Hematological responses of common carp *Cyprinus carpio* administered with Neem (*Azadirachta indica*) leaf extraction

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**ABSTRACT**

The study investigated the effect of Neem Leaf Extract (NLE)-a herbal product on hematological parameters of common carp, *Cyprinus carpio*. Common carp fry were fed on diets having different levels of NLE [no NLE (D1), 0.25(D2), 0.5(D3), 1.0(D4), 1.5(D5), 2.0(D6) g/kg in basal diet containing rice bran and mustard] for 180 days in outdoor tanks. The results revealed an increase in TEC, TLC and PCV in groups D2-D6 compared to control (P<0.05), but increased PCV was observed only in D2, D3 and D4 groups. Further, increasing trend for Hb content was observed from D2-D6 irrespective of NLE levels. The MCV values decreased with increase in levels of NLE in contrast to MCHC, whereas MCH considerably varied among the treatments. Significantly increased total protein and globulin was also observed while albumin to globulin ratio did not show any response in any of the treatments. Collectively, these observations support the hypothesis that the response of hematological parameters to NLE treatment is regulated via both positive and negative feedback mechanisms, with differential modulations based on the NLE levels. The results imply that NLE can boost the immune system via hematological parameters by increasing TEC, TLC, PCV, Hb at certain levels.

**Key words:** *Azadirachta indica*, Aquaculture, Common carp, Hematology, Herbal extract.

**INTRODUCTION**

Aquaculture has a potential to preserve aquatic biodiversity by decreasing the pressure on wild stocks and producing animal protein for growing global population (Bostock *et al*., 2010). Aquaculture industry in India is mainly carp based and it has made a long leap of over tenfold increase in last five decades. Currently, the inland sector is growing more rapidly than marine sector and 95% of inland production comes from aquaculture (Katiha *et al*., 2005).

With increasing intensity of culture practices, and profitability pressures in farms, incidences of disease outbreaks have also increased, resulting in poor survival and growth ultimately leading to lower profits. Factors such as overcrowding, periodic handling, high or sudden changes in temperature, poor water quality, and poor nutritional status contribute to physiological changes such as stress or immunosuppression and thus, heighten susceptibility to infection.

To overcome this problem, various prophylactic and chemotherapeutic agents such as antibiotics and disinfectants have been increasingly used for disease and health management in aquaculture. In order to avoid economic losses, several antimicrobials drugs are administered regularly in fish food or sometimes in baths and injections (Rico *et al*., 2013). Improper and continuous use of these products potentially lead to development of antibiotic resistance in bacteria, immune-suppression, environmental pollution and the accumulation of residues in fishes (Syahidah *et al*., 2015) and hence are not recommended. Therefore, scientists have intensified efforts to exploit natural products such as herbs in developing alternative dietary supplements that enhance growth, health and immune system of fish.

Among various potential herbs, neem, *Azadirachta indica* is the most studied worldwide and the crude extracts of *A. indica* are effectively used to improve the immune status, boost the antioxidant properties and to reduce or stop prolific breeding in fish (Obaroh and Nzeh, 2013). Among culturable carps, common carp, *Cyprinus carpio* is the third most important farmed freshwater species in the world (Ljubojevic *et al*., 2015). It is a rustic, omnivorous, fast growing, having commercial value and cosmopolitan in nature. Stress and disease incidences especially in intensive farming could affect the survival and growth of fish (Biswas *et al*., 2006). Currently, culture of common carp is also facing a serious problem due to its prolific breeding, which has led to over population and stunting in growth of the fish due to its early age maturity (5-6 months). Thus for efficient and sustainable development of common carp culture especially in composite fish culture, the prolific breeding needs to be checked. Several plant materials are reported that possess antifertility property when

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administered orally. Keeping these aspects in view, the specific objective of the study was designed and focused to investigate the response of neem leaf extract on select hematological parameters of common carp.

**MATERIALS AND METHODS**

The experiment was conducted in out-door cemented tanks at the fish farm of College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Punjab, India.

