

Circulatory physiology and haematopoiesis of *Channa punctatus* under piscicidal toxicity of *Acacia auriculiformis* pericarp

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Abstract

Acacia auriculiformis pericarp preparation (APP) has been recently introduced as a piscicide of choice to substitute Mohua (*Bassia latifolia*) oil cake (MOC). The present study aims to evaluate its effect on circulatory physiology and haematopoiesis of *Channa punctatus* to justify its mode of action as a piscicide. Profound alterations in the circulatory physiology of *Channa punctatus* with oligocythemic hypochromic anaemia and significant mobilization of immature cells from the erythropoietic lineage into circulation are evident under APP exposure. A relationship of hypoxia-induced *Epo* expression with erythropoietic dynamics has been drawn under LC₀ APP exposure. However, at both LC₅₀ and LC₁₀₀ exposure erythropoietic insufficiency and subsequent erythropenia are recorded. The principal secondary metabolite of APP, saponin is held responsible for membrane destabilization, pore formation, and several topographical alterations of erythrocytes. The exocytosis of haemoglobin is confirmed quantitatively and qualitatively. Retention of plasma Hb among the treated fish is substantiated through direct and indirect bilirubin content of blood and the physiological relevance of such condition has been interpreted. Leucocytosis and more specifically neutrocytosis at the initial exposure period observed as an immediate defensive mechanism which is ultimately broken down with exposure concentration and duration. The treated fish develops thrombocytosis as a haemostatic mechanism to check haemorrhage, but the circulatory deficiency ultimately leads to death of the fish.

Introduction

Composite fish culture is a method of polyculture of fish species, usually the fast-growing carps, through optimum niche utilization for obtaining the best yield (Saha and Saha 2022a). As a step towards the pre-stocking pond preparation for culturing fish through this technique, it is imperative to exclude the unwanted fish species from the system to restrict to the desirable composition. From the ecological consideration, the plant origin piscicides are the best option for eradication of those undesirable species referred to predators and weed fishes. Mohua oil cake (MOC), obtained from the seeds of *Bassia latifolia* (Bhatia 1970) and rotenone containing *Derris* spp. (Rickard and Cox 1986) are two traditionally used piscicides. Very recently *Acacia auriculiformis* pericarp preparation (APP) has been introduced as a piscicide of choice to substitute MOC by Saha and Saha (2022a) in terms of its efficacy, availability, and cost efficiency. A detailed phytochemical analyses (Saha and Saha 2022b) detected and quantified seven triterpenoid saponins (acaciasides), twenty two flavonoids, eight tannins and one phenolic rich fraction- Gallyl glucose from the methanolic extract of APP through LC-ESI-MS. Among those, acaciasides have been identified as the principal secondary metabolites.

Preliminary investigations on different saponin group like- Petersaponin, Ginsenoside, Digitonin, Holothurinoside, and Holothurins showed their effects on cell membrane and gill. El Izzi et al (1992) showed higher concentrations of Petersaponins, from *Petersianthus macrocarpus* plant promoted numerous holes in the cell membrane. Choi et al. (2001) proved Ginsenosides, from the root of *Panax ginseng*, were used as an anti-stress agent for its selective regulation efficacy of voltage-dependent Ca²⁺ channel subtypes in bovine chromaffin cells. Menin et al. (2001) proved that the cell membrane permeabilization capacity of Digitonin occurs due to its high affinity for cholesterol. Eeckhaut et al. (2015) proved that Holothurinosides obtained from echinoderms *Bohadschia atra* and *Holothuria forskali* body wall was detrimental to the secondary gills lamellae of free-living fish species and caused the detachment of the epithelia, edema, and pore formation at the level of the epithelial cells and pillar cells leading to their destruction. Seeman et al. (1973) proved that Holothurin, from sea cucumber *Actinopyga agassizi*, cause small holes 40–50 Å in the membrane of human erythrocyte.

Although the biological effects of different saponin groups are underscored, the bioactivities of acaciasides are not well documented. The present study is thus aimed towards evaluation of its effect on circulatory physiology of a murrel, *Channa punctatus* to justify its use as an efficient piscicide.

Materials and methods

Collection of plant material

Fully matured fruit pericarps of *Acacia auriculiformis* (A. Cunn. ex Benth) were collected from Sonajhuri forest (23°67'76"N 87°68'52"E), nearby Santiniketan, Bolpur, West Bengal. The dust was cleared from the pericarps with a brush, then sundried and pulverized in powdered form and sieved through the uniform mesh (no. 40). A preparation (APP) from the dried matured fruit was used for experimentations.

Collection and maintenance of experimental fish

An indigenous murrel, *Channa punctatus* (Bloch), a potential predator was used for haematological and haematopoietic evaluation under exposure to APP. A group of about 800 *C. punctatus* specimens (16.96 ± 0.27 cm length and 61.12 ± 2.7 gm body weight) were collected from local fish farms around Sriniketan (23°40'33"N 87°39'37"E) and were acclimated under laboratory condition for two weeks providing *Tubifex* sp. *ad libitum* twice daily as a live feed. Hydrological parameters were determined according to the standard procedures (APHA/AWWA/WPCF, 1998). During both acclimation and experimentations following hydrological parameters were maintained- temperature 28 ± 2°C, pH 7.2 ± 0.06, dissolved oxygen 5.24 ± 0.005 ppm, total hardness 71 ± 2.53 ppm, and Photoperiod (hours light) 12–14 h. Animal maintenance and experimental procedures were in accordance with the animal welfare laws, guidelines, and policies of the Institutional Animal Ethics Committee of Visva-Bharati (IAEC approval number: IAEC/III-19/ 2020).

Haematological assay

For haematological study of *Channa punctatus*, the fish was anaesthetized using Tricaine methanesulfonate (MS-222; 50 mg/L) immediately before blood collection (Stetter 2001; Neiffer and Stamper 2009). Blood samples were collected by severing the caudal peduncle following the method proposed by Hesser (1960). Blood was collected into 1.5 mL microtubes (Tarsons, India) containing ethylenediamine tetraacetic acid (EDTA; 4 mg/mL) as an anticoagulant for whole blood assay and then mix it for 10 min. For plasma samples, total blood was collected in frozen microtubes containing EDTA. The blood samples were centrifuged 2 times at 4°C at 3000 rpm for 20 min each time and supernatants were collected carefully for assay. For serum samples, the collected blood

samples were left undisturbed without anticoagulant at room temperature for 4 hours to clot. After complete clotting, serum was carefully separated and subsequently centrifuged at 2000 x g at 4°C for 10 min. For experimentation, the fish were exposed to three different lethal concentrations of APP according to Saha and Saha (2022a) viz. 15 ppm (LC₀), 24 ppm (LC₅₀), and 40 ppm (LC₁₀₀) with experimental periods of 6h, 12h, 18h, and 24h. A total of four experimental groups denoted as C for control, T₁ for LC₀ exposure, T₂ for LC₅₀ exposure, and T₃ for LC₁₀₀ exposure are maintained. Total erythrocyte count (TEC) and total leucocyte count (TLC) were done by Neubauer's improved double haemocytometer (Fein-OPTIK, Blankenburg, G.D.R.) using modified Dacie's fluid (Dacie and Lewis 1984) as diluent. Haemoglobin content was estimated from both the whole blood and serum samples through Cyanmethaemoglobin method, using Drabkin's reagent (Blaxhall and Daisley 1973). Haematocrit (Hct) was measured using the method of McLeay and Gordon (1977). The Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH), and Mean Cell Haemoglobin Concentration (MCHC) were calculated using standard formulae as mentioned by Dacie and Lewis (2001).

$$\text{MCV (fl)} = \text{Haematocrit (\%)} / \text{TEC (cu mm)} \times 10$$

$$\text{MCH (pg)} = \text{Haemoglobin (g \%)} / \text{TEC (cu mm)} \times 10$$

$$\text{MCHC (g/dl)} = \text{Haemoglobin (g \%)} / \text{Haematocrit (\%)} \times 10$$

For differential cell count blood smear was prepared on glass slides. The smears were stained with haemoglobin specific peroxidase stain o-Dianisidine following the method of O'Brien (1961) and Giemsa was used as a counterstain for the nucleus (Mahajan and Dheer 1979). A differential count of WBC was done from the stained preparation under an optical microscope (Olympus-BX41). The presence of different maturity stages of RBCs was also done separately from the same smear preparation and their relative abundance was calculated and presented in percentage (Mahajan and Dheer 1979).

Bilirubin estimation

Both direct and indirect bilirubins of serum were estimated following Jendrassik and Grof (1938) using chemical reagents kit from Delta lab laboratory and analyzed with a semi-automatic Robonik Prietest touch plus biochemistry analyzer. Serum samples were collected from all the experimental groups at 6h, 12h, 18h, and 24h experimental time points. The values are presented in mg/dl units. Total bilirubin was estimated by summation of direct and indirect bilirubin.

Native polyacrylamide gel electrophoresis (Native PAGE)

Native PAGE of serum samples was performed (Davis 1964) in discontinuous buffer system with an Amersham Pharmacia Biotech vertical gel electrophoresis apparatus at 200V and constant current supply of 20mA. Serum samples were collected from all the experimental groups at 24h experimental time point. The gel was stained with haemoglobin specific peroxidase stain o-Dianisidine (O'Brien 1961) followed by Coomassie brilliant blue R250 (Malik and Berrie 1972) staining. Images were captured in Bio-Rad Gel Doc EZ imager using both silver filter and coomassie filter.

Scanning Electron Microscope (SEM) of erythrocytes

Scanning electron microscopy (SEM) of erythrocytes has been performed from collected from all the experimental groups at 6h, 12h, 18h, and 24h experimental time points. For sample preparation, blood samples were collected and then fixed with 2.5% glutaraldehyde and finally with isoamyl acetate. The air-dried erythrocyte suspension on cover glass were sputter coated with gold and examined under a scanning electron microscope (Zeiss EVO LS10, Carl Zeiss, Germany) at an accelerating voltage of 15–20 kV.

Erythropoiesis and Neutropoiesis

To study the erythropoietic and neutropoietic lineage of the fish, tissue imprints of the pronephric kidney and spleen were prepared from all the experimental groups at 6h, 12h, 18h, and 24h time points following Dheer et al. (1986) and Maiti et al. (2000). The tissue imprints were stained with o-Dianisidine and counterstained with Giemsa (O'Brien 1961) as described earlier. The cells of the erythropoietic lineage were identified under an optical microscope (Olympus-BX41) according to Mahajan and Dheer (1980b) and Kulkeaw and Sugiyama (2012). The neutropoietic lineages were identified following Mahajan and Dheer (1980a) and Tak et al. (2013). The relative abundance of the cells of the erythropoietic and neutropoietic lineage was calculated and presented in percent value.

Erythropoietin (EPO) assay

Erythropoietin (EPO) of plasma and haematopoietic tissues viz. pronephric kidney and spleen was measured using Fish EPO ELISA kit (Bioassay Technology Laboratory) based on biotin double antibody sandwich technology. Absorbance of each well was measured at 450 nm using a microplate reader (Thermo Scientific Multiskan Go) and concentration of EPO was calculated from a standard curve and presented as ng/l.

