

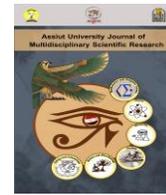
Assiut University Journal of Multidisciplinary Scientific Research (AUNJMSR)
Faculty of Science, Assiut University, Assiut, Egypt.

Printed ISSN: 2812-5029

Online ISSN: 2812-5037

The 7th Conference for Young Scientists in Basic and Applied Sciences,
May 10 – 11th (2022), Faculty of Science – Assiut University

<https://aunjournals.ekb.eg/>



Amelioration effect of propolis supplementation on haematological indices and histopathological alterations of *Clarias gariepinus* during heat stress

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ARTICLE INFO

Article History:

Received: 2022-05-19

Accepted: 2022-06-23

Online: 2022-08-14

Keywords:

Heat stress, propolis, histopathology, haematology, catfish

ABSTRACT

The purpose of this study was to look into the ability of a propolis-supplemented diet on some haematological indices and histological alterations in the gill, kidney, and testes of catfish (*Clarias gariepinus*) to cope with heat stress (HS), which could alter the physiology, homeostatic systems, and survival of catfish, indicating that the effects of heat stress on warrant further investigation.

In this study, male catfish were separated into three groups: control at 28°C, HS group at 36°C, and HS group at 36°C supplemented with propolis for up to 21 days. The treatment group at 36°C showed that, RBCs decreased considerably while WBCs did not decrease appreciably during the research period when compared to the control one. Microscopically, all tissues revealed changes in the morphological structure: the most massive difference in the gills was curving tips and edema, the most major change in the kidney was vacuolated tubular cells, and the most major change in the testes was a degraded cyst. However, the propolis group showed enhanced RBC and WBC counts, and most histological and histochemical markers were returned to the normal structure when compared to the control one.

INTRODUCTION

Temperature increase caused by global climate change is regarded as one of the most damaging stressors to species. High temperatures generate a wide range of varied and

frequently negative reactions in organisms, with damage manifesting itself at all levels of structural structure [1]. Changes in ambient water temperatures have a significant impact not only on metabolic characteristics, but also on growth, physiological fitness, reproduction, immune defenses, and the likelihood of disease in fish, which ultimately sets ecological patterns related to species distribution, abundance, and biological interactions [2,3]. Because most poikilothermic species, such as fish, cannot adjust their internal body temperature, so external temperature plays a significant role in managing the creatures' overall performances as a symbol of physical wellness [4]

It should also be considered that these temperature oscillations can indirectly cause haematological changes [5]. Changes in haematological parameters are easily detected and are regarded as effective bio-indicators of the health status and welfare of fish being able to represent an early diagnosis of fish pathological disorders [6]. Therefore, the classic analysis of the variables in haematology is a reasonable approach to assessing the health indicators for teleost fish [7].

Histological analyses of the target tissues may reveal the effects of chronic levels of exposure, and the results of histopathological studies are valuable in developing water quality guidelines. Fish gills are primarily a respiratory organ and they are also an essential immunological organ. Because of their close interaction with the external environment, gills are more sensitive to high water temperatures than other organs of fish, making them more susceptible to heat stress [8,9]. The kidney is a primary route of xenobiotic metabolite excretion and gets the greatest amount of post branchial blood; therefore, it is more prone to develop histopathological abnormalities under stress [10]. Temperature also influences reproduction by regulating the reproductive cycle, either by encouraging or decreasing gametogenesis [11,12].

Nutritional approaches, such as adding functional components to diets, have the potential to assist farmed fish cope with temperature stress. Propolis is a viscous waxy substance found in beehive extract that is antiseptic, antibacterial, anti-inflammatory, and antioxidant. The main components responsible for propolis' biological activity appear to include flavonoids, aromatic acids, diterpenic acids, and phenolic chemicals [13]. Because this functional ingredient improves animal development, physiological performance, antioxidants, and immunological support, it has a lot of promise in farmed fish diets [14].

Clarias gariepinus is the most commonly utilized freshwater fish in toxicological investigations. They are a prominent protein source in Africa and other parts of the world. Our study aimed to correlate pathological changes with elevated temperature in different tissues of catfish (*C. gariepinus*) and estimate the amelioration capacity of diets supplemented with propolis under heat stress.