**Experimental fish:** The fish species common carp, *Cyprinus carpio* was used for the feeding experiment using Neem Leaf Extract (NLE) as an herbal supplement. Each treatment had 3 replicates and every replicate was stocked with 80 fish having same age and similar size (Average length: 6.2 cm and weight: 3.5 g).

**Experimental design:** The experiment was carried out in 80 m² out-door cemented tanks. Approximately, two-inch thick layer of soil was spread at the bottom of each tank to hasten the decomposition process.Liming and manuring was performed as required.

**Preparation of experimental feed:** The Neem Leaf Extract (NLE) was incorporated in basal diet (D1; rice bran; mustard meal:: 1:1) at different levels of NLE viz, 0.25(D2), 0.5(D3), 1.0(D4), 1.5(D5), 2.0(D6) g/kg. Sinking pellets of different experimental feeds were made in an electric lab pelletizer. Fish were fed daily in the morning at 1.5% of fish BW (after sunrise) to different treatments (D1-D6) for 180 days.

**Proximate analyses:** The proximate analyses of NLE, other feed ingredients and formulated feeds with respect to crude protein (CP), ether extract (EE), ash (A), moisture and nitrogen free extract (NFE) content was performed on dry matter (DM) basis by following AOAC (2000) methods (Table 1).

**Water quality parameters:** Water samples were collected monthly in the morning hours for the analyses of selected physico-chemical parameters.

**Blood collection:** The blood samples were withdrawn from the caudal vein and were placed in two different micro centrifuge tubes, one with anticoagulant vials for hematological parameters and other without anticoagulant vials for serum protein analysis. The blood samples for protein analysis were centrifuged at 3000 rpm for 15 minutes and the supernatant was collected and stored at -20 °C until further use.

**Hematological parameters:** Hematological status of fish in terms of TEC and TLC count, hemoglobin and serum total proteins was estimated at termination of the experiment. Parameters include TEC, TLC count, hemoglobin and serum total proteins were estimated at the termination of the experiment according to the standard protocols. Briefly, total TEC, TLC, Packed Cell Volume or Hematocrit value, MCV, MCH, MCHC was calculated according to Haney et al. (1992). The hemoglobin was estimated by acid haematin method (Sahli, 1962) using 0.1N HCl and total protein concentration in the plasma of the fish was quantified using the Biuret method, and Serum albumin and globulin was estimated with the help of bromocresol green binding method (Dumas et al., 1971).

**Statistical analysis:** Statistical analysis of the data was performed with a Statistical Package for the Social Sciences (SPSS v16.0). One-way ANOVA was applied to understand the effect of *A. indica* supplemented diets on water quality parameters and hematological parameters of fish at 0.05 level of significance, followed by Duncan’s multiple comparison to determine significant differences among the treatments.

**RESULTS AND DISCUSSION**

During the experiment, all water-quality parameters were within the optimal range for rearing common carp. No significant mortalities occurred in any of the treatments during the experimental period.

**Hematological parameters:** Due to great importance of blood in pathological studies, hematological parameters are used as an index of fish health status in fish to detect the physiological changes following different stress conditions. In the present study, the impact of NLE incorporated diet was observed on different hematological parameters of *C. carpio*. The current study and earlier reports on hematological parameters suggest that the responses of neem vary depending on the species, size, age of the fish, concentration or dose of the herb, and length or duration of the treatment.

**Total Erythrocyte Counts (TEC):** Fish fed with NLE showed significantly higher TEC counts compared with that of control. In different treatments, TEC (x10⁶ mm⁻³) was 0.73 (D1), 0.89 (D2), 2.12 (D3), 3.27 (D4), 1.28 (D5), 0.98 (D6). The results showed that D4 treatment had highest counts of erythrocytes followed by other treatments (Table 2 and Fig. 1 A). The response of erythrocytes, elevated until D4 along with increase in the dose of NLE and then it declined at the highest doses (D5 and D6). Collectively, the results of TEC, showed a sigmoidal pattern of response and reveals that, the ideal dose is D3 and D4 to enhance the TECs, whereas D5 and D6 could be a negative feedback for the RBC synthesis. TECs (erythrocytes) normally constitute a major part of blood cells and number varies with species but they generally range between 1.05 and 3.0 (x10⁶ mm⁻³). In the present study, TEC in D4 showed significantly higher numbers compared to other treatment groups. Similar results were also found in rohu fed with *Allium sativum* and goldfish fed on Azadirachta (Sahu et al., 2007; Kumar et al., 2013). In addition to earlier reports on other herbs and spices, which showed increased erythrocyte counts in *Oreochromis mossambicus* fed with diets supplemented having thyme,
rosemary and fenugreek (Gültepe et al., 2014), garlic and ginseng (Shelby et al., 2006) compared to control. Similarly, increased erythrocyte counts in *O. niloticus* challenged with *Aeromonas hydrophila* and treated with aqueous NLE (Ismael et al., 2011) was observed.