Statistical analyses

All the results are presented as mean ± standard deviation of the mean. Statistical analyses were done through a variance analysis (one-way ANOVA test) using Tukey's method in Minitab 17 software. In every analysis $p < 0.05$ value was considered significant.

Results

Haematological profile

Total and Differential erythrocyte count

Total and differential circulatory erythrocyte count presented in Fig. 1, Fig. 7 and Table 1 showed a significant initial increase of total erythrocyte count (TEC) in the T₁ group. In T₁ group, TEC became surplus by increase of young erythrocyte (YE) and mature reticulocyte (MR) although mature erythrocyte (ME) and young reticulocyte (YR) were decreased. Such a surplus reached its peak at 12h exposure.

Table 1
Comparative score of haematological profile of *Channa punctatus* under exposure to APP versus control

Haematological parameters	T ₁ group				T ₂ group				T ₃ group		
	6h	12h	18h	24h	6h	12h	18h	24h	6h	12h	18h
TEC	+ 2.56%	+ 9.26%	+ 6.17%	+ 3.84%	-3.3%	-8.19%	-10.29%	-18.19%	-5.3%	-14.19%	-20.35%
Mature erythrocyte	-0.25%	-0.86%	-1.64%	- 2.18%	-2.04%	-2.92%	-2.92%	-11.82%	-12.74%	-20.85%	-32.08%
Young erythrocyte	+ 57.94%	+ 113.36%	+ 183.56%	+ 217.96%	+ 164.57%	+ 194.79%	+ 248%	+ 300.87%	+ 315.55%	+ 296.45%	+ 252.2%
Mature reticulocyte	+ 117.08%	+ 138.95%	+ 179.15%	+ 214.91%	+ 361.53%	+ 532.74%	+ 859.78%	+ 2598.61%	+ 3147.29%	+ 5737.2%	+ 9073.48%
Young reticulocyte	-54.85%	-38.94%	-26.12%	-9.83%	-29.12%	-18.4%	-0.75%	+ 33.56%	+ 15.36%	-18.46%	-35.31%
polychromatophilic erythroblast	0	0	0	0	0	0	0.21%	0.4%	0	0	0
Haematocrit	+ 2.07%	+ 7.61%	+ 5.08%	+ 2.41%	-6.7%	-17.05%	-21.09%	-28.85%	-10.93%	-23.74%	-32.16%
Haemoglobin	+ 0.49%	+ 1.45%	+ 4.32%	+ 7.75%	-2.33%	+ 7.24%	+ 18.36%	+ 24.37%	+ 3.71%	+ 16.82%	+ 31.72%
Serum haemoglobin	+ 23.23%	+ 58.36%	+ 198.99%	+ 290.83%	+ 65.82%	+ 354.29%	+ 473.62%	+ 819.03%	+ 419%	+ 817.63%	+ 1169.54%
MCV	+ 0.13%	+ 0.83%	+ 1.65%	+ 2.99%	+ 0.86%	+ 2.23%	+ 3.72%	+ 6.38%	+ 1.5%	+ 6.36%	+ 8.9%
MCH	-0.24%	-0.46%	-0.6%	-0.74%	-0.64%	-0.81%	-1.29%	-1.61%	-1.22%	-1.79%	-2.6%
MCHC	-0.98%	-1.72%	-3.06%	-4.67%	-1.42%	-3.77%	-5.03%	-7.52%	-3.69%	-7.75%	-12.08%
Total bilirubin	+ 2.7%	+ 11.2%	+ 21.71%	+ 23.32%	+ 6.05%	+ 1.59%	-4.26%	-11.04%	+ 9.18%	-8.35%	-22.48%
Direct bilirubin	+ 0.09%	+ 4.06%	+ 8.17%	+ 11.07%	+ 3.75%	+ 1.16%	-4.57%	-8.37%	+ 6.88%	-4.13%	-29.87%
Indirect bilirubin	+ 4.49%	+ 15.7%	+ 30.3%	+ 31%	+ 7.5%	+ 1.85%	-4.06%	-12.71%	+ 10.64%	-11.02%	-17.79%
TLC	+ 10.73%	-3.26%	-19.98%	-26.84%	+ 7.41%	-14.4%	-35.91%	-45.2%	+ 4.79%	24.94%	-51.98%
Thrombocyte	+ 1.6%	+ 1.3%	+ 1.4%	+ 1.74%	+ 17.11%	+ 18.07%	+ 20.66%	+ 22.89%	+ 48.48%	+ 49.83%	+ 53.53%
Neutrophil	+ 3.55%	+ 3.41%	+ 3.32%	+ 3.68%	-13.88%	-16.02%	-19.27%	-21.64%	-40.09%	-41.87%	-46.29%
Small lymphocyte	+ 5.34%	+ 9.82%	+ 13.88%	+ 17.26%	-26.3%	-25.6%	-29.6%	-30.35%	-68.69%	-68.23%	-72.02%
Large lymphocyte	-27.27%	-27.27%	-30.73%	-33.03%	-52.08%	-52.29%	-53.94%	-56.1%	-97.51%	-98.91%	0
Eosinophil	-21.28%	-24.81%	-29.21%	-34.23%	-33.41%	-36.43%	-40.92%	-45.33%	-93.25%	-97.95%	0
Basophils	+ 7.85%	+ 9.77%	+ 10.55%	+ 12.65%	+ 35.87%	+ 38.69%	+ 40.33%	+ 43.02%	-13.43%	-20.06%	-27.96%
Monocytes	-32.33%	-41.69%	-37.49%	-21.66%	- 69.14%	-72.93%	-70.96%	- 64.05%	0	0	0
T ₁ group- treatment group exposed to LC ₀ concentration; T ₂ group- treatment group exposed to LC ₅₀ concentration; T ₃ group- treatment group exposed to LC ₁ concentration. '+' denote increment in comparison with control group; '-' denote reduction in comparison with control group.											

In the T₂ group, TEC decreased because of the cumulative decrease of both ME and YR which was not compensated by cumulative increase of YE and MR at all exposure time periods. Mobilization of polychromatophilic erythroblast (PE) was also recorded in T₂ group at 18h and 24h exposure period.

In T₃ group, decrease of TEC was specifically caused due to decrease in ME, which was not replenished by the combined increase of YE, and MR.

Haematocrit (Hct)

Alteration in the Haematocrit (Hct) parameter depicted in Fig. 2 and Table 1 represents a somewhat similar trend like that of TEC ie. increased in T₁ group and consistently decreased in T₂ and T₃ group.

Haemoglobin (Hb)

Blood haemoglobin (Hb) and serum haemoglobin (Hb) of the experimental fish represented in Fig. 2 and Table 1 showed a significant increase among all the treated groups.

Derived haematological parameters

The derived variables were calculated after subtracting serum Hb from whole blood Hb and the result is presented in Fig. 2 and Table 1. It showed that MCV consistently increased while both MCH and MCHC were decreased among all the treated groups.

Bilirubin concentration

Total bilirubin, direct bilirubin and indirect bilirubin presented in Fig. 3 and Table 1 showed their consistent increment in T₁ group. These three parameters increased in T₂ group up to 12h exposure, thereafter reduced significantly. In T₃ group, these parameters initially increased at 6h exposure, thereafter showed reduction.

Total and Differential leucocyte count

Total and differential leucocytes count presented in Fig. 6, Fig. 7 and Table 1 showed a similar trend in changes of total leucocyte count (TLC) in all treated groups, initial increase followed by a decrease. These changes were mainly contributed by thrombocyte (THR) followed by Neutrophil (NEU). In T₁ group, TLC became surplus due to the increment of THR, NEU, small lymphocyte (sLYM) and basophil (BSP) at 6h exposure. TLC decreased because of large lymphocyte (LYM), eosinophil (ESP) and monocyte (MNC) though THR, NEU, sLYM and BSP increased at the subsequent observation periods.

In T₂ group, however, surplus TLC became contributed by THR and BSP at 6h exposure. In the subsequent observation periods TLC decreased as a consequence of cumulative decrease in NEU, sLYM, ESP, LYM and MNC count though THR and BSP count increased.

In T₃ group, the initial increase in TLC is resulted due to the increment of THR only. In the subsequent observation periods TLC decreased because of the cumulative decrease of NEU, sLYM, ESP, LYM, MNC and BSP though THR showed an increased abundance.

Native gel staining

Comparative photographs of NATIVE PAGE have been presented in Fig. 4 stained with o-Dianisidine and Coomassie brilliant blue. O-Dianisidine stain identified haemoglobin protein from three stained lanes bearing serum from all treated groups but remained unstained in the fourth lane containing serum of the control group of fish. Whereas, Coomassie stain identified the four lanes irrespective of haemoglobin protein.

SEM of Erythrocytes

Photographs of scanning electron microscopy of erythrocytes of control as well as treated groups have been presented in Fig. 5. The micrograph of control samples showed regularly shaped erythrocytes with the usual smooth surface. RBCs from the treated groups showed various abnormalities in morphology such as irregularly shaped RBCs, elongated cells, protuberances on the surface of the irregular boundary of cells, marginal folding, lobopodial projections, cytoplasmic bleb, fused cells, notched, the shape of erythrocytes change from elliptical to semi-spherical (spherocytes), crenated cells having serrated margins, membrane internalization, tear drop shaped cells. Shrinkages are often present on the cell membrane of RBC and some pores are prominent on the membrane. Membrane pores are so extended that oozing out of cytoplasmic contents is occurring.

Erythropoiesis

Differential cell types of the erythropoietic lineage from the pronephric kidney and spleen have been presented in Fig. 8, Fig. 11 and Table 2.

