST and urea levels, likely by mitigating the oxidative stress and systemic inflammation that typically follow pathogenic challenges **(Michalak *et al*. 2022).** By being as free radical remove and change inflammatory pathways like NF-κB, kelp-derived metabolites help maintain cellular membrane stability, thereby preventing the leakage of intracellular enzymes into the bloodstream **(Siddik *et al*., 2023).** This maintenance of lower biochemical markers indicates that KP supplementation effectively preserves the functional architecture of the liver and kidneys, enhancing the host's resilience against infection-induced tissue damage **(Marmelo *et al*. 2024; Siddik *et al*. 2023).** The reduction in kidney function markers (urea and uric acid) in the KP-4 group suggests that kelp may mitigate the nephrotoxicity often associated with systemic bacterial infections. The superior hepatoprotection observed in the KP-2 group post-infection indicates that there may be an optimal inclusion threshold beyond which the metabolic load of processing supplements might slightly offset the protective benefits during active disease.

The histopathological findings support the biochemical data, showing that KP supplementation (particularly KP-3 and KP-4) significantly reduced necrosis, vascular congestion, and inflammatory infiltration in the gills, liver, and intestines post-challenge. This tissue-level resilience is further explained by PCNA immunohistochemistry. The high PCNA expression in the KP-1 group post-infection reflects an intense attempt at cellular repair following severe damage. In contrast, the mild-to-moderate PCNA expression in the KP-3 and KP-4 groups suggests that these fish experienced less initial damage and were able to maintain homeostasis without the need for massive compensatory cellular proliferation. This sparing effect confirms that KP reinforces the structural barriers of the fish, preventing the systemic spread of *A. hydrophila*. Several research studies demonstrated that dietary supplementation with marine macroalgae significantly enhances the histological health of fish by strengthening the intestinal barrier, mitigating liver damage, and improving overall tissue architecture. For instance, a commercial blend of macroalgae including (Ulva sp. and *Gracilaria gracilis*) and microalgae (*Chlorella vulgaris* and *Nannochloropsis oceanica*) has been shown to increase the anterior intestinal absorption area of *D. labrax* juveniles **(Mota *et al*. 2023).** Furthermore, the brown macroalgae Saccharina japonica has been found to ameliorate liver injury induced by high-carbohydrate diets in swamp eels (*Monopterus albus*) by significantly reducing the accumulation of lipid droplets, glycogen, and collagen fibers in hepatic tissues **(Yu *et al*. 2022).** These improvements are largely attributed to the antioxidant properties of algal polysaccharides, such as ulvans and fucoidans, which modulate gut microbiota and enhance the resilience of internal organs against metabolic stress **(Øverland *et al*. 2019).**

## 5. Conclusions

In conclusion, the dietary KP significantly enhanced the growth performance, physiological resilience, and immune competence of *P. hypophthalmus* fries in a dose-dependent manner. Over a 60-day period, the KP-4 inclusion level optimized growth indices and feed efficiency while providing the most robust antioxidant capacity and renal protection. Importantly, KP supplementation acts as a potent immunomodulator during an *A. hydrophila* challenge; while KP-4 promoted the strongest humoral response

via elevated GLB levels, the KP-3 formulation appeared to offer superior physiological priming for enzymatic antioxidant defenses and serum bactericidal activity during infection. These biochemical improvements are further corroborated by histopathological and immunohistochemical findings, where KP-treated groups particularly at higher inclusion levels exhibited marked reductions in tissue degradation, inflammatory infiltration, and PCNA-mediated cellular stress in the gills, hepatopancreas, and intestines. Collectively, these results demonstrate that KP is a safe, highly palatable functional ingredient that mitigates oxidative stress and organ damage, thereby improving the overall health and survivability of fish against bacterial pathogens. These results suggest that the bioactive polysaccharides, focuoids, and alginates of *A. nodosum* functioned as biological and functional bioactives that stabilized the internal environment of the fry during the critical developmental period.

**Table (1).** Effects of dietary supplementation with kelp powder (KP) for 60 days on the growth performance parameters, feed efficiency and survival rates of *P. hypophthalmus* fries before bacterial infection.