MATERIALS AND METHODS

1. Experimental animals

Male *Clarias gariepinus* (average body weight 250-300 g and average total length 30-35 cm) were provided from Nile River, Assiut, Egypt. A total of 45 individuals were equally distributed among three indoor tanks (15 fish /per tank) and the size of each tank was (80 cm × 35 cm × 40 cm). Catfish were maintained in continuous aerated de-chlorinated water and fed with commercial fish food twice daily.

2. Experimental procedures and sample collection

After three week's acclimatization under laboratory conditions before the experiment, the water temperature was increased gradually from 28°C to 36°C for 2h. daily for three weeks; controls were maintained at 28°C, experimental fish stressed at 36°C and another group was kept at 36°C with supplementation with propolis (1.5 gm. dissolved in 10 ml of 70% ethanol then filtration and add 10ml of the solution to 10L of water tank).

3. Blood sampling

Five catfish were sampled from each group. Catfish were anesthetized using clove oil at the rate of 3 mg/L and blood samples were promptly collected from the caudal vein using a heparinized plastic syringe to avoid the consequence of stress and blood coagulation. The blood samples were stored in sterilized microfuge tubes containing 20 mM EDTA to retard further coagulation. During laboratory analysis, any unexpected blood clotting was minimized by gently shaking of the microfuge tube.

4. Histopathological and histochemical analysis

Formal alcohol glacial acetic acid solution was used to fix samples of gill, trunk kidney (TK) and testes samples. Then it is dehydrated in upgraded alcohol and processed for paraffin techniques. Samples were sectioned into 4 µm .Sections were stained for general histology by haematoxylin and eosin (H&E), Masson's trichrome and Sirius red stains for collagen fibers. Finally examined stained sections and selected region were determined under light microscope.

5. Statistical analysis

All data were presented as mean ± standard error (SE) and subjected to a one-way analysis of variance (one-way ANOVA) followed by multiple comparisons (Tukey's test) to determine significant differences among the treatments and controls. $P < 0.05$ was considered statistically significant. Using Graph Pad Prism software version 5.

RESULTS

1. Haematological parameters

Several hematological parameters including the number of RBCs ($\times 10^6/\text{mm}^3$) HGB (g/dl), hematocrit (HCT) percent and WBCs ($\times 10^3/\text{mm}^3$) were measured in samples collected at each of the temperature regimes. Administration of HS in catfish led to a significant decrease in RBCs count of blood compared to control. The supplementation with propolis in HS-group showed a significant rise in the RBCs count compared to HS-group, reverting relatively to the direction to control values **Fig. 1(A)**. Also, administration of HS in catfish led to a significant decrease in HGB compared to control. The supplementation with propolis in HS group revealed a significant increase when compared with to HS-group, reverting their values to near-control values **Fig.1 (B)**.

On the other hand, the value of HCT percentage showed that HS and HS + propolis led to insignificant change **Fig.1(C)** and the values of WBCs ($\times 10^3/\text{mm}^3$) in fish blood showed insignificant decrease in HS in comparison with control group. Also, Co-treatment with propolis caused insignificant change in WBCs count compared with either positive or negative corresponding control values **Fig.1 (D)**.