Table 2
Comparative score of haematopoietic parameters of *Channa punctatus* under exposure to APP versus control

Haematopoietic parameters	T ₁ group				T ₂ group				T ₃ group			
	6h	12h	18h	24h	6h	12h	18h	24h	6h	12h	18h	24h
Proerythroblast	+ 3.91%	+ 5.42%	+ 7.85%	+ 10.59%	+ 20.38%	+ 25.14%	+ 30.79%	+ 38.78%	+ 46.2%	+ 50.37%	+ 60.26%	+ 71.28%
Basophilic erythroblast	+ 10.69%	+ 7.3%	+ 3.23%	-1.4%	-18.53%	-23.58%	-29.54%	-40.9%	-53.43%	-59.46%	-71.62%	-90.08%
polychromatophilic erythroblast	+ 43.17%	+ 29.8%	+ 14.03%	-2.11%	-23.66%	-36.12%	-48.34%	-64.02%	0	0	0	0
Acidophilic erythroblast	+ 8.71%	+ 7.25%	+ 4.83%	+ 0.72%	-7.08%	-15.6%	-30.15%	-48.52%	-65.46%	-69.24%	-78.01%	-85.3%
Young reticulocyte	-2.84%	-3.17%	-3.53%	-4.28%	+ 1.23%	+ 3.11%	+ 5.11%	+ 7.45%	+ 8.41%	+ 10.33%	+ 12.46%	+ 15.75%
Mature reticulocyte	-1.12%	-0.48%	+ 0.16%	+ 1.09%	+ 1.23%	+ 3.11%	+ 5.11%	+ 7.45%	+ 8.41%	+ 10.33%	+ 12.46%	+ 15.75%
Young erythrocyte	-29.78%	-34.65%	-35.21%	-37.03%	-96.73%	-98.78%	-99.79%	0	-97.21%	-98.6%	0	0
Mature erythrocyte	-4.52%	-5.34%	-6.43%	-7.82%	-18.9%	-25.44%	-39.72%	-55.74%	-58.95%	-66.47%	-81.49%	-95.99%
Myeloblast	+ 5.08%	+ 6.48%	+ 9.22%	+ 13.19%	+ 15.81%	+ 21.52%	+ 30.08%	+ 41.9%	+ 43.83%	+ 48.11%	54.74%	73.1%
Promyelocyte	-4.76%	-6.86%	-9.1%	-12.34%	-13.6%	-19.49%	-28.23%	-41.75%	-44.17%	-51.27%	-61.14%	-83.78%
Mesomyelocyte	+ 0.65%	+ 1.01%	+ 1.32%	+ 1.7%	+ 2.01%	+ 2.59%	+ 3.41%	+ 4.95%	+ 5.23%	+ 6.7%	+ 9.03%	+ 11.52%
Metamyelocyte	+ 0.24%	+ 0.58%	+ 0.8%	+ 1.24%	+ 1.5%	+ 2.01%	+ 3%	+ 4.36%	+ 4.77%	+ 5.23%	+ 6.18%	+ 7.86%
Mature neutrophil	-4.63%	-6.59%	-10.89%	-18.26%	-24.43%	-29.51%	-39.29%	-50.44%	-53.58%	-57.27%	-68.99%	-79.71%
Plasma EPO	+ 44.27%	+ 84.93%	+ 72.43%	+ 35.01%	+ 30.81%	+ 18.66%	-25.38%	-50.34%	+ 7.66%	-23.08%	-46.84%	-61.6%
Pronephric EPO	+ 47.91%	+ 111.55%	+ 58.73%	+ 31.38%	+ 36.92%	+ 8.61%	-24.91%	-41.34%	+ 23.5%	-13.7%	-35.86%	-51.77%
Splenic EPO	+ 57.37%	+ 146.07%	+ 90.64%	+ 63.21%	+ 35.77%	+ 17.02%	-18.91%	-39.5%	+ 30.04%	-5.68%	-27.26%	-44.69%
T ₁ group- treatment group exposed to LC ₀ concentration; T ₂ group- treatment group exposed to LC ₅₀ concentration; T ₃ group- treatment group exposed to LC ₁₀₀ concentration. '+' denote increment in comparison with control group; '-' denote reduction in comparison with control group.												

Erythroblasts

Relative abundance of the earliest stage of the erythropoietic lineage, Proerythroblast (PRE) increased consistently throughout experimentation among the three treated groups. Relative abundance of the succeeding stages viz. Basophilic erythroblast (BE), Polychromatophilic erythroblast (PE) and Acidophilic erythroblast (AE) increased up to 18h of exposure in T₁ group; thereafter BE and PE decreased but AE increased. In the T₂ and T₃ groups, unlike that of the T₁ group PRE, BE, PE and AE reduced at all exposure period and PE remained unrecognized in T₃ group.

Reticulocytes

In T₁ group relative abundance of YR decreased throughout the experimentation, MR decreased up to 12h exposure but thereafter it increased. In the T₂ and T₃ groups, AE abundance decreased but YR and MR consistently increased.

Erythrocytes

Unlike the trend followed by reticulocytes, both YE and ME both showed a throughout decreasing trend among all the treated groups. YE remained unnoticed at 24h exposure in T₂ group and at 18h and 24h exposure in T₃ group.

Neutropoiesis

Relative abundance of the cells in neutropoietic lineage of the experimental fish has been presented in Fig. 10, Fig. 11 and Table 2.

Myeloblast (MB)

In the pronephric tissue and spleen the granulocyte committed progenitor cell MB increased consistently throughout the experimentation among all the treated groups.

Myelocytes

Promyelocyte (PM), the first committed stage in neutrophil lineage, declined consistently in all treated groups unlike MB.

Mesomyelocyte (MES), the first differential stage of the neutrophilic lineage, following a different trend unlike that of the PM and increased consistently in all the treated groups.

Metamyelocyte (MET), the penultimate neutropoietic stage, followed a similar pattern of MES, and increased consistently in all treated groups.

Mature neutrophils (MN)

Relative abundance of the ultimate stage of this lineage is mature neutrophils (MN), which declined consistently unlike that of MET in all the treated groups.

Erythropoietin (EPO) level

EPO levels of plasma, pronephric kidney and spleen presented in Fig. 9 and Table 2 showed a similar pattern with each other throughout the experimentation among all the treated groups. Plasma, pronephric and splenic EPO increased in T₁ group at all observation time points. In T₂ group, EPO levels of the three different tissues increased up to 12h exposure thereafter decreased at remaining observation time points. In T₃ group, EPO levels of the tissues initially increased up to 6h exposure, thereafter the values declined.

Discussion

The circulatory profile of *Channa punctatus* exhibits profound alterations under treatment with APP. Initial increases of TEC in the T₁ group up to 12h exposure was due to immediate mobilization of excess immature erythrocytes viz. YE and MR from the haematopoietic pool as it has been substantiated by differential count (Fig. 1 and Table 1). TEC showed a declining trend after this period of exposure but remained higher than the control value. Likewise, the Hct value also exhibited higher value throughout the experimentation. A significant loss of ME in this group of fish was evident throughout the experimentation and a concomitant increase in immature erythrocyte count to >4% at 24 h exposure was recorded. In control fish, immature erythrocyte count remains within 2%. Although YR significantly decreased with time of exposure (Fig. 1 and Table 1), it contributes >1% to TEC. The count of both the circulatory MR and YE enhanced at equal proportions and each showed >3 times increment. This phenomenon is accountable for erythrocytosis in this treatment group and can be interpreted as an effort to accelerate erythropoiesis to cope up with toxic stress. Erythropoietic acceleration is evident through a rise in EPO level in three different tissues viz. blood, pronephric kidney, spleen and concomitant alteration in erythropoietic dynamics. In this group, the relative abundance of all the blast cells of this lineage increased whereas the propenultimate MR, penultimate YE, and ultimate ME developmental stages decreased in the haematopoietic tissue for their mobilization into circulation.

In both T₂ and T₃ groups, erythropenia was evident throughout the experimental period due to regression of ME from circulation (Fig. 1 and Table 1) as evident through lower values of TEC and Hct. In the control group, a major contribution to TEC is ME which comprises >98% of the erythrocytes. In both T₂ and T₃ groups, the contribution of ME significantly declined to 87% and 45% in the respective groups. Whereas, the contribution of immature cells to TEC increased to >13% and >55% in the respective groups after 24 h of exposure. A circulatory deficient condition demands mobilization of cells from haematopoietic pool even before maturation, mobilization of MR and YE is confirmatory to that. Although in the T₁ group, both YE and MR equally mobilized in the circulation, in T₂ and T₃ groups circulatory MR increased >26 times and >154 times respectively. However, erythropoietic insufficiency is evident in both T₂ and T₃ groups with a decrease in the relative abundance of all the blast cell lineages except PRE. Although reticulocytes count increased, both the YE and ME significantly decreased and the fact can be interpreted as mobilization of MR prior to transformation into successive stages. Failure in the maintenance of EPO level and normal time lag for differentiation of PRE into erythroblasts, quick turnover of erythroblasts into reticulocytes, and physiological need based mobilization of immature cells in circulation ultimately jeopardized the erythropoietic dynamics of the fish under this acute toxic exposure.

Haemoglobin (Hb) content of the blood of all the treated groups of fish increased throughout the experimental period (Fig. 2 and Table 1). The derived variable MCV also significantly increased within 12 hours onward exposure. Within this period, the abundance of immature erythrocytes increased significantly in circulation and the fact is reflected in higher MCV (Fig. 2 and Table 1). The two other derived values MCH and MCHC are calculated at the higher side in all the treated groups. However, these results are unusual as the immature erythrocytes are supposed to have less haemoglobin. Such an unusual increase of these two parameters would not be directly correlated with the increase of Hb, TEC, and Hct because of the presence of serum Hb in all the treated groups as measured quantitatively (Fig. 2 and Table 1) and qualitatively (Fig. 4). Subtracted serum Hb gives a different result for both MCH and MCHC (Fig. 2 and Table 1). The only answer to the presence of Hb in plasma is leakage of it from the damaged cells. LC-ESI-MS analyses revealed seven triterpenoids saponin in APP (Saha and Saha 2022b) as the major secondary metabolite. Saponin interacts with both membrane lipids at their polar head region and cholesterol molecule through OH groups at C3 or C28. The hydrophobic aglycone backbone of the saponin molecule also intercalates into the hydrophobic interior of the lipid bilayer (Francis et al. 2002). Such interactions result in membrane destabilization which is evident through SEM studies of erythrocytes. A range of abnormalities is observed in the surface topography of erythrocytes including pore formation, membrane shrinkage, lyses, etc (Fig. 5) under APP treatment. In normal physiological conditions, Hb is released in plasma from the senescent RBCs. However, Hb does not accumulate in the plasma because it is converted into unconjugated (indirect) bilirubin after joining with albumin and finally released from the body through an excretory pathway after conversion into conjugated (direct) bilirubin into the liver (Dorcas and Solomon 2014; Hastuti et al. 2019). In all the treated groups plasma Hb levels increased significantly due to saponin-induced preterm RBC breakdown. In the T₁ group, both direct and indirect bilirubin concentration in serum increased concomitantly with an increase in serum Hb indicating restoration of hepatic function for conversion of Hb into bilirubin. However, in the T₂ and T₃ groups both forms of bilirubin decreased within 12h and 6h exposure respectively in the T₃ group both direct and indirect bilirubin concentrations declined more than the control value. Under exposure to higher APP concentration, hepatic ability for conversion of Hb into bilirubin declined and results in the accumulation of plasma Hb. This plasma

Hb has no importance in respiratory physiology and virtually an anaemic condition is developed in both the T₂ and T₃ groups. The anaemia developed in these treated groups could be categorized as oligocythemich hypochromic type.

Circulatory deficiency, particularly anaemia, develops tissue hypoxia. Hypoxia is known to induce *Epo* gene expression (Wang and Semenza 1996). Hypoxia-induced elevation of EPO level is known in fish like rainbow trout (Kakuta and Randall 2002; Datta et al. 2022). Erythropoietin is a glycoprotein hormone that regulates erythropoiesis in fish like other vertebrates by promoting the differentiation of erythroblasts from erythropoietic stem cells (Gordon et al. 1967) as reflected by an increase in PRE. In developing erythrocytes pyridoxal phosphate is activated by EPO and induces hemoglobin synthesis (Reddy et al. 1992) and the rapid transformation of blast cells into reticulocytes and immature erythrocyte is confirmatory to the fact. EPO levels in both circulation and haematopoietic tissue increased throughout the experimentation in the T₁ group and at the initial period of exposure in both the T₂ & T₃ groups. In the T₁ group acceleration in erythropoietic dynamics is evident from the increase in relative abundances of the blast cells and leads to greater mobilization of immature erythrocytes into circulation to cope up with the hypoxic condition developed. Hypoxia could lead to mobilization of immature RBCs in circulation of fish (Sawhney and Johal 2000) as a function of triggering the sympathetic system and production of adrenalin (Porges 1997). Acceleration of the erythropoiesis was also observed at the initial hours of treatment among the two other treated groups, but those fish ultimately failed to maintain *Epo* expression and consequently failed to restore their erythropoietic dynamics with increasing exposure time. The fish thus succumbs due to the loss of blood cells and their inability to restore erythropoiesis to compensate for that loss.