| KP levels | Initial weight (g) | Final weight (g) | WG (g) | WG % | SGR (%/ day) | FI (g feed/fish) | FCR | Survival (%) |
|---|---|---|---|---|---|---|---|---|
| **KP-0** | 6.35 | 36.45 e | 30.10 e | 474.02 e | 1.942 e | 58.48 d | 1.94 a | 100.0 |
| **KP-1** | 6.46 | 43.75 d | 37.29 d | 577.24 d | 2.125 d | 63.18 cd | 1.69 b | 100.0 |
| **KP-2** | 6.49 | 48.67 c | 42.18 c | 649.92 c | 2.239 c | 66.12 bc | 1.57 c | 100.0 |
| **KP-3** | 6.42 | 54.39 b | 47.97 b | 747.20 b | 2.374 b | 69.92 ab | 1.46 d | 100.0 |
| **KP-4** | 6.61 | 63.43 a | 56.82 a | 859.61 a | 2.513 a | 73.43 a | 1.29 e | 100.0 |
| Pooled SE | 0.043 | 2.471 | 2.451 | 36.151 | 0.053 | 1.508 | 0.606 | 0.0 |
| *P* value | 0.477 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | - |

Means with different letters are significantly different at $P < 0.05$ by Tukey's HSD test.
WG: Weight gain, SGR: Specific growth rate, FI: Feed intake, FCR: Feed conversion rate

**Table (2).** Effects of dietary supplementation with kelp (*A. nodosum*) powder (KP) for 60 days on the oxidative stress biomarkers of *P. hypophthalmus* fries before and after bacterial infection.

| Bacterial infection | KP levels | SOD (U/mg protein) | CAT (U/mg protein) | GPX (U/mg protein) | MDA (nmol/mg protein) |
|---|---|---|---|---|---|
| **Before infection** | **0** | 6.50 d | 9.35 g | 96.42 g | 27.92 d |
| | **1** | 9.91 cd | 10.32 fg | 98.10 fg | 25.41 e |
| | **2** | 11.43 c | 12.68 de | 100.08 ef | 25.13 e |
| | **3** | 15.40 ab | 15.53 bc | 106.78 cd | 23.81 e |
| | **4** | 12.87 bc | 13.93 cd | 101.77 e | 23.29 e |
| **After infection** | **0** | 7.80 d | 10.74 fg | 106.11 d | 34.90 a |
| | **1** | 11.89 bc | 11.87 ef | 107.97 cd | 31.76 b |
| | **2** | 13.60 bc | 14.58 bcd | 110.08 bc | 31.41 bc |
| | **3** | 18.48 a | 17.86 a | 117.38 a | 29.77 bcd |
| | **4** | 15.44 ab | 15.99 b | 112.04 b | 29.12 cd |
| **Pooled SE** | | 0.712 | 0.512 | 1.202 | 0.708 |
| **Two-way ANOVA** | | *P* value | | | |
| **Bacterial infection** | | 0.007 | <0.0001 | <0.0001 | <0.0001 |
| **Kelp levels** | | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| **Infection × kelp levels** | | 0.954 | 0.942 | 0.995 | 0.962 |

Means followed by different letters are significantly different at $P < 0.05$ by Tukey's HSD test.

**Table (3).** Effects of dietary supplementation with kelp powder (KP) for 60 days on blood protein profile of *P. hypophthalmus* fries before and after bacterial infection.

| Bacterial infection | KP levels | Total protein (g/dL) | Albumin (g/dL) | Globulin (g/dL) | Albumin / Globulin ratio |
|---|---|---|---|---|---|
| **Before infection** | **0** | 5.46 c | 3.40 abc | 2.06 b | 1.67 a |
| | **1** | 5.82 ab | 3.54 ab | 2.28 b | 1.57 abc |
| | **2** | 5.79 ab | 3.57 ab | 2.22 b | 1.61 ab |
| | **3** | 6.01 a | 3.68 a | 2.33 b | 1.59 abc |
| | **4** | 5.79 ab | 3.46 ab | 2.33 b | 1.50 abcd |
| **After infection** | **0** | 5.17 d | 2.85 de | 2.31 b | 1.25 bcde |
| | **1** | 5.50 c | 2.96 de | 2.54 ab | 1.18 de |
| | **2** | 5.49 c | 3.03 cde | 2.45 ab | 1.24 cde |
| | **3** | 5.65 bc | 3.16 bcd | 2.49 ab | 1.29 bcde |
| | **4** | 5.49 c | 2.64 e | 2.85 a | 0.96 e |
| **Pooled SE** | | 0.049 | 0.066 | 0.055 | 0.050 |
| **Two-way ANOVA** | | | | | |
| **Bacterial infection** | | <0.0001 | <0.0001 | 0.008 | <0.0001 |
| **Kelp levels** | | <0.0001 | 0.002 | 0.161 | 0.269 |
| **Infection × kelp levels** | | 0.997 | 0.387 | 0.810 | 0.873 |

Means followed by different letters are significantly different at $P < 0.05$ by Tukey's HSD test.