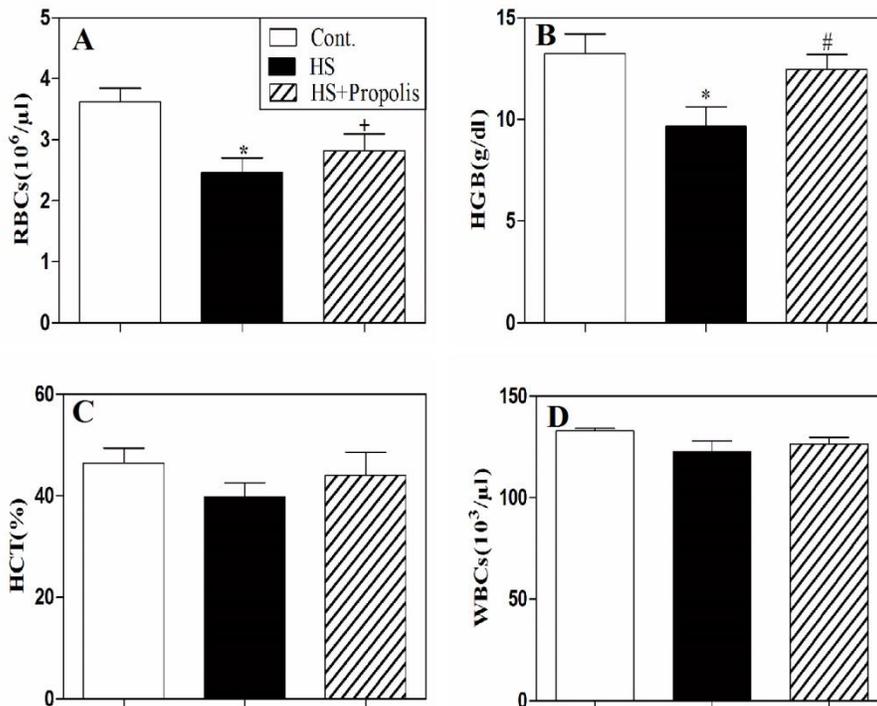


Figure 1: (A) the Red-Blood-Cells (RBCs) count, (B) the Hemoglobin (HGB), (C) hematocrit (HCT) percent, (D) White-Blood-Cells (WBCs) count as Mean \pm SEM in blood of control and different treated catfish. Columns with different superscript are significantly different at * $P < 0.05$ for HS vs. Cont; # $P < 0.05$ for HS+propolis vs. HS; + $P < 0.05$ for HS+propolis vs. Cont.

2. Histopathological studies:

Gills:

Fig.2 (A-C): Section of gill of all experimental groups stained by H&E X-400. The control group showed normal histological appearance of gill structures for both filament primary and secondary lamellar (PL,SL). SL perpendicular to the axis of the gill filament, these filament consists of loose connective tissues matrix and blood vessels and covered by stratified epithelium while SL on each side consists of a thin layer of a single layer of flattened epithelial cells which rests on a basement membrane covering the pillar cells which alternate with blood capillaries system (PC-BC) which consist main vascular component of the gills and appeared as ladder shapes which surrounded externally by pavement cells **Fig.2 (A)**. In the present investigation histological alterations of gills HS groups showed severe deformation in gills histomorphology, epithelial detachment curving shape and curling tips at ending of SL lamellae, Disorganization of (PC-BC) system and sub-epithelial edema were seen in SL **Fig.2 (B)**. Meanwhile, gills after exposed to HS + propolis revealed restoration of regular arrangement of secondary lamellae height which contains normal (PC-BC). Increase hyperplasia between secondary lamella which is more or less similar to control group were observed **Fig.2 (C)**.

Fig.2 (D-F): Section of gills of all experimental groups stained by Application of combined periodic acid Schiff's reaction + Alcian blue (PAS&AB-PH:2.5) (X-400). In the control groups showed polysaccharides contents were marked observed in basement membrane and presence few numbers of mucous secreting cells at the top of inter-lamellar epithelium between SL **Fig.2 (D)**. Hyperactivity of mucous cells at the top of stratified epithelium between SL which stained deep violet indication of containing acid mucopolysaccharides was noticed **Fig.2 (E)**. While HS treated + propolis showed decrease in number of mucous secreting cells in the top of inter-lamellar epithelium between (SL) and marked polysaccharides contents in basement membrane were noticed **Fig.2 (F)**.