Changes in the erythrocyte profile observed in the current study and earlier research in fish indicates that a compensation of oxygen deficit in the body due to gill damage particularly in high doses (Drastichova et al., 2004). The reduction trend in RBC at higher doses (D5 and 6) in the present study could be caused either by the inhibition of erythropoiesis or by the destruction of red cells and destruction of hematopoietic tissue in kidney and spleen (Iwama et al., 1976).

**Total Leukocyte counts (TLC):** TLC in fish fed with NLE was significantly increased compared with the control. Among the treatments, significant differences were found at P<0.5 level of significance (Table 2 & Fig. 1 B). The responses of leukocytes showed increase in trend like erythrocytes until D4 along with increase in the dose of NLE and then it declined at the highest doses (D5 and D6). Similarly, Uthayakumar et al. (2014) reported that WBCs significantly increased in *A. invadans* infected fish and administered with neem soluble fractions against fungal pathogen. Talpur and Ikhwanuddin (2013) also obtained similar trend of increase in WBCs in Asian seabass, *Lates calcarifer* upon administration with *A. indica* leaf supplement. Collectively, the results of the current study corresponded with those reported earlier on the counts of WBC using various herbs and herbal extracts. Therefore, the immunity in fish, particularly non-specific is mediated by leukocytes, and raise in leukocyte count is an evidence of immune-stimulation and which is stimulated by herbs and perhaps could boost the immune system.

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<th>Table 1: Proximate composition of NLE, feed ingredients and pelleted feeds.</th>
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<td>Parameter</td>
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<tr>
<td>Total Protein (g 100 g⁻¹)</td>
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<td>Total Lipid (g 100 g⁻¹)</td>
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<td>Total Carbohydrate (g 100 g⁻¹)</td>
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<td>Ash (g 100 g⁻¹)</td>
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<td>Nitrogen Free extract</td>
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<th>Table 2: Hematological parameters of fish in different treatments of NLE in <em>Cyprinus carpio</em>.</th>
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<tr>
<td>Parameter</td>
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<td>RBC (10⁶mm⁻³)</td>
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<td>WBC (10⁶mm⁻³)</td>
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<td>PCC (%)</td>
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<td>Serum total protein (g dl⁻¹)</td>
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<td>Serum globulin (g dl⁻¹)</td>
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<td>Albumin Globulin ratio</td>
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![Fig 1: A) Response of Total Erythrocyte Count (x10⁶ mm⁻³), F_{(6,17), P<0.05} = 2.882, and B) Total Leucocyte Count (x10⁶ mm⁻³), F_{(6,17), P<0.05} = 58.758]
Packed Cell Volume (PCV) or Hematocrit Value (Ht):
Throughout the study, elevated PCV (%) was observed in fish fed with NLE compared to control and significant differences were observed among the treatments (Table 2 and Fig. 2). The PCV was significantly increased in the treatment groups (D2, 3 and 4) compared to control, however the levels were equal to control in D5 and 6. The study shows the increasing response trend is in agreement with the findings of Mona et al. (2015), where 2.0% administration of neem leaf supplemented diet significantly increased Ht in silver carp against A. hydrophila and Uthayakumar et al. (2014) reported significantly increased Ht in A. invadans infected fish and administered with neem soluble fractions against fungal pathogen. Interestingly, Harikrishnan et al. (2003) in C. carpio following herbal treatment with A. indica and Kumar et al. (2013) in Carassius auratus, did not find any significant differences or no response of Azadirachtin among the treatment groups compared with the control.