**Table (4).** Effects of dietary supplementation with kelp powder (KP) for 60 days on serum immune indices of *P. hypophthalmus* fries before and after bacterial infection.

| Bacterial infection | KP levels | LYZ (µg/mL) | MPO (OD at 540 nm) | Serum bactericidal activity |
|---|---|---|---|---|
| **Before infection** | **0** | 19.39 c | 1.00 b | 6.31 de |
| | **1** | 20.03 c | 1.02 b | 7.69 abc |
| | **2** | 20.47 c | 0.99 b | 6.53 cde |
| | **3** | 19.71 c | 1.02 b | 8.50 a |
| | **4** | 19.90 c | 1.00 b | 7.74 ab |
| **After infection** | **0** | 25.21 b | 1.20 a | 5.70 e |
| | **1** | 26.03 ab | 1.22 a | 6.93 bcd |
| | **2** | 26.61 a | 1.18 a | 5.87 de |
| | **3** | 25.62 ab | 1.21 a | 7.66 abc |
| | **4** | 25.87 ab | 1.20 a | 6.97 bcd |
| **Pooled SE** | | 0.562 | 0.019 | 0.186 |
| **Two-way ANOVA** | | *P* value | | |
| **Bacterial infection** | | <0.0001 | <0.0001 | 0.005 |
| **Kelp levels** | | <0.0001 | 0.241 | <0.0001 |
| **Infection × kelp levels** | | 0.959 | 0.997 | 0.998 |

Means followed by different letters are significantly different at $P < 0.05$ by Tukey's HSD test.

**Table (5).** Effects of dietary supplementation with kelp (*A. nodosum*) powder (KP) for 60 days on serum biochemical indices of *P. hypophthalmus* fries before and after bacterial infection.

| Bacterial infection | KP levels | AST (U/L) | ALT (U/L) | ALP (U/L) | Uric acid (mg/dL) | Urea (mg/mL) | Creatinine (mg/dL) |
|---|---|---|---|---|---|---|---|
| **Before infection** | **0** | 6.59 bc | 4.91 b | 10.50 bc | 1.09 cd | 3.64 bcd | 0.63 c |
| | **1** | 5.96 cd | 4.44 b | 9.50 c | 1.10 c | 3.67 bcd | 0.63 c |
| | **2** | 5.06 d | 3.77 c | 8.07 d | 1.02 d | 3.40 cd | 0.58 cd |
| | **3** | 6.23 bc | 4.64 b | 9.94 c | 0.93 e | 3.12 d | 0.55 d |
| | **4** | 6.01 cd | 4.47 b | 9.58 c | 0.91 e | 3.04 d | 0.54 d |
| **After infection** | **0** | 8.73 a | 6.04 a | 11.92 ab | 1.37 a | 4.73 b | 0.88 a |
| | **1** | 8.34 a | 5.77 a | 11.44 ab | 1.36 a | 5.76 a | 0.88 a |
| | **2** | 7.09 b | 4.90 b | 9.68 c | 1.24 b | 4.42 bc | 0.82 ab |
| | **3** | 9.22 a | 6.38 a | 12.61 a | 1.17 c | 4.05 bcd | 0.77 b |
| | **4** | 8.41 a | 5.82 a | 11.49 ab | 1.14 c | 3.96 bcd | 0.76 b |
| **Pooled SE** | | 0.263 | 0.159 | 0.271 | 0.028 | 0.170 | 0.024 |
| **Two-way ANOVA** | | *P* value | | | | | |
| **Bacterial infection** | | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| **Kelp levels** | | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.011 | <0.0001 |
| **Infection × kelp levels** | | 0.903 | 0.955 | 0.988 | 0.818 | 0.388 | 0.753 |

Means followed by different letters are significantly different at $P < 0.05$ by Tukey's HSD test.

**Table (6).** Lesion scoring from different experimental groups of *P. hypophthalmus* fries fed diets supplemented with kelp powder (KP) before and after bacterial infection.

| Bacterial infection | KP levels | Gill lesions | | | | Hepatic lesions | | | | Intestinal lesions | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Inflammatory infiltrates | Epithelial hyperplasia | Lamellar necrosis | Telangiectasis | Congestion | Vacuolization | Inflammatory infiltrates | Hepatic & acinar necrosis | Congestion | Villar necrosis | Inflammatory infiltrates |
| **Before infection** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **After infection** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 4 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |

0=Normal, 1–25%=mild (1), 25–50%=moderate (2), 50–100%=severe (3).