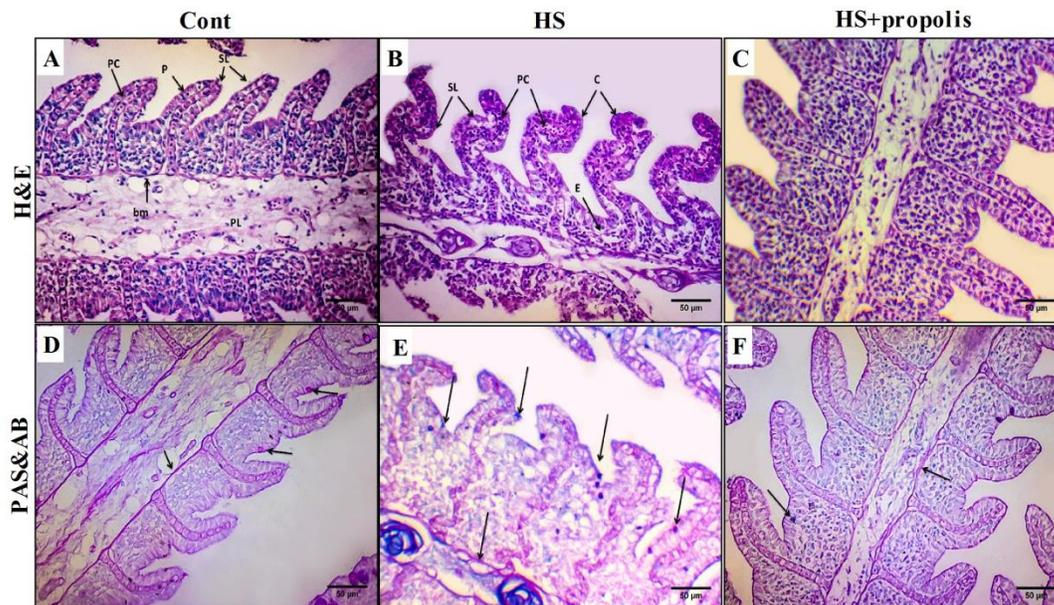


Figure 2: (A) Section of catfish gills of control group showed regular arrangement of (PL) and (SL). PL delimited by basement membrane (bm) and contains connective tissues matrix. SL contains regular ladder shape pillar cells (PC) alternative with blood capillaries which constitutes the main vascular component of gills. All these structures are surrounded by elongated spindle shape pavement cells (P) which covered by cuboidal cells, between (SL) stratified squamous epithelium was noticed, (B) HS group showed disappearance and collapse of the (PC-BC) system Epithelial cells up-toward the (1/3) length of (SL) with edema and congestion of blood capillaries. Curly shape (C) appearance of (SL), (C) HS + propolis group showed restoration of normal morphology more or less than control with increase of hyperplasia between (SL) (H&E, X400). (D) Control group revealed polysaccharides contents stained pink at basement membranes and faint reaction on boundary of ladder shape systems, (E) HS group revealed marked increase in both polysaccharides contents also number and density of mucous cells (dark arrow), (F) HS+ propolis group revealed marked decreased of polysaccharides contents were seen (PAS &AB PH:2.5, X400).

3. Histopathological studies Trunk kidney (TK)

Fig.3 (A-C): Section of trunk kidney of all experimental groups stained by H&E X-400. The control groups showed collecting tubules with different shapes from elongated to rounded and renal corpuscle. Each tubule surrounded by basement membrane and lined by cubic epithelial cells containing homogenous acidophilic cytoplasm and rounded vesicular and centrally located nuclei, few pyknotic nuclei were observed near apical brush border. Renal corpuscle consists of Bowman's capsule (BC) and glomerulus which appeared spherical or ovoid in shape surrounded by double membrane capsule enclosing a tuft of blood capillaries (glomerulus). There is a space in between the glomerulus and BC, which called the Bowman's space. Haematopoietic tissue was located between tubules which includes all types of granular leukocytes cells, red blood cells and melanomacrophage centers (MMC) **Fig.3 (A)**.

In contrast, HS catfish revealed deformation of epithelial cells of tubules which contains large vacuoles and increased of pyknotic nuclei beside brush border, increased of melanomacrophage cells and interstitial hematopoietic tissue exhibited marked depletion were observed **Fig.3 (B)**. Propolis + HS showed almost restoration of the renal histoarchitecture more or less similar to control groups **Fig.3 (C)**.

Fig.3 (D-F): Section of TK of all experimental groups stained by Masson trichrome's stain (X-400). Control groups revealed normal amount of collagen fibers around renal both tubules, corpuscles and blood vessel **Fig.3 (D)**. In HS showed increased collagenous fibers around both tubules, corpuscles and blood vessels. Fine fibers in ground substances of kidney were noticed **Fig.3 (E)**. In HS + propolis showed marked increased but less than HS **Fig.3 (F)**.