Total leukocyte count (TLC) increased significantly within 6h of exposure among all the treated groups. This increase in TLC could be interpreted as a defensive strategy of the fish to enhance the innate immunity of the fish. Similar phenomena have been found in exposure to different plant-derived active compounds viz. saponin (Ninomiya et al. 1995), glycyrrhizin (Jang et al. 1995), aloe (Kim et al. 1999), and azadirachtin (Harikrishnan et al. 2009). In exposure to saponin, the principal secondary metabolite content of APP (41%) can induce a cell-mediated immune system by producing cytokines like interleukins and interferons in mammals (Kensil 1996; Abe et al. 1982) as well as can enhance antibody production (Rajput et al. 2007). Saponin interacts with the lipoprotein domain of HDL/bGBP (b-glucan binding protein and high-density lipoprotein) in macrophages and haemocytes of fish which increased α2-Macrophage activity, respiratory burst, phagocytic activity with resistance against any infection in fish (Su and Chen, 2008). A strong adjuvant effect of saponin is known to induce both T cell-dependent as well as T cell-independent antigens. According to Barr et al. (1998) and Sjölander et al. (2001), saponin can interact with antigen-presenting cells through incorporation into cell or endosomal membranes after the formation of the pore through interaction with membrane cholesterol (Bangham et al. 1962) which might expose the incorporated antigen to cytosolic proteases. Saponin also induces strong cytotoxic CD8 + lymphocyte responses to induce cytotoxic T-lymphocyte (Kensil 1996). Differential leukocyte count was also found to be altered in circulation due to increased mobilization of neutrophils, small lymphocytes, and basophils from the haematopoietic pool of the T₁ group and only the basophils in the T₂ group. In the T₁ group neutrophils and lymphocyte counts consistently escalated to accomplish the phagocytic function and cell-mediated immunity of the fish. Consistently increased basophil in the blood of both the T₁ and T₂ groups again indicate rapid differentiation and mobilization of this cell type from the haematopoietic pool in response to inflammatory stimuli by the increment of inflammatory cytokines like IKK α, β, and γ. Among the secondary metabolites of APP, tannin also contributes a significant amount of 19.3% (Saha and Saha 2022b) which can increase the production of the majority of the pro-inflammatory cytokines leading to the signaling pathway for the production of basophils in fish (Li et al. 2020). Unlike mammalian basophils, fish basophil contains no heparin or histamine (Li et al. 2020) which could be correlated with the reaction cascade leading to inflammation (Jerzyńska et al. 2000). However, after 12h of exposure, TLC decreased in circulation than the control value. The differential count showed that TLC decreased because of the fact of decrease in eosinophil and large lymphocytes in the T₁ group; neutrophil, eosinophil, small lymphocyte, and large lymphocyte in the T₂ group; all the WBCs without thrombocyte in the T₃ group. Some of the leucocytes increased throughout the experimental period viz. neutrophil, small lymphocyte, basophil, and thrombocyte in the T₁ group, basophil, and thrombocyte in the T₂ group, and only thrombocyte in the T₃ group. After 12 h of exposure decline in TLC in all the treated groups indicates defence system of the fish is compromised in a time of exposure dependant manner. Thrombocytes showed a consistent increase in all the treated groups throughout the experimentation with an extreme condition in the T₃ group as a haemostatic mechanism to prevent blood loss due to branchial endothelial tissue damage. Jain (1986) reported that a reduction in eosinophil count may be a manifestation of toxic stress through stress hormone-sensitive cortisol production (Wojtaszek et al. 2002; Datta et al. 2021). Primary stress response with increased mucus secretion has been reported by Saha and Saha (2022a) in *Channa punctatus* under APP exposure. Neutrophil, the second largest contributor of TLC, showed a significant increase in the T₁ group but a decrease in both T₂ and T₃ groups. To interpret the result neutropoiesis study has been taken under consideration.

Neutropoiesis

Neutropoietic acceleration in the T₁ group is evident from the increase in the relative abundance of the cell types of this lineage viz. myeloblast (MB), mesomyelocyte (MES), and metamyelocyte (MET) in the haematopoietic tissue of the treated fish. A decrease in promyelocyte (PM) of this lineage indicates the need-based rapid transformation of this cell type into MES. A decrease in the ultimate developmental stage, mature neutrophil (MN) in the haematopoietic tissue indicates their rapid mobilization into the blood stream as it has been reflected in differential count. Like mammals, the most important cytokine stimulator of granulopoiesis in zebrafish is identified as the granulocyte-colony stimulating factor (GCSF) (Hermans et al. 2003). GCSF plays its role through both GCSFR-dependent and GCSFR-independent pathways and regulates the proliferation, survival, and differentiation of neutrophil precursors and thereby helping in neutropoiesis (Liongue et al. 2009). A complex network of transcription factors plays a critical role in the commitment of hematopoietic stem cells to the myeloid lineage by acting independently, co-operatively, or antagonistically (Havixbeck and Barreda 2015). Saponin a known immune stimulator in fish (Su and Chen 2008; Nadal et al. 2018) induces neutropoiesis in the T₁ group of *C. punctatus* to increase phagocytic cells in circulation to boost the defence mechanism. However, in both the T₂ and T₃ groups neutropenia was noticed though the relative abundance of MB, MES, and MET increased significantly like that of T₁. However, HSD Tukey's test indicates that the MET population becomes significantly higher in T₂ and T₃ groups in comparison to that of T₁. It indicates that at exposure to higher concentration, final stage of maturation from MET to MN is interrupted. Thus it can be interpreted that under toxic stress

the fish exhibit an effort to cope up with the situation by strengthening the defence system but when the level of stress increased further the defence mechanism is compromised.

Conclusion

The secondary metabolites of APP, particularly the triterpenoid saponin results in profound alterations in circulatory physiology of *Channa punctatus* with oligocythemic hypochromic anaemia. Mobilization of immature cells from haematopoietic tissue to circulation is evident among the treated fish. Hypoxia-induced *epo* gene expression through a rise in circulatory, pronephric, and splenic EPO levels and subsequent acceleration in erythropoiesis is evident in the fish at exposure to LC₀. However, at exposure to LC₅₀ and LC₁₀₀ APP erythropenia and erythropoietic insufficiency were observed due to the failure in maintenance of EPO level. Saponin is responsible for RBC membrane destabilization and exocytosis of haemoglobin in plasma which is enumerated as whole blood Hb content. However, the conversion rate of plasma Hb into bilirubin is a factor of time and results in the retention of Hb in plasma. Acute toxic exposure also resulted in neutrocytosis as an immediate defensive mechanism. Thrombocytosis is another circulatory outcome manifested in the exposed fish as a haemostatic mechanism to check blood loss through ruptured capillaries of the gill. The Outcome of this experiment provides evidences in support of the piscicidal efficiency and suitability of APP in pre-stocking pond management for composite fish culture.

Declarations

Author contribution

Samar K Saha conceptualized the study and edited the manuscript. Beauty Saha designed and performed the experiments; generated, analysed and validated data; prepared the draft manuscript. Funding acquisition was done by Beauty Saha. Project administration and Supervision were done by Samar K Saha. All authors read and approved the final manuscript.

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Data availability

The datasets provided will be available from the corresponding author on reasonable request.

Ethical approval

Animal maintenance and experimental procedures were in accordance with the animal welfare laws, guidelines, and policies of the Institutional Animal Ethics Committee of Visva-Bharati (IAEC approval number: IAEC/III-19/ 2020).

With the submission of this manuscript "Circulatory physiology and haematopoiesis of *Channa punctatus* under piscicidal toxicity of *Acacia auriculiformis* pericarp" I would like to confirm that the above-mentioned manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institutes' (Visva Bharati University) representatives are fully aware of this submission.

Consent for publication

All authors agree to submit the manuscript entitled "Circulatory physiology and haematopoiesis of *Channa punctatus* under piscicidal toxicity of *Acacia auriculiformis* pericarp" to Fish Physiology and Biochemistry for publication consideration.

Competing interests

The authors declare no competing interests

References

1. APHA (1998) Standard Methods for the Examination of Water and Waste Water. American Public Health Association, American Water Works Association and Water Pollution Control Federation, twentyth ed. Washington.
2. Bhatia HL (1970) Use of mahua oil cake in fishery management. Indian Farming 20: 39-40.
3. Abe N, Ebina T, Ishida N (1982) Interferon induction by glycyrrhizin and glycyrrhetic acid in mice. Microbiol Immunol 26(6): 535-539. <https://doi.org/10.1111/j.1348-0421.1982.tb00207.x>.
4. Bangham AD, Horne RW, Glauert AM, Dingle JT, Lucy JA (1962) Action of saponins on biological membranes. Nature 196(4858): 952-955. <https://doi.org/10.1038/196952a0>.
5. Barr IG, Sjolander A, Cox JC (1998) ISCOMs and other saponin based adjuvants. Adv Drug Deliv Rev 32: 247-271. [https://doi.org/10.1016/S0169-409X\(98\)00013-1](https://doi.org/10.1016/S0169-409X(98)00013-1).
6. Blaxhall PC, Daisley KW (1973) Routine haematological methods for use with fish blood. J Fish Biol 5(6): 771-781. <https://doi.org/10.1111/j.1095-8649.1973.tb04510.x>.