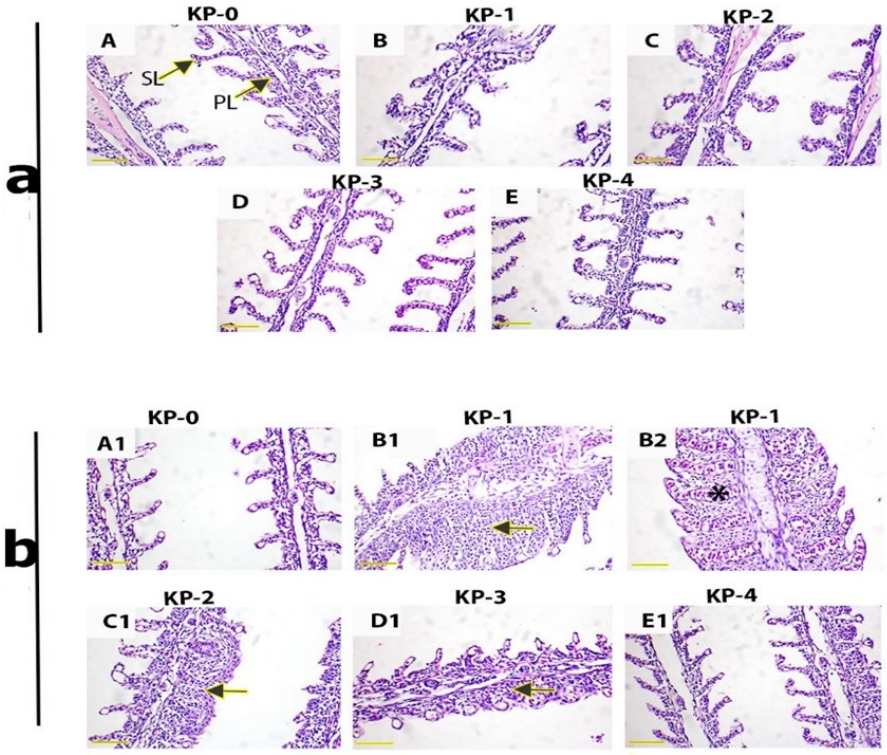



**Fig. (1).** Comparative of the histopathological changes in the gills of *P. hypophthalmus* fed diets supplemented with Kelp powder (KP) before ( a ) (A-E) and after (b) *A. hydrophila* infection (A1-E1).
a: Gills showing normal histoarchitecture primary filaments (PL) and secondary filaments (SL) (arrows). H&Estain, X250
b: Gills showing variable degrees of epithelial hyperplasia among different groups associated with round infilammatory cells (arrows), telengiectasis and congestion( star). H&Estain, X250