Fig.3(G-I): Section of TK of all experimental groups stained by Sirius red stain for collagen (Type I&III) (X-400). In control groups showed normal amount collagenous fiber around the renal tubules and the blood vessel **Fig.3 (G)**, while marked increased of collagen fibers in HS located around blood vessels **Fig.3 (H)**. In HS+ propolis showed marked increased but less than HS groups **Fig.3 (I)**.

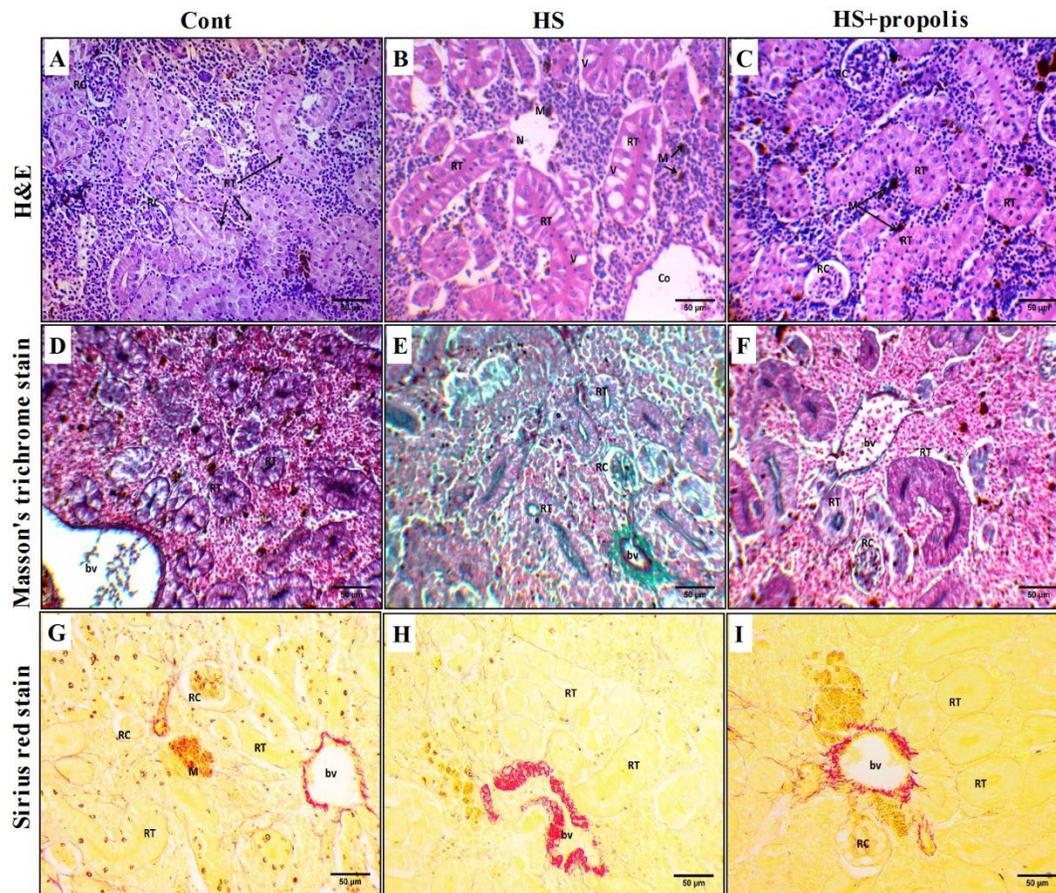


Figure 3: (A) Section of catfish TK of control group showed normal (RC) &(RT), (B) HS group showed vacuolation in renal tubule (V), blood congestion (Co), proliferation of melanomacrophage cells, (C) HS + propolis group showing restoration of (RC) and (RT) morphology **H&E, X400**. (D) Control group showed collagen fibers in both (RC)& (RT) and around the blood vessel (bv), (E) HS group showed increased collagenous fibers in renal structures, (F) HS+ propolis showed faint collagenous fibers (**Masson's Trichrome, X400**). (G) Control showed faint red stain collagen in renal structures around (bv) &(RT), (H) HS group showed increase in collagen fibers contents, (I) HS + propolis group showed pale red stain collagen fibers in renal structures (**Sirius red stain, X400**).