7. Choi S, Jung SY, Kim CH, Kim HS, Rhim H, Kim SC, Nah SY (2001) Effect of Ginsenosides on voltage-dependent Ca²⁺ channel subtypes in bovine chromaffin cells. *J Ethnopharmacol* 74: 75-81. [https://doi.org/10.1016/S0378-8741\(00\)00353-6](https://doi.org/10.1016/S0378-8741(00)00353-6).
8. Dacie JV, Lewis SM (1984) Practical haematology. 5th edn. Churchill Livingstone, London.
9. Dacie JV, Lewis SM (2001) Practical haematology. 9th edn. Churchill Livingstone, London, pp 633.
10. Datta N, Kar PK, Saha SK (2021) Circulatory physiology and erythropoiesis in freshwater fish *Labeo rohita* experimentally parasitized by *Argulus bengalensis*. *J Appl Ichthyol* 38: 63-72. <https://doi.org/10.1111/jai.14287>.
11. Davis BJ (1964) Disc electrophoresis—II method and application to human serum proteins. *Ann NY Acad Sci* 121(2): 404-427. <https://doi.org/10.1111/j.1749-6632.1964.tb14213.x>.
12. Dheer JMS, Dheer TR, Mahajan CL (1986) Haematological and haematopoietic response to sodium chloride stress in a freshwater air-breathing fish *Channa punctatus* Bloch. *J Fish Biol* 28(1): 119-128. <https://doi.org/10.1111/j.1095-8649.1986.tb05148.x>.
13. Dorcas IK, Solomon RJ (2014) Calculation of liver function test in *Clarias gariepinus* collected from three commercial fish ponds. *Nat Sci* 12(10): 107-123.
14. Eeckhaut I, Caulier G, Brasseur L, Flammang P, Gerbaux P, Parmentier E (2015) Effects of holothuroidichtyotoxicsaponins on the gills of free-living fishes and symbiotic pearlfishes. *Biol Bull* 228 (3): 253-265.
15. El Izzi A, Benie T, Thieulant ML, Men-Oliver LL, Duval J (1992) Stimulation of LH release from cultured pituitary cells by saponins of *Petersianthus macrocarpus*: a permeabilising effect. *Planta Med* 58(3): 229-233. <https://doi.org/10.1055/s-2006-961441>.
16. Francis G, Kerem Z, Makkar HPS, Becker K (2002) The biological action of saponins in animal system: a review. *Br J Nutr* 88(6): 587-605. <https://doi.org/10.1079/BJN2002725>.
17. Gordon AS, Cooper GW, Zanjani ED (1967) The kidney and erythrooiesis. *Semin. Hematol* 4(4): 337-358.
18. Harikrishnan R, Balasundaram C, Dharaneedharan S, Moon YG, Kim MC, Kim JS, Heo MS (2009) Effect of plant active compounds on immune response and disease resistance in *Cirrhina mrigala* infected with fungal fish pathogen, *Aphanomyces invadans*. *Aquac Res* 40(10): 1170-1181. <https://doi.org/10.1111/j.1365-2109.2009.02213.x>.
19. Hastuti S, Subandiyono S, Windarto S (2019) Blood performance of jaundice catfish *Clarias gariepinus*. *AACL Bioflux* 12(2): 480-489.
20. Havixbeck JJ, Barreda DR (2015) Neutrophil Development, Migration, and Function in Teleost. *Biology* 4(4): 715-734. <http://dx.doi.org/10.3390/biology4040715>.
21. Hermans MHA, van de Geijn GJ, Antonissen C, Gits J, van Leeuwen D, Ward AC, Touw IP (2003) Signaling mechanisms coupled to tyrosines in the granulocyte colony-stimulating factor receptor orchestrate G-CSF-induced expansion of myeloid progenitor cells. *Blood* 101(7): 2584-2590. <https://doi.org/10.1182/blood-2002-07-2062>.
22. Hesser EF (1960) Methods for routine fish hematology. *Prog Fish Cult* 22(4): 164-171. [https://doi.org/10.1577/1548-8659\(1960\)22\[164:MFRFH\]2.0.CO;2](https://doi.org/10.1577/1548-8659(1960)22[164:MFRFH]2.0.CO;2).
23. Jain NC (1986) Schalm's veterinary hematology. Lea and Febiger, 4th edn. Philadelphia.
24. Jang SI, Marsden MJ, Kim YG, Choi MS, Secombes CJ (1995) The effect of glycyrrhizin on rainbow trout, *Oncorhynchus mykiss* (Walbaum), leucocyte responses. *J Fish Dis* 18(4): 307-315. <https://doi.org/10.1111/j.1365-2761.1995.tb00307.x>.
25. Jendrassik L, Grof P (1938) Vereinfachte photometrische Methoden zur Bestimmung des blutbilirubins. *Biochem Z* 297: 81-89.
26. Jerzyńska J, Stelmach I, Kuna P (2000) The role of heparin in allergic inflammation. *Pol Merkur Lekarski* 8(47): 341-346.
27. Kakuta I, Randall D (2002) Effects of hypoxia on erythropoietin levels in trout kidney and spleen. In *Responses of Fish to Aquatic Hypoxia*. International Congress on the Biology of Fish. pp. 15-20.
28. Kensil CR (1996) Saponins as vaccine adjuvant. *Crit Rev Ther Drug Carrier Syst* 13(1-2): 1-55.
29. Kim KH, Hwang YJ, Bai SC (1999) Resistance to *Vibrio alginolyticus* in juvenile rockfish (*Sebastes schlegelii*) fed diets containing different doses of aloe. *Aquaculture* 180(1-2): 13-21. [https://doi.org/10.1016/S0044-8486\(99\)00143-X](https://doi.org/10.1016/S0044-8486(99)00143-X).
30. Kulkeaw K, Sugiyama D (2012) Zebrafish erythropoiesis and the utility of fish as models of anemia. *Stem Cell Res Ther* 3(55): 1-11. <http://dx.doi.org/10.1186/scrt146>.
31. Li M, Feng L, Jiang WD, Wu P, Liu Y, Jiang J, Zhou XQ (2020) Condensed tannins decreased the growth performance and impaired intestinal immune function in on-growing grass carp (*Ctenopharyngodon idella*). *Br J Nutr* 123(7): 737-755. <https://doi.org/10.1017/S0007114519003295>.
32. Liongue C, Hall CJ, O'Connell BA, Crosier P, Ward AC (2009) Zebrafish granulocyte colony-stimulating factor receptor signaling promotes myelopoiesis and myeloid cell migration. *Blood* 113: 2535-2546. <https://doi.org/10.1182/blood-2008-07-171967>.
33. Mahajan CL, Dheer JS (1979) Cell types in the peripheral blood of an air-breathing fish *Channa punctatus*. *J Fish Biol* 14(5): 481-487. <https://doi.org/10.1111/j.1095-8649.1979.tb03546.x>.
34. Mahajan CL, Dheer JMS (1980a) Origin and Development of neutrophils in an air breathing fish, *Channa punctatus* Bloch. *Acta Zool* 61: 221-224. <https://doi.org/10.1111/j.1463-6395.1980.tb01310.x>.
35. Mahajan CL, Dheer JMS (1980b) An autoradiographic and cytochemical study of erythropoiesis in a fresh water fish, *Channa punctatus* Bloch. *J Fish Biol* 17: 641-648. <https://doi.org/10.1111/j.1095-8649.1980.tb02797.x>.
36. Maiti M, Saha SK, Homechaudhuri S, Banerjee S (2000) Characterization of erythropoietic cells in *Oreochromis niloticus* (L) and *Cirrhinus mrigala* (HAM). *J Aquacult Trop* 15(1): 81-90.
37. Malik N, Berrie A (1972) New staining fixative for proteins separated by gel isoelectric focusing, based on coomasie brilliant blue. *Anal Biochem* 49(1): 173-176. [https://doi.org/10.1016/0003-2697\(72\)90255-2](https://doi.org/10.1016/0003-2697(72)90255-2).

38. McLeay DJ, Gordon MR (1977) Leucocrit: A simple hematological technique for measuring acute stress in salmonid fish, including stressful concentrations of pulpmill effluent. J Fish Res Board Can 34(11): 2156-2163. <https://doi.org/10.1139/f77-284>.
39. Menin L, Panchichkina M, Keriel C, Olivares J, Braun U, Seppert EK, Saks VA (2001) Macrocompartmentation of total creatine in cardiomyocytes revisited. Mol Cell Biochem 220: 149-159. <https://doi.org/10.1023/A:1010960309898>.
40. Nadal AL, Peggs D, Wiegertjes GF, Brugman S (2018) Exposure to antibiotics affects saponin immersion-induced immune stimulation and shift in microbial composition in zebrafish larvae. Front Microbiol 9: 2588. <https://doi.org/10.3389/fmicb.2018.02588>.
41. Neiffer DL, Stamper MA (2009) Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. ILAR J 50(4): 343-360. <https://doi.org/10.1093/ilar.50.4.343>.
42. Ninomiya M, Hatta H, Fujiki M, Kimi M, Yamamoto T, Kusuda R (1995) Enhancement of chemotactic activity of yellowfish (*Seriola quinqueradiata*) leucocytes by oral administration of quillaja saponin. Fish Shellfish Immunol 5(4): 325-328. <https://doi.org/10.1006/fsim.1995.0031>.
43. O'Brien BRA (1961) Identification of haemoglobin by its catalase reaction with peroxide and o-dianisidine. Stain Technol 36(2): 57-61. <https://doi.org/10.3109/10520296109113244>.
44. Porges SW (1997) Emotion: An evolutionary by-product of the neural regulation of the autonomic nervous system. Ann N Y Acad Sci 807: 62-77.
45. Rajput ZI, Hu SH, Xiao CW, Arijó AG (2007) Adjuvant effects of saponins on animal immune responses. J Zhejiang Univ Sci B 8(3): 153-161. <https://doi.org/10.1631/jzus.2007.B0153>.
46. Reddy DC, Vijayakumari P, Kalarani V, Davies RW (1992) Changes in erythropoietic activity of *Sarotherodon mossambicus* exposed to sublethal concentrations of the herbicide diuron. Bull Environ Contam Toxicol 49(5): 730-737. <https://doi.org/10.1007/BF00200787>.
47. Rickard PP, Cox PA (1986) Use of Derris as a fish poison in Gadualcanal, Solomon Islands. Econ Bot 40(4): 479-484. <https://doi.org/10.1007/BF02859661>.
48. Sadeghi P, Kazerooni F, Savari A, Movahedinia A, Safahieh A, Ajdari D (2015) Application of biomarkers in Epaulet grouper (*Epinephelus stoliczkae*) to assess chromium pollution in the Chabahar Bay and Gulf of Oman. Sci Total Environ 518: 554-561. <https://doi.org/10.1016/j.scitotenv.2015.03.017>.
49. Saha B, Saha SK (2022a) *Acacia auriculiformis* pericarp- a substitute of Mohua oil cake as piscicide in pre-stocking pond preparation. Aquaculture 548: 737586. <https://doi.org/10.1016/j.aquaculture.2021.737586>.
50. Saha B, Saha SK (2022b) Phytochemical Analysis of *Acacia auriculiformis* Pericarp through Liquid Chromatography-Electrospray Ionization-Mass Spectrometry. Indian J Pharm Sci 84(3): 660-668. [10.36468/pharmaceutical-sciences.961](https://doi.org/10.36468/pharmaceutical-sciences.961).
51. Sawhney AK, Johal MS (2000) Erythrocyte alterations induced by malathion in *Channa punctatus* (Bloch). Bull Environ Contam Toxicol 64(3): 398-405. <https://doi.org/10.1007/s001280000014>.
52. Seeman P, Cheng D, Iles GH (1973) Structure of membrane holes in osmotic and saponin hemolysis. J Cell Biol 56: 519-527. <https://doi.org/10.1083/jcb.56.2.519>.
53. Stetter MD (2001) Fish and Amphibian Anesthesia. Vet Clin North Am Exot Anim Pract 4(1): 69-82. [https://doi.org/10.1016/S1094-9194\(17\)30052-X](https://doi.org/10.1016/S1094-9194(17)30052-X).
54. Sjölander A, Drane D, Maraskovsky E, Scheerlinck J, Suhrbier A, Tennent J, Pearce M (2001) Immune responses to ISCOM formulations in animal and primate models. Vaccine 19(17-19): 2661-2665. [https://doi.org/10.1016/S0264-410X\(00\)00497-7](https://doi.org/10.1016/S0264-410X(00)00497-7).
55. Su BK, Chen JC (2008) Effect of saponin immersion on enhancement of the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Fish Shellfish Immunol 24(1): 74-81. <https://doi.org/10.1016/j.fsi.2007.09.002>.
56. Tak T, Tesselaar K, Pillay J, Borghans JAM, Koenderman L (2013) What's your age again? determination of human neutrophil half-lives revisited. J Leukoc Biol 94: 595-601. <https://doi.org/10.1189/jlb.1112571>.
57. Wang GL, Semenza GL (1996) Molecular basis of hypoxia-induced erythropoietin expression. Curr Opin Hematol 3(2): 156-162. <https://doi.org/10.1097/00062752-199603020-00009>.
58. Wojtaszek J, Dziejulska-Szwajkowska D, Lozinska-Gabska M, Adamowicz A, Dzugaj A (2002) Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): cortisol effect on the carp blood. Gen Comp Endocrinol 125: 176-183. <https://doi.org/10.1006/gcen.2001.7725>.