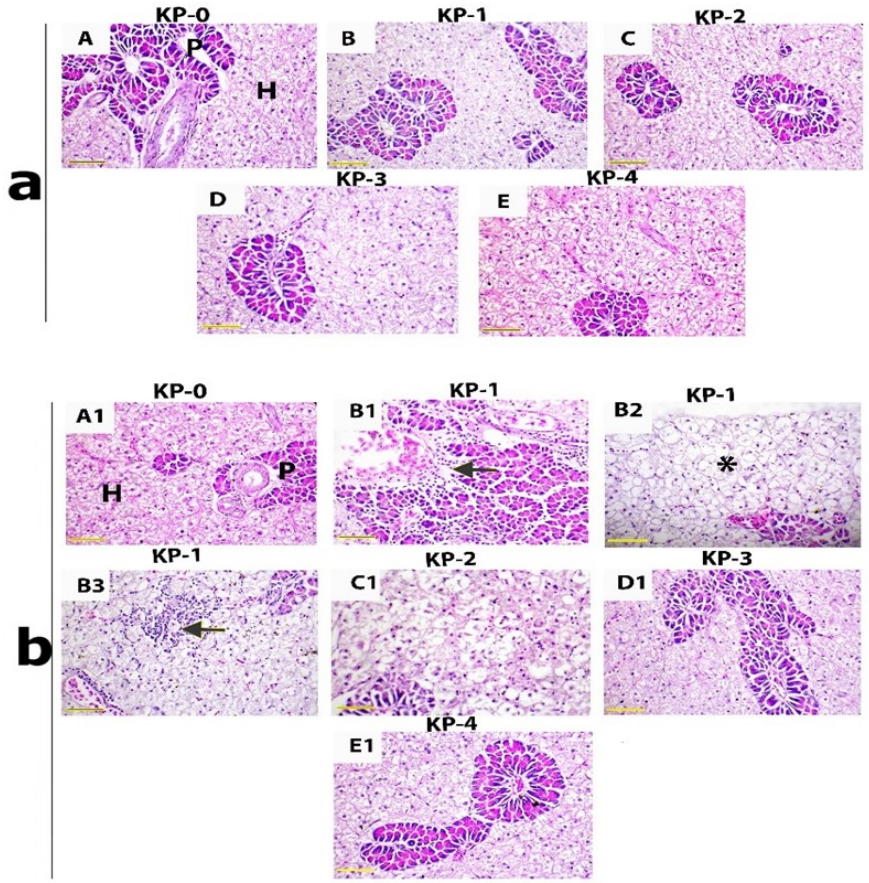



**Fig. (2).** Comparative of the histopathological changes in the hepatopancreas of *P. hypophthalmus* fed diets supplemented with Kelp powder (KP) before (a) (A-E) and after (b) *A. hydrophila* infection (A1-E1).
a: hepatopancreatic tissue showing normal histoarchitecture of hepatocytes (H&Estain ,X250)
b: hepatopancreatic tissue showing congestion ,degeneration of pancreatic cells associated with polymorph and round infilammatory cells( arrows) , X 250 and vaccular degeneration of hepatocyte (star) (B2&C1), H&Estain , X400