4. Histopathological studies:

Testes

Fig.4 (A-C): Section of testes of all experimental groups stained by H&E X-400. In control groups seminiferous tubules (ST) vary in size and shape which consists of all stages of spermatogenesis which rest on basement membrane primary spermatogonia cysts (PSG), secondary spermatogonia (SSG), and primary spermatocytes (PSC), secondary spermatocytes (SSC), spermatids (SP) and free spermatozoa (SZ), and Sertoli cells (S). Few large PSG rounded cell contain faint acidophilic cytoplasm with centrally located and vesicular nuclei. Cysts of SSG containing small size cells with acidophilic cytoplasm and vesicular nuclei were seen. Cysts of PSC consist of small number of cells

with faint staining cytoplasm and deeply stained chromatin. Cysts of SSC contain large number of cells with faint acidophilic cytoplasm and vesicular nuclei. Cysts of spermatids (SP) contain large number of smallest cells with small condensed nuclei. Aggregation of free and small size spermatozoa (SZ) were observed in lumen of lobules which appeared round and deeply stained. Sertoli cells with elongated to oval shaped nuclei. Interstitial tissue (I) connects neighboring lobules which contains round to oval shape leydig cells with distinct nucleus are noticeable **Fig.4 (A&D)**. HS exhibited deformation of seminiferous tubule with irregular and thick basement membrane. Main tubules contain both degenerated cysts and vacuolated cells. Lumen of all tubules containing spermatozoa was seen. Widening of interstitial space and increase of interstitial connective tissues matrix was seen **Fig.4 (B&E)**. In HS + propolis revealed that testicular tissue was almost restored to normal structure **Fig.4(C&F)**.

Fig.4 (G-I): Section of testes of all experimental groups stained by Masson trichrome's stain (X-400). Control groups revealed normal of amounts of collagenous fibers around seminiferous tubules and in intertubular connective tissues **Fig.4 (G)**. While accumulation in HS and decreased in HS + propolis of collagen fibers around the seminiferous tubules and in intertubular connective tissues were seen **Fig.4 (H&I)**.

Fig.4 (J-L): Section of testes of all experimental groups stained by Sirius red stain (X-400). Control groups exhibited fine deposition of red staining collagenous fibers (Type I& III) around the somniferous tubules and in intertubular connective tissues **Fig.4 (J)**. Accumulation in HS and decreased in HS + propolis of collagen fibers (Type I&III) around the seminiferous tubules and in intertubular connective tissues were seen **Fig.4 (K&L)**.