Figures

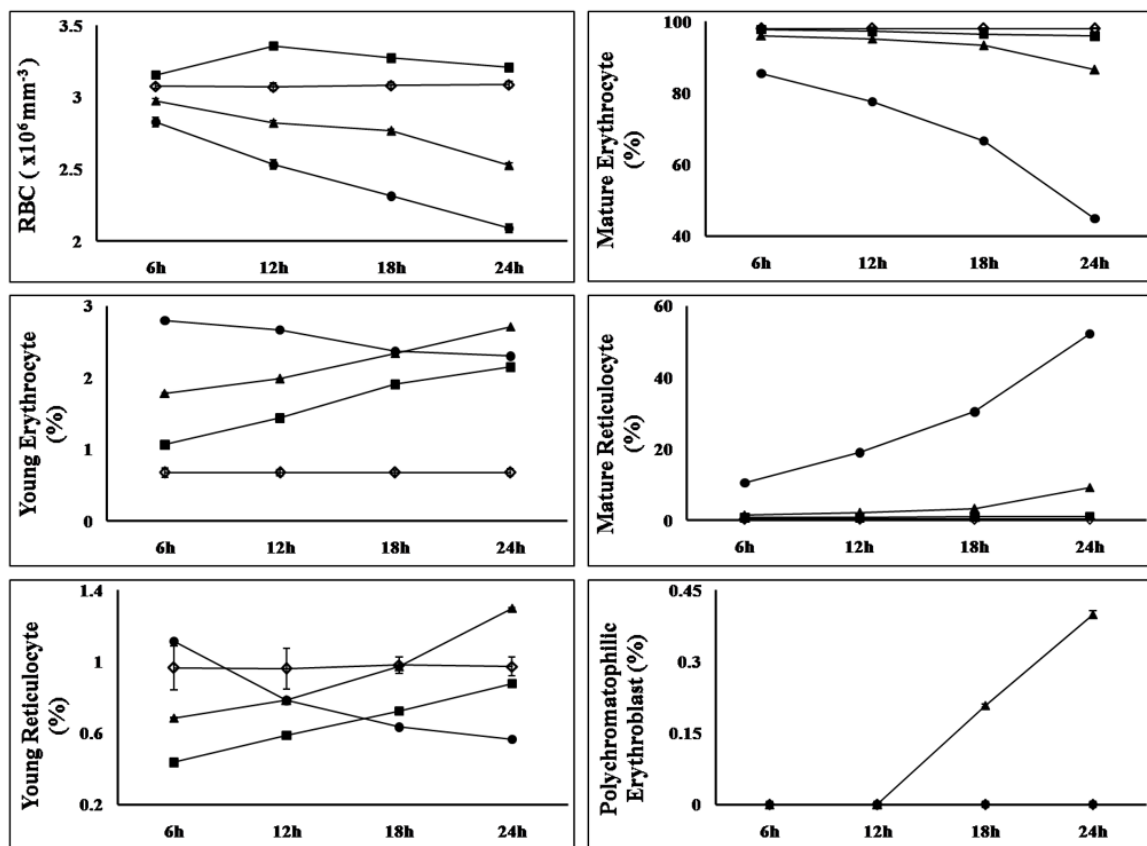


Fig. 1 Total and differential erythrocyte count of *Channa punctatus* under lethal and sublethal exposure to APP.

Control —◆— ; LC₀ exposure —■— ; LC₅₀ exposure —▲— ; LC₁₀₀ exposure —●—

Figure 1

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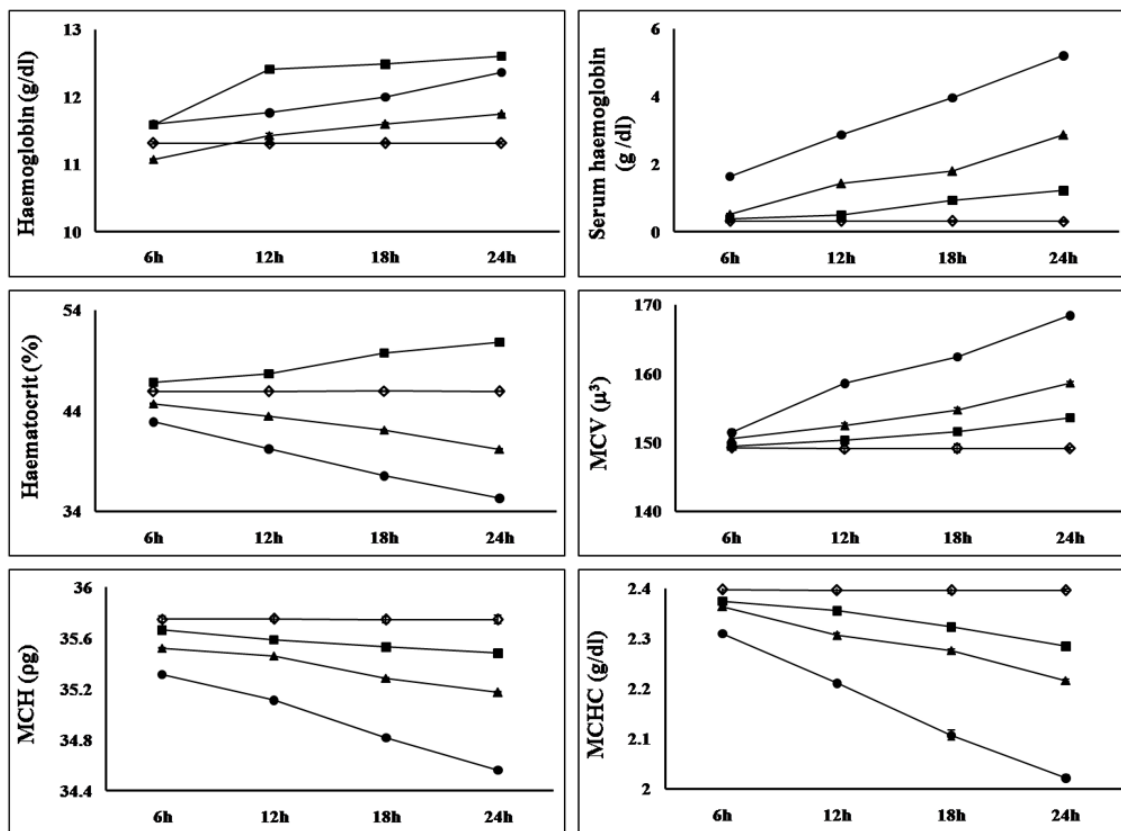


Fig. 2 Haematological parameters from peripheral blood of *Channa punctatus* under lethal and sublethal exposure to APP. Control ◇ ; LC₀ exposure ■ ; LC₅₀ exposure ▲ ; LC₁₀₀ exposure ●

Figure 2

See image above for figure legend.

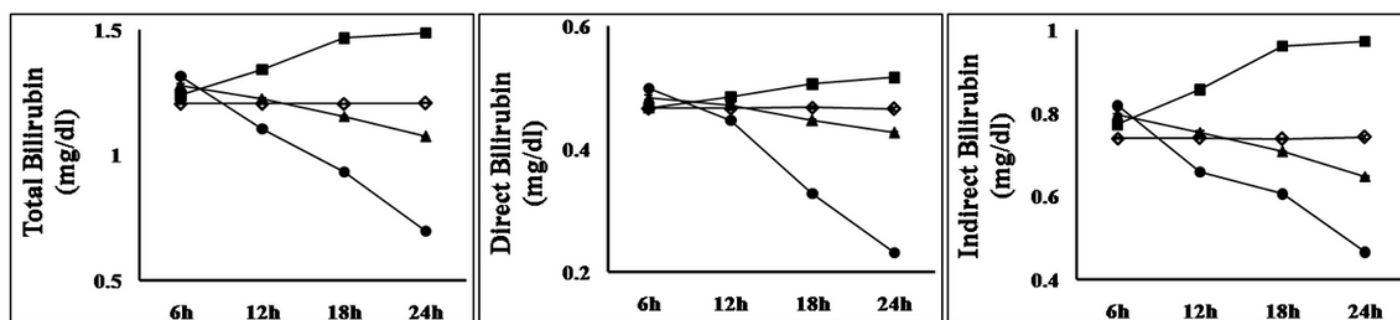


Fig. 3 Total bilirubin, direct and indirect bilirubin concentration present in peripheral blood of *Channa punctatus* under lethal and sublethal exposure to APP. Control ◇ ; LC₀ exposure ■ ; LC₅₀ exposure ▲ ; LC₁₀₀ exposure ●

Figure 3

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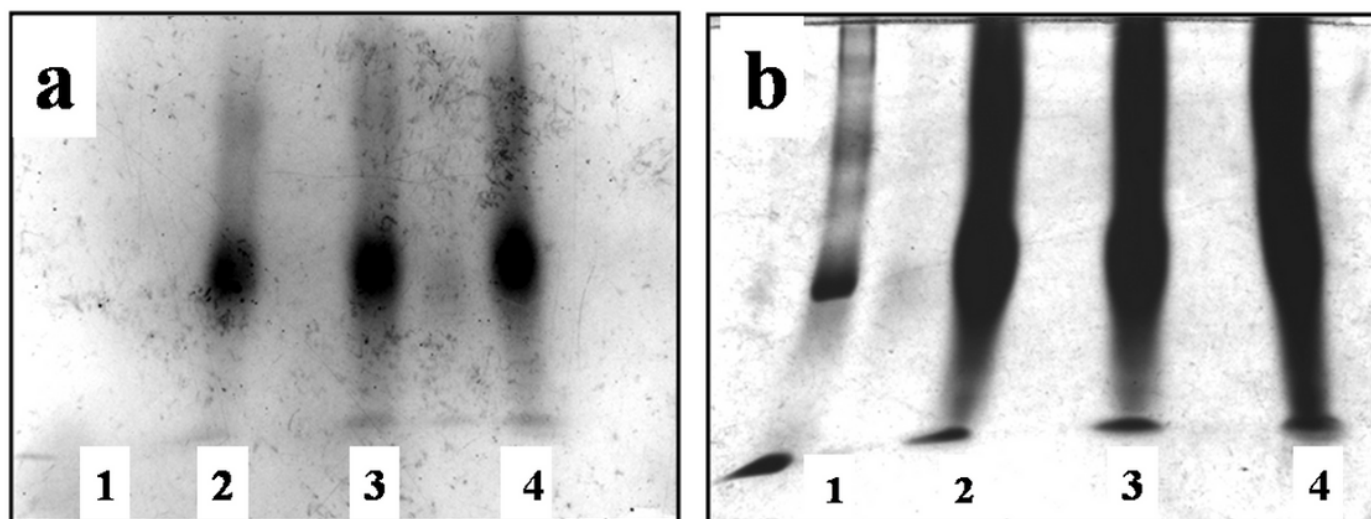