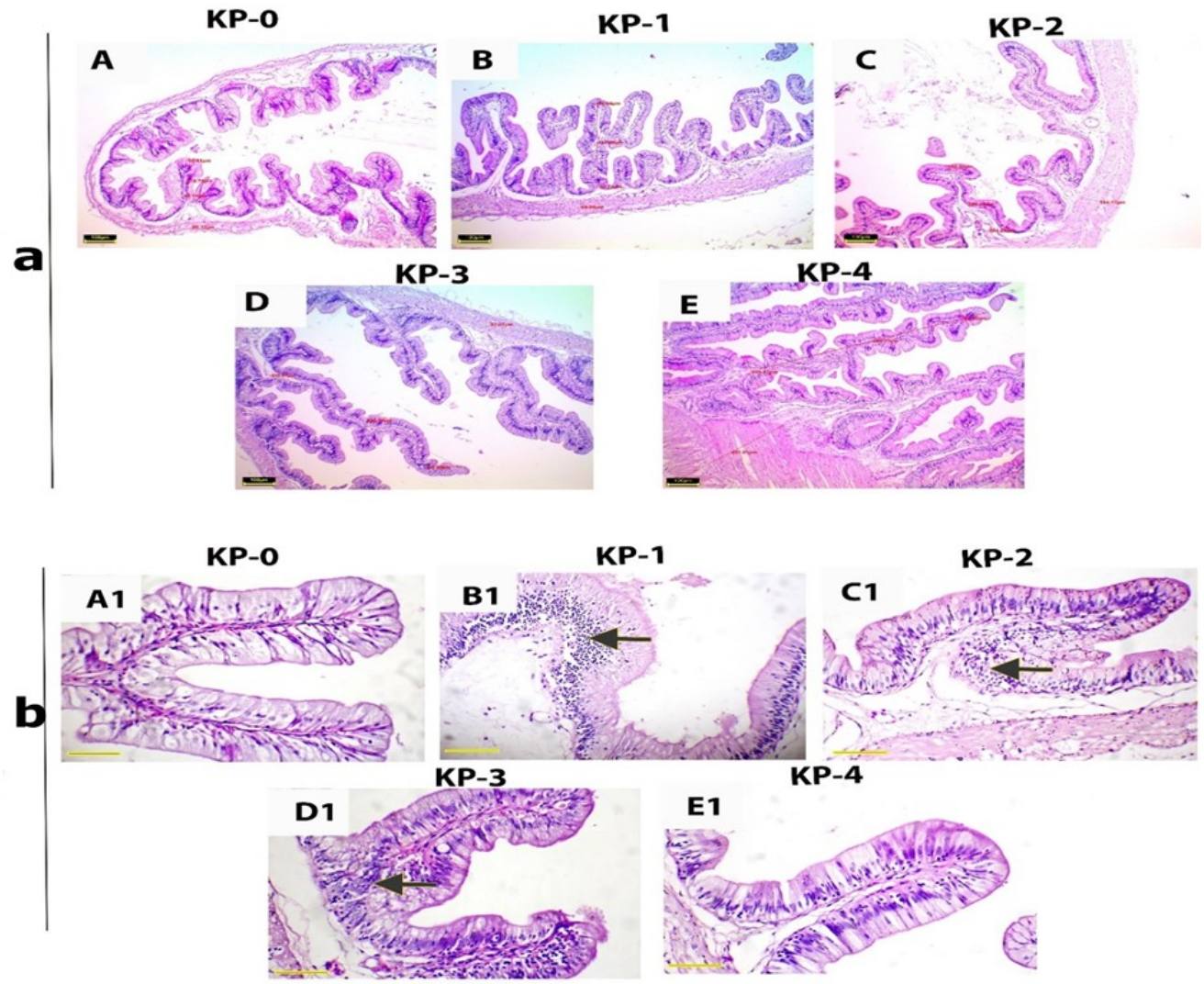



**Fig. (3).** Comparative histopathological changes in the intestinal tissue of *P. hypophthalmus* fed diets supplemented with Kelp powder (KP) before (a) (A-E) and after( b) *A. hydrophila* infection (A1-E1).
a: different parts of intestinal tissue showing normal histoarchitecure. H&Estain ,X 250 (A-E).
b: different parts of intestinal tissue showing in KP1&KP2 epithelial hyperplasia associated with polymorph and round infilammatory cells ,KP3&KP4 showing minimal histopathological changes( no epithelial hyperplasia ,very few round cells invading between intestinal epithelium (arrows). KP0 control negative. H&Estain , X,400 (A1-E1).

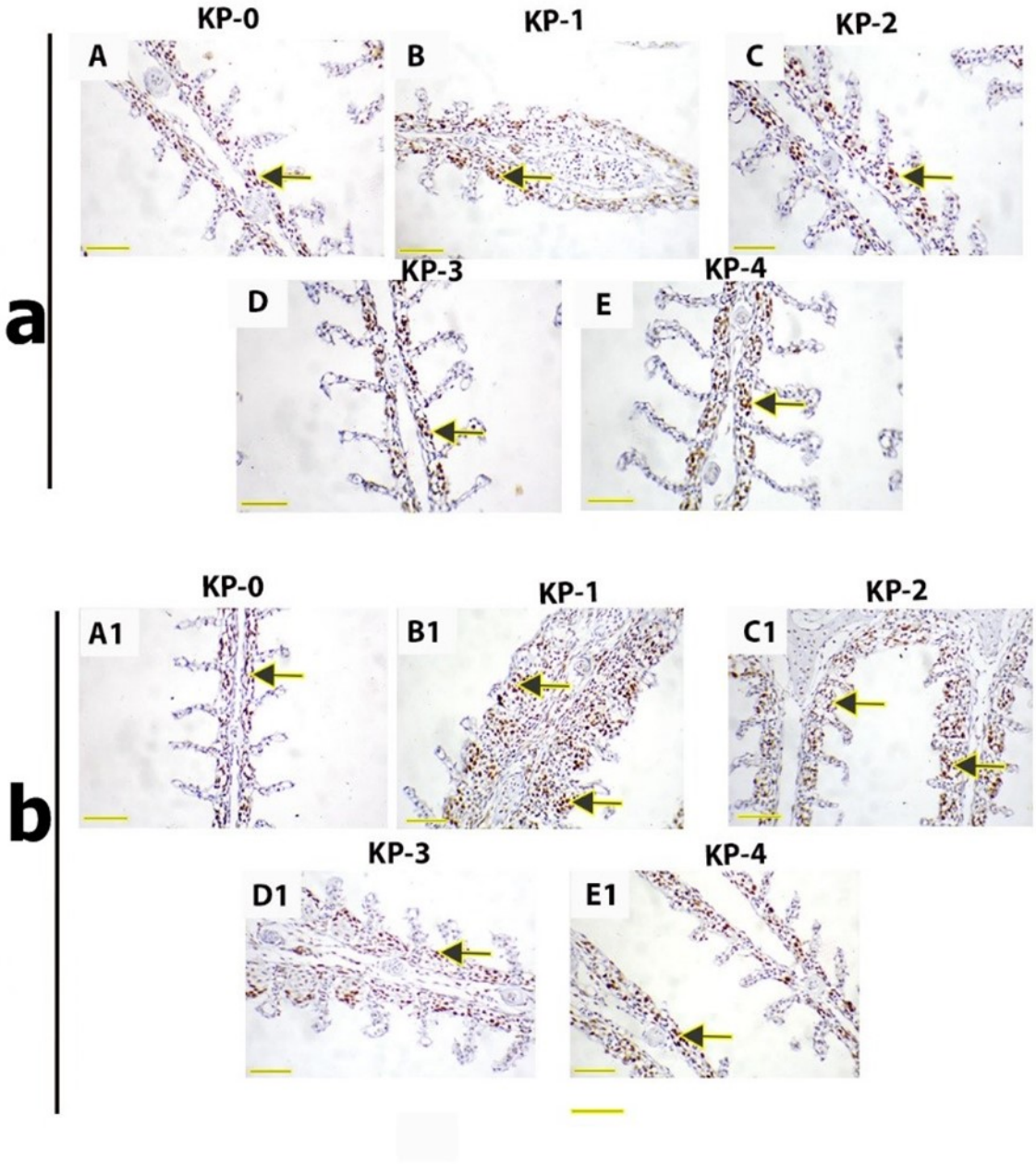



**Fig. (4).** Gills showing variable degrees (mild, moderate and marked ) of specific granular brown immunoperoxidase reaction against PCNA within the epithelial cells lining lamellae and within pillar cells a ( befor infection) (A-E) and b (after infection) (A1-G1) notice thatKP1 group after infection showing marked reaction compared to other groups (arrow),minimal reaction appears in KP4( E1) group. DAB technique ,Mayer's Haematoxylin ,X250