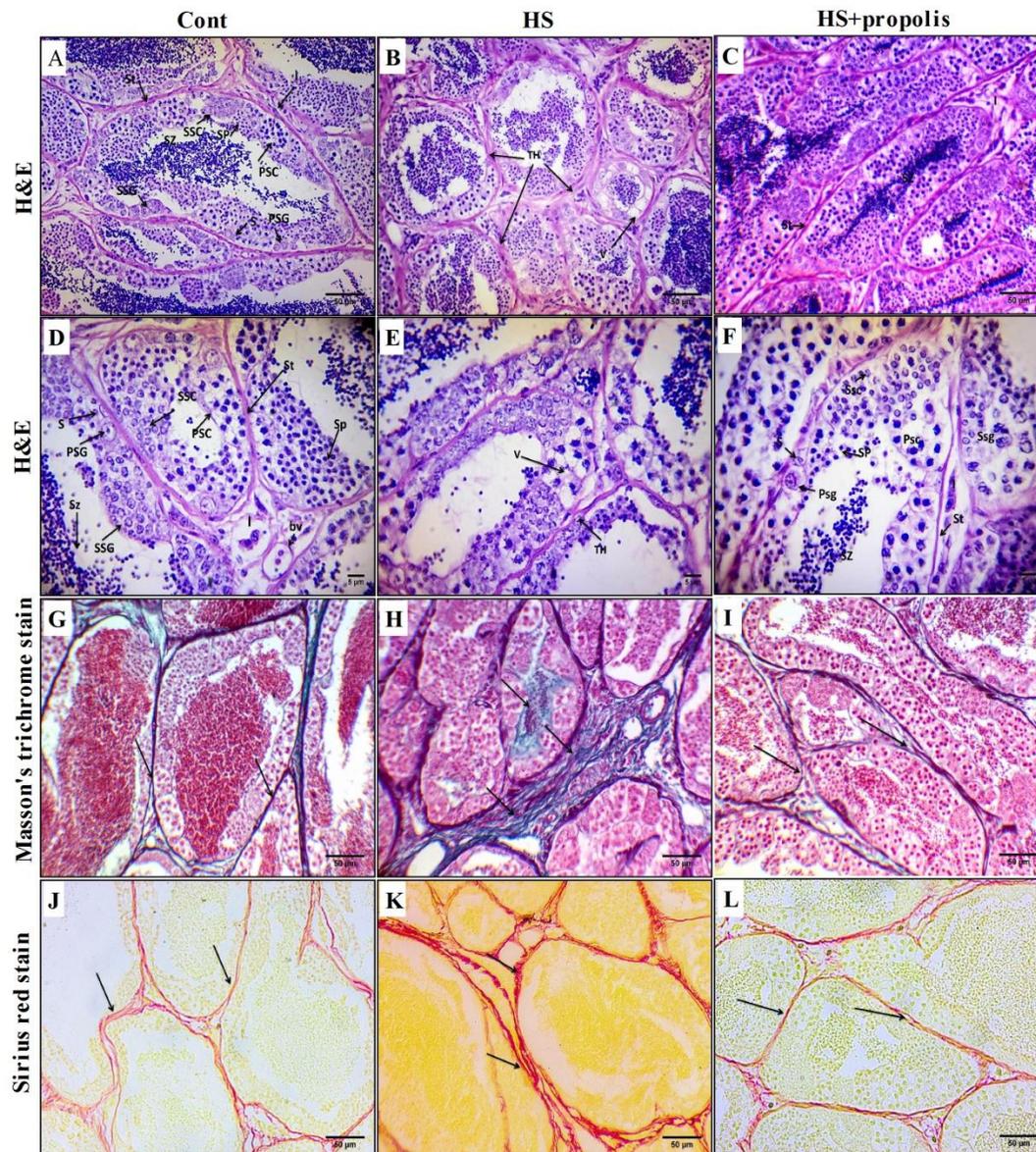


Figure 4: (A&D) Section of catfish testes of control group showed (S) & (PSG,SSG), (PSC&SSC) (Sp) (Sz) (I), (B&E) Sections of heat-stressed catfish testes revealed deformation of the seminiferous tubule shape ,irregular and thick basement membrane. Few tubules contain degenerated cysts and vacuolated (V) cells were clear observed, (C&F) Section of heat stress catfish testes of treated with propolis showed restoration of normal architecture of seminiferous tubules (St) (H&E, X400 and X1000). (G) Control group showed fine deposition of collagenous fibers around the seminiferous tubules and in intertubular connective tissues, (H) HS group showed marked increase in deposition of collagenous fibers around& inside the somniferous tubules, (I) HS+ propolis group showed marked decrease as compared with (HS)and appeared more or less similar to control (Masson trichrome's stain, X400). (J) Control group showed fine deposition of red staining collagenous fibers (Type I& III) around the somniferous tubules and in intertubular connective tissues, (K) HS group showed thickening of red staining

collagenous fibers (Type I & III) around the seminiferous tubules and in between tubules, (L) HS+ propolis group showed faint red color of collagen when compared with (HS) and appeared more or less similar to control (**Sirius red stain, X400**).

DISCUSSION

Fish depend on the temperature of their water-based habitat for their survival, production, and correct metabolic activities. When fish are stressed, physiological processes and internal structural changes can occur that are either harmful or beneficial, and if these abnormalities are not corrected or compensated for, the organism weakens, losing the ability to cope with subsequent pressures [4]. To help fish overcome the physiological challenges posed by a rapidly changing environment, researchers may look into developing new aquafeed compositions. The impact of propolis supplementation on fish diet to deal with heat stress was demonstrated in our research.