Hemoglobin: The response of NLE showed increased Hb content among the treatments (up to D4) compared to control (D1). The Hb content of D2-D4 treatments was significantly increased compared to D1 (Control). However, there were significant differences observed among the treatments irrespective of increase in dosage of NLE.

Results on hemoglobin suggest that, its quantitative estimation provides an index of erythrocytes function and health status of the animal. In the present study, Hb content was significantly different (increased) in treated groups over control; this demonstrates that the fish were not under stress due to NLE, and NLE had no negative impact on Hb levels. Further, our results, was in similar trend with the findings of Harikrishnan et al., (2009) in goldfish fed with a diet containing A. indica extract, where Hb content showed increasing trend in RBC count. Uthayakumar et al. (2014) was also found increased Hb levels with increase in hexane soluble fraction of A. indica in Channa striatus.

Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC): The results showed that MCV values decreased with increase in dose of NLE and the differences were significant among treatments (Table 2 & Fig. 4A). Whereas, MCH result showed considerable changes among the treatments and the differences among treatments were significant (Table 2 & Fig. 4 B). However, The MCHC values in all NLE treated groups were significantly high compared with the control except for that of D4, where D4 was in par with the control (Table 2 and Fig. 5 C). The ratio of albumin to globulin did not show any trend and lowest value being recorded in D2 (Table 2& Fig. 5 D). In the present study, the highest plasma protein, albumin and globulin content recorded in D4 and D5 group. Lower albumin to globulin ratio indicates the presence of more amounts of globulin. Since the gamma fraction makes the largest portion of globulin, it can be inferred that A. indica may enhance the immune response of C. carpio. Sahu et al.

![Fig 2: Response of Packed Cell Volume (%) of fish in different treatments of NLE at the end of the experiment. Values described by the same letter on the bars are not significantly different from each other (F0.05, df: 5, 17 =137.961, P<0.05).](image)

![Fig 3: Response of Hemoglobin (g dl⁻¹) of fish in different treatments of NLE at the end of the experiment. Values described by the same letter on the bars are not significantly different from each other (F0.05, df: 5, 17 =3.952, P<0.05).](image)
Fig 4: Response of A) MCV (fl; femto litre), (F<sub>0.05; 5, 17</sub> = 99.918), B) MCH (pg; pico gram), (F<sub>0.05; 5, 17</sub> = 69.087), C) MCHC (g/dL), (F<sub>0.05; 5, 17</sub> = 6.404) of fish in different treatments of NLE at the end of the experiment. Values described by the same letter on the bars are not significantly different from each other (P<0.05).

Fig 5: Response of Blood serum A) total protein (g dl<sup>-1</sup>, F<sub>0.05; 5, 17</sub> = 144.927), B) Albumin (g dl<sup>-1</sup>), (F<sub>0.05; 5, 17</sub> = 65.826), C) Globulin (g dl<sup>-1</sup>), (F<sub>0.05; 5, 17</sub> = 55.152) and D) Albumin/globulin ratio (F<sub>0.05; 5, 17</sub> = 10.024) of fish in different treatments of NLE at the end of the experiment. Values described by the same letter on the bars are not significantly different from each other (P<0.05).
(2007) reported that the serum total protein, after long term feeding with mango seed kernel, increased compared to control and similar trend was reported by Kumar et al. (2013). The results of the current study reveals that NLE could increase the immune indices like lysozyme, catalase and superoxide dismutase activity and other positive effect that could stimulate immune system of the fishes and the study reveals that the hematological profile of an animal reflects the immunological status of the animal.

In conclusion, our results suggest that NLE enhanced the hematological parameters of the common carp and perhaps it could help in improving the development, metabolism, and control reproduction thereby sex ratio of the fish. However, this study examined the response of NLE only on hematological parameters, meticulous investigations are warranted on other parameters to elucidate the clear mechanisms of actions involved development, metabolism, control of prolific breeding and sex ratio of common carp.

REFERENCES


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