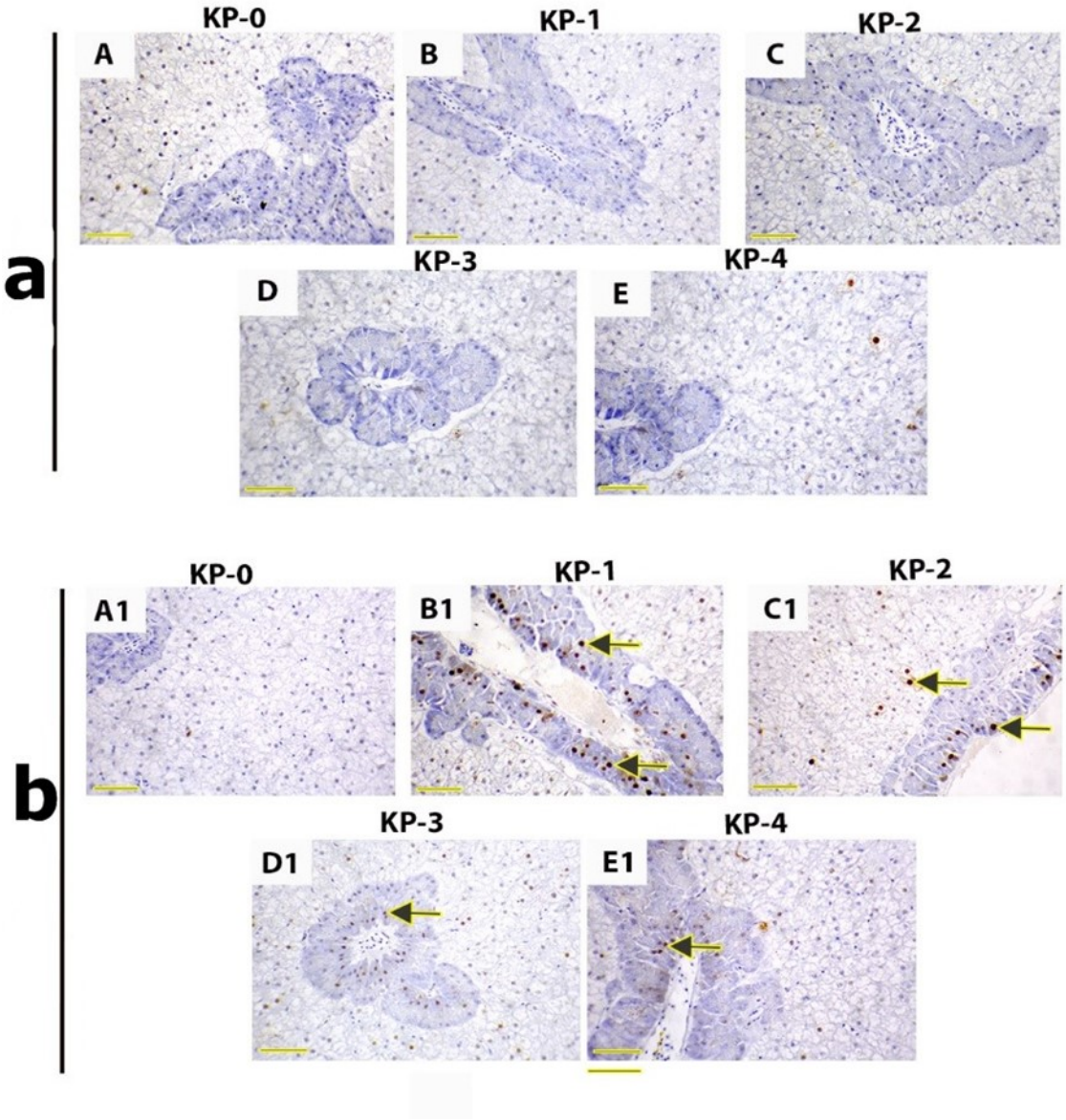



**Fig. (5).** Hepatopancrease showing comparative immunohistochemical reaction against PCNA antigen , in b (after infection) noticing mild to moderate specific brown immunoperoxidase reaction against PCNA antigen within the exocrine pancreatic tissues and hepatic paranchyma .In a ( befor infection) no detected reaction noticed. DAB technique ,Mayer's Haematoxylin ,X250