The analysis of haematological indicators is essential for evaluating the health status of fish under varied stress conditions. Blood components are extremely temperature sensitive, and if there is a physiological variation, it will be reflected in the criteria of various blood characteristics. This might effect on both the natural physical process of reproduction and the overall growth of the fish population. Several studies have been conducted to investigate changes in catfish blood parameters as a result of stress caused by changes in water temperature [15,7] Extreme warm exposure, significantly decreased RBC, hematocrit, hemoglobin and decreased WBC were observed in catfish. The decrease in these values may result from relatively higher erythrophagocytosis of damaged RBC or shrinking of RBC from warm stress [16]. This could be the result of the poor hematopoietic system function during extreme thermal stress [7]. And therefore, low quality and small amounts of red blood cells as well as low level of hemoglobin leads to a decline in the availability of oxygen in the body. Apart from transportation of oxygen, red blood cells perform other important functions in the body and inadequate amounts and quality of red blood cells would have a chain of consequences on metabolic activities other than just the provision of oxygen for respiration. In the current investigation, the addition of dietary propolis resulted in outstanding efficacy outcomes, promoting a considerable improvement in RBCs count, hemoglobin, and hematocrit when compared to the control group. These similar several studies that reported the supplementation of direct propolis, and ethanolic extract propolis, in fish diets significantly increases the hematocrit values [17,14,18]. Because propolis contains a diverse spectrum of flavonoids, vitamins, and other compounds with diverse biochemical structures and bioactivities, it may have a function in increasing erythropoiesis in fish hematopoiesis. White blood cells (WBCs) play a key part in nonspecific or innate immunity, and the leucocyte count/activity of a fish signals its overall health. WBCs play a role in the management of immunological processes, and changes in WBC counts following toxicant exposure suggest a decline in the fish's nonspecific immunity [19]; the WBC count was lower in the HS group, this agrees with the results authors [20,21].

In addition, high temperatures affect homeostasis in fish, causing abnormalities of critical organs such as the gills, kidneys, and testes. Gill remodeling is required to strategically maximize gas exchange while maintaining homeostatic balance [22]. In this study, the swelling of epithelial cells in the gill lamellae was first observed in the group heat-

stressed. This change in gill-tissue structure can block the stress factors from infiltrating into the deep tissues, and has a certain protective effect for the fish gills, yet it occurs at the cost of reducing gas and material exchange [23]. Curling of the secondary lamellae reported in our study at heat stress group which may be to improve oxygen diffusion and serve as a defense mechanism to protect lamellae [24,25]. Mucous cells in the catfish gills, located mostly on the primary lamellar epithelium, and a few mucous cells, were placed on the epithelium of secondary lamellae. In this study, the histochemical characteristics of the mucous cells in the gills of *C. gariepinus* showed the presence of acidic mucosubstances which were stained purple with PAS and blue with AB, pH 2.5, respectively. The number of mucous cells increased in HS group compared with control group.

Vacuolization of the kidney showed vacuolar degeneration of tubular epithelium, loss of tubular structure and necrosis of the renal tubules were discovered to occur in kidney tissue as a result of an imbalance between the rate of synthesis of chemicals in parenchymal cells and the rate of release into the circulatory system. The present results are in agreement with those detected in *Cyprinus carpio communis* acclimated to various temperatures (Ahmed *et al.*, 2011). Because MMCs are known to mediate innate immunity in fish, their increase amount may signal that the defenses are compromised by high temperatures.

In furthermore, by destroying testicular tissue, HS lowers sperm production and increases the number of malformed sperm [27]. Histological investigation of testicular tissue sections in our study revealed that HS generated abnormalities in the spermatogenic cell layers in seminiferous tubules, but similar abnormalities were seldom found in the HS supplementation with propolis group.

CONCLUSION

Supplementing the food with extract of propolis boosted the economic efficiency of catfish production by refining haematological parameters and improving histological damage and dysfunction of different tissue during heat stress.

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