Figure 4

Native PAGE photograph of serum of *Channa punctatus* under lethal and sublethal exposure to APP. (A) stained with haemoglobin specific peroxidise stain o-Dianisidine, (B) stained with Coomassie brilliant blue R250. Lane 1 - Control fish; Lane 2- LC₀ exposure fish; Lane 3- LC₅₀ exposure fish; Lane 4- LC₁₀₀ exposure

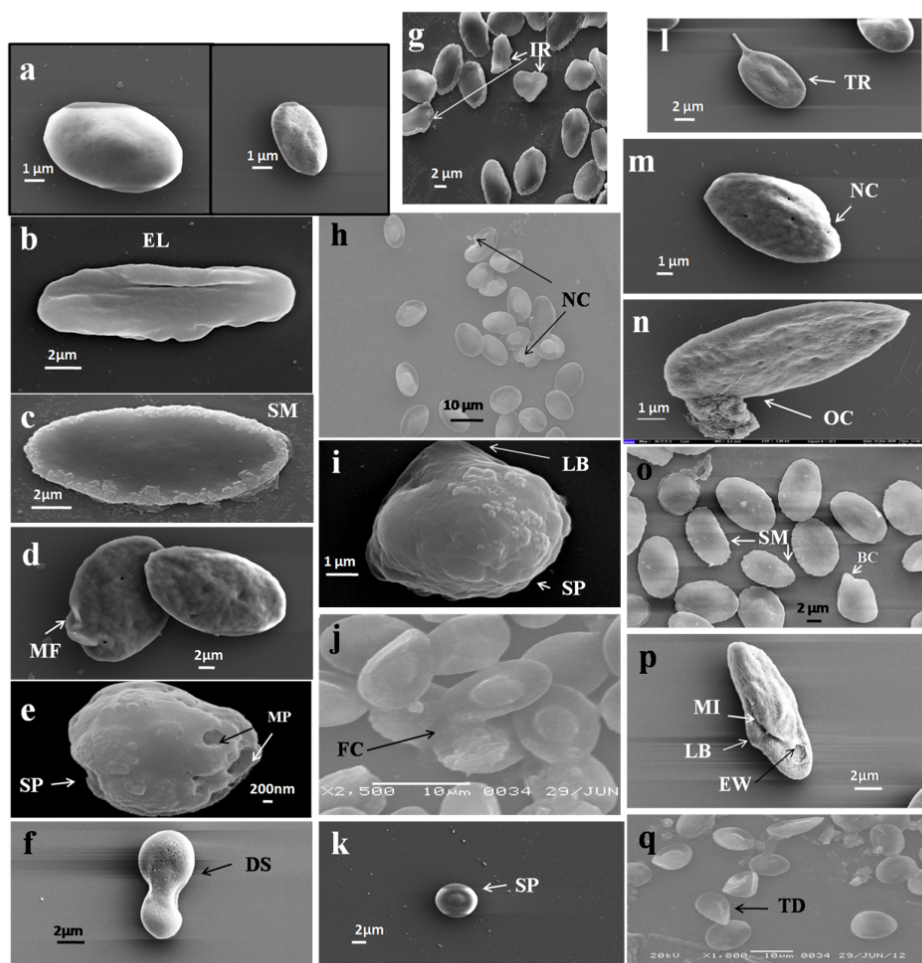


Figure 5

Scanning electron microscopy (SEM) of erythrocytes of *Channa punctatus* under lethal and sublethal exposure to APP. (a) control; (b-q) APP exposed; IR- irregular shape cells, NC- notched cell, EL- elongated cell, BC- blebed cell, SP- spherocytes, LB- lobopodial projections, DS- dumbbell shaped cell, SM- cells having serrated margins, MP- membrane pores, MF- marginal folding, OC- oozed out of cytoplasmic content, FC- fused cells, MI- membrane internalization, EW- elongated membrane whole, TD- tear drop, TR- tennis racket shaped

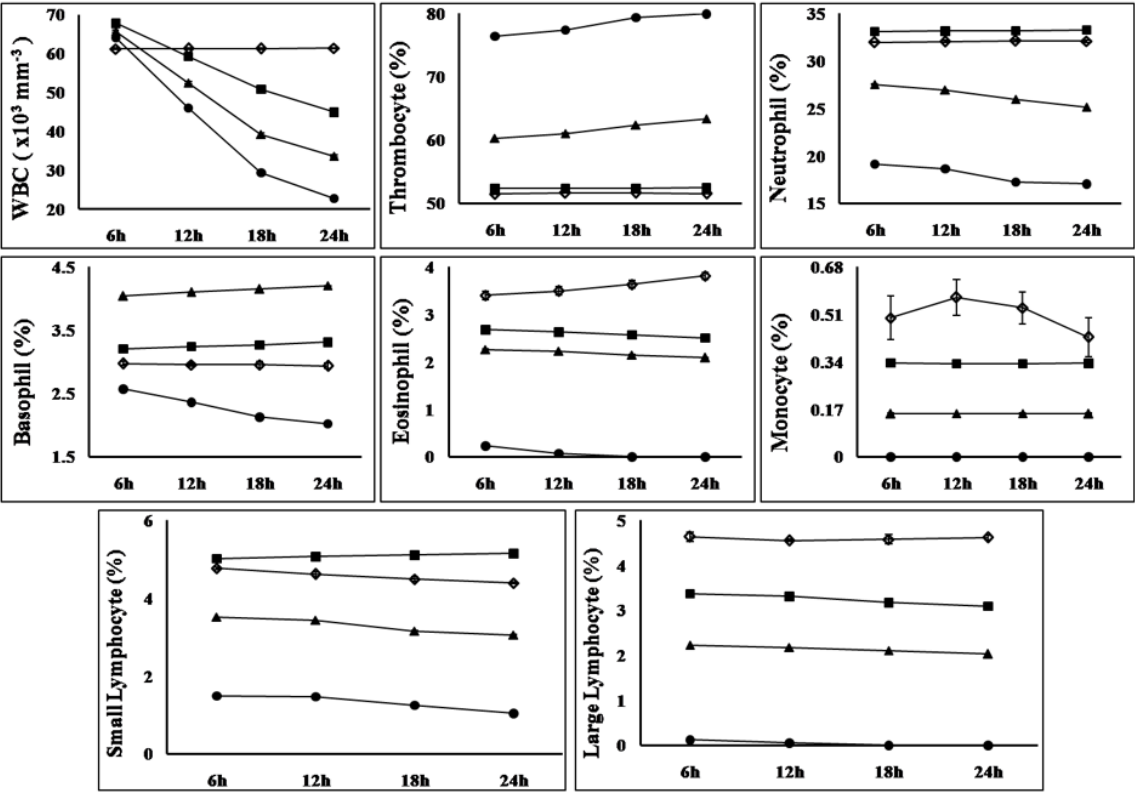


Fig. 6 Total and differential leucocyte count of *Channa punctatus* under lethal and sublethal exposure to APP.
Control ◇ ; LC₀ exposure ■ ; LC₅₀ exposure ▲ ; LC₁₀₀ exposure ●

Figure 6
See image above for figure legend.

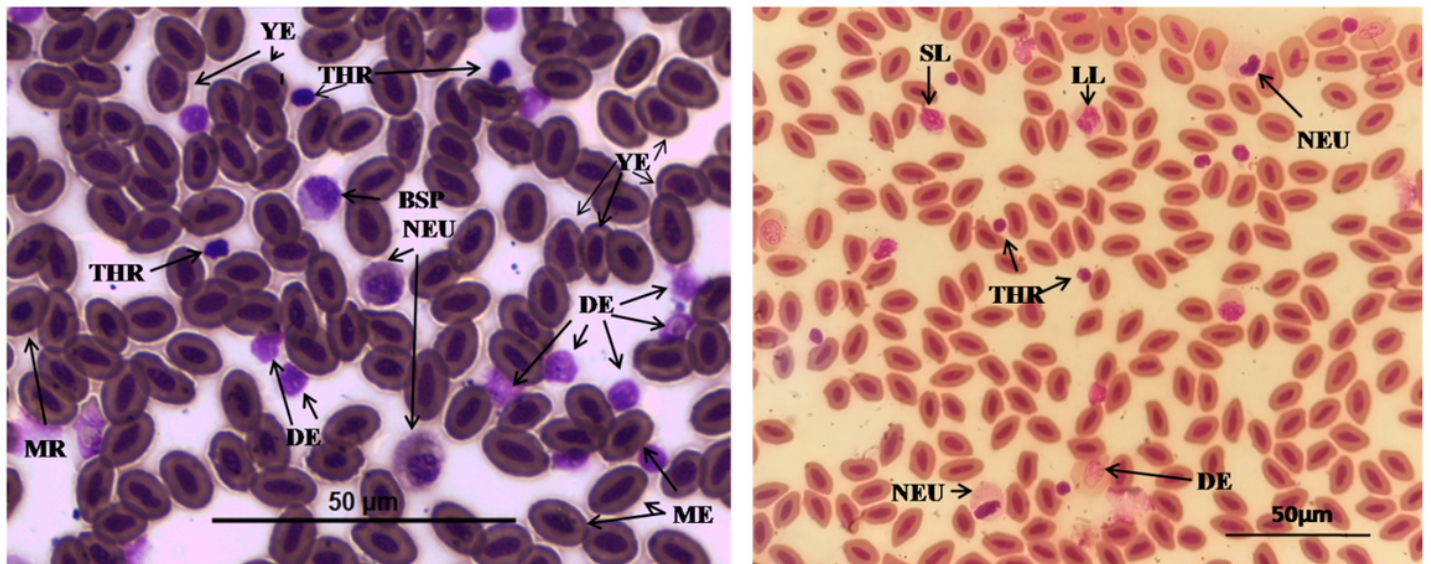


Figure 7

Light microscopy of the circulatory cells of *Channa punctatus* under lethal and sublethal exposure to APP (o-Dianisidine and Giemsa staining). YE- Young erythrocyte, ME- Mature erythrocyte, MR- Mature reticulocyte, DE- Destroying erythrocyte, THR- Thrombocyte, NEU- Neutrophil, BSP- Basophil, SL- Small lymphocyte, LL- Large lymphocyte