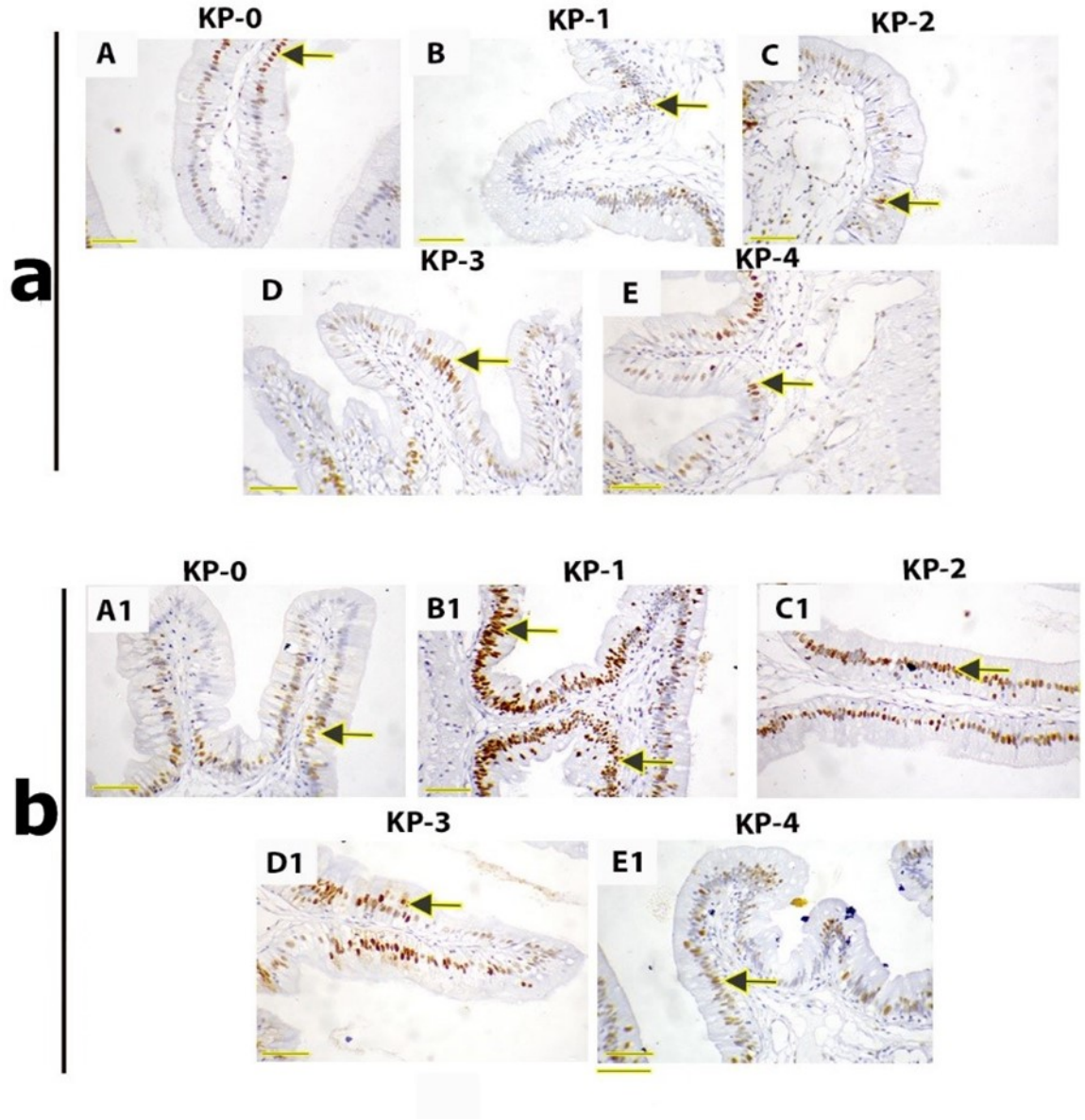



**Fig. (6).** Intestine showing comparative immunohistochemistry reaction against PCNA protein within the cytoplasm of epithelial lining the intestinal villi as agolden brown stain (arrows) indicates presence of PCNA protein, reflecting active and proliferating cells . Notice that KP2(C1) group after infection showing moderate reaction ,KP1 (B1) showing marked reaction. DAB technique ,Mayer's Haematoxylin, X400

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