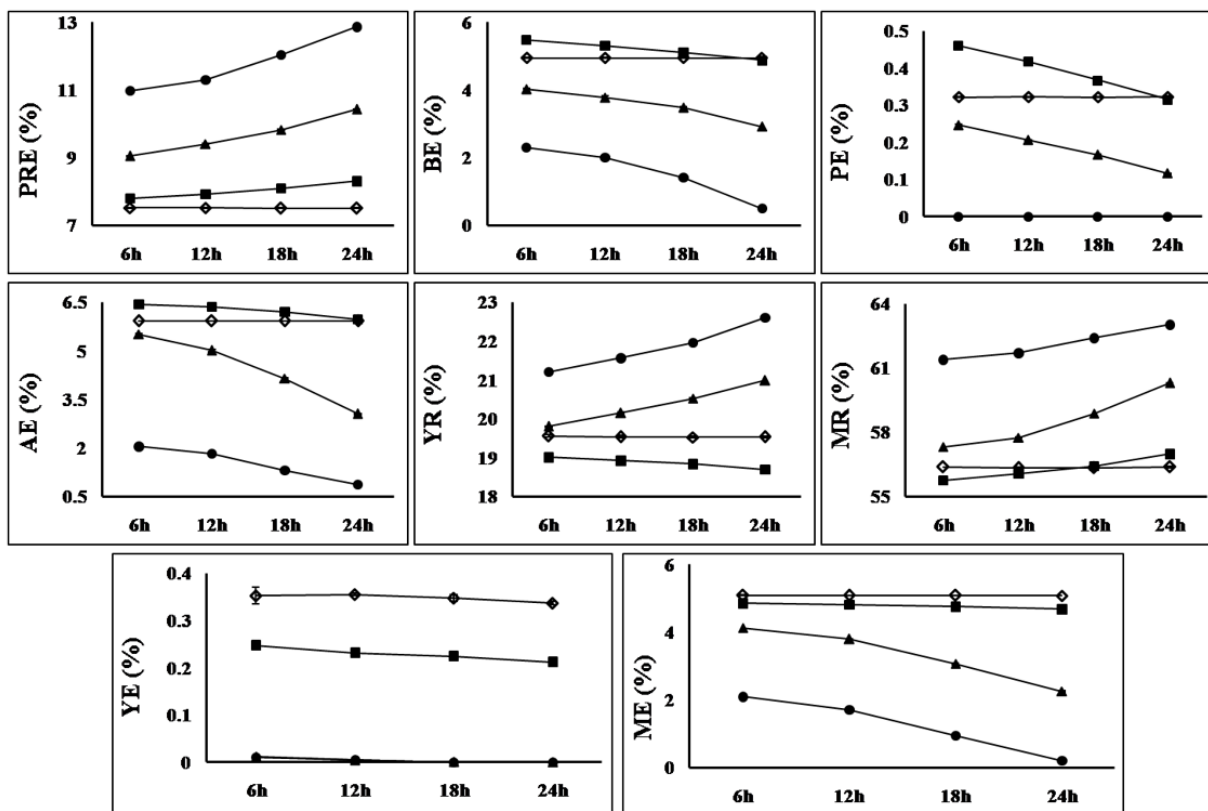


Fig. 8 Erythropoietic lineages from pronephric kidney of *Channa punctatus* under lethal and sublethal exposure to APP. Control ◇ ; LC₀ exposure ■ ; LC₅₀ exposure ▲ ; LC₁₀₀ exposure ●

Figure 8

See image above for figure legend.

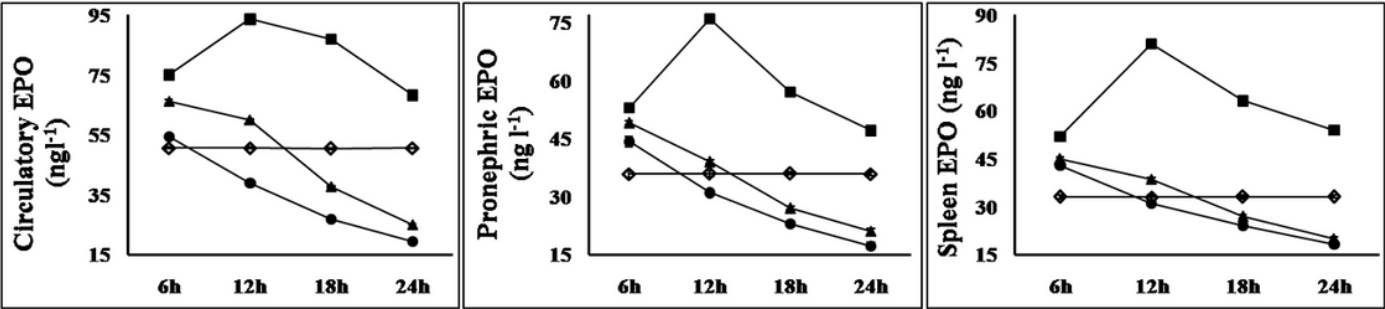


Fig. 9 Circulatory, pronephric and splenic EPO concentration of *Channa punctatus* under lethal and sublethal exposure to APP. Control ◇ ; LC₀ exposure ■ ; LC₅₀ exposure ▲ ; LC₁₀₀ exposure ●

Figure 9

See image above for figure legend.

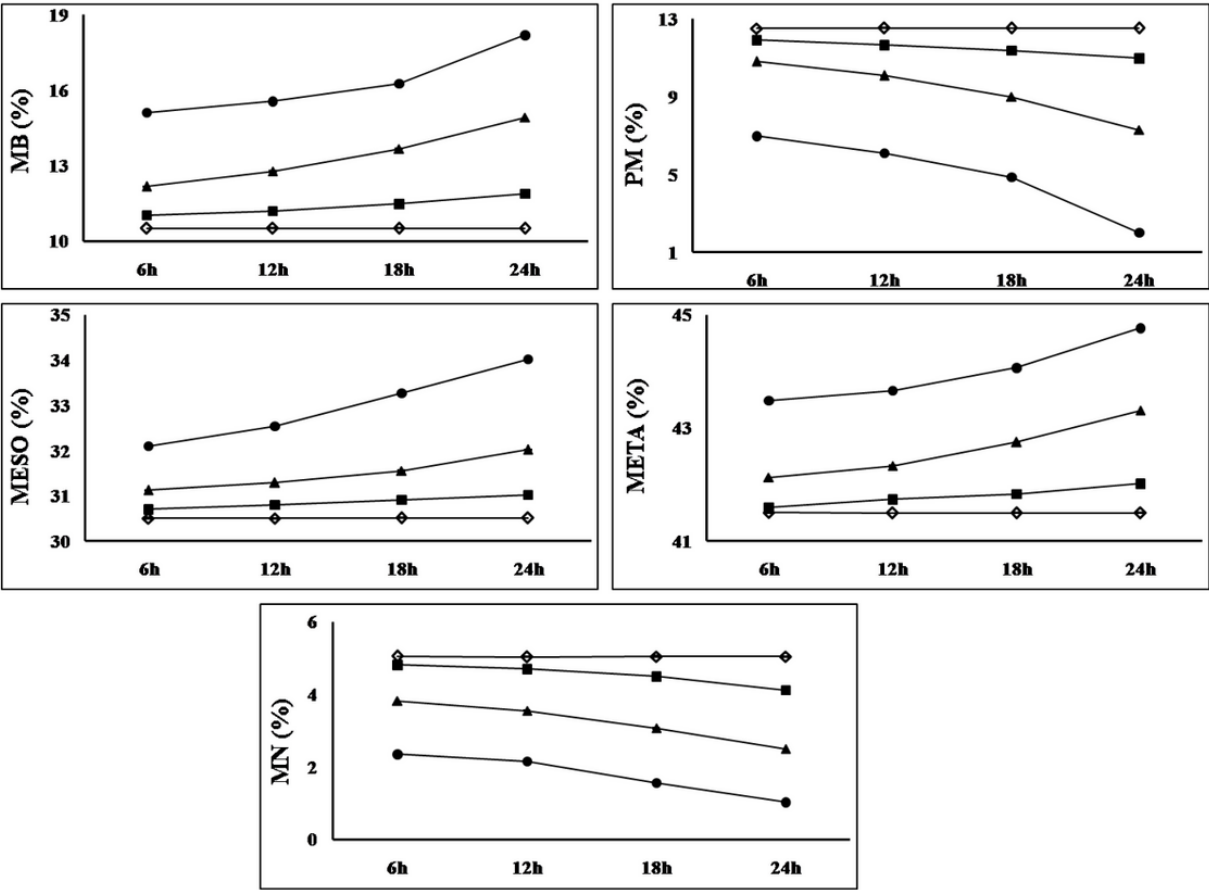


Fig. 10 Neutropoietic lineages from pronephric kidney of *Channa punctatus* under lethal and sublethal exposure to APP. Control ◇ ; LC₀ exposure ■ ; LC₅₀ exposure ▲ ; LC₁₀₀ exposure ●

Figure 10

See image above for figure legend.

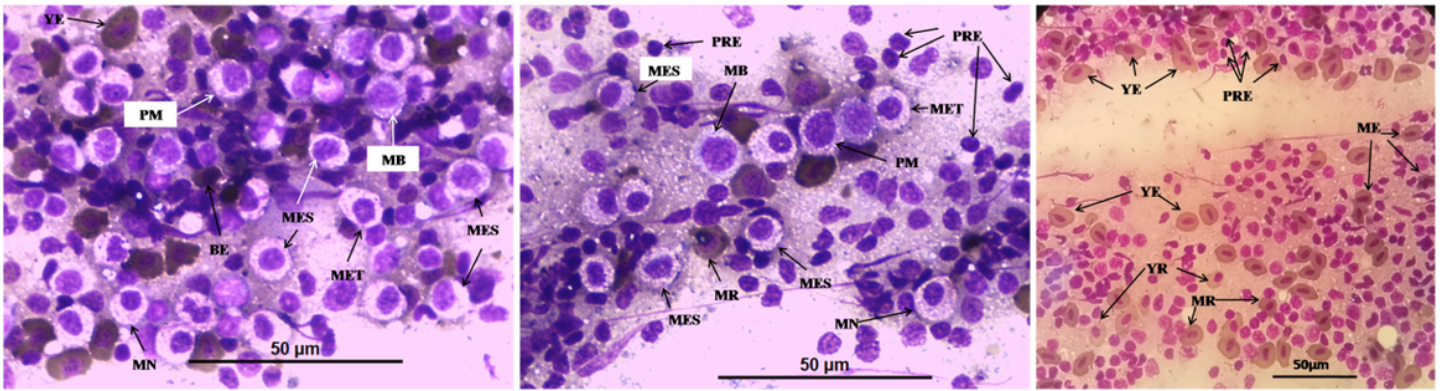


Figure 11

Light microscopy of haematopoietic lineage in pronephric kidney and spleen of *Channa punctatus* under lethal and sublethal exposure to APP (o-Dianisidine and Giemsa staining). PRE- Proerythroblast, BE- Basophilic erythroblast, YR- Young reticulocyte, MR- Mature reticulocyte, YE- Young erythrocyte, ME- Mature erythrocyte, MB - Myeloblast, PM- Promyelocyte, MES- Mesomyelocyte, MET- Metamyelocyte, MN- Mature neutrophil