

Effect of azadirachtin on haematological and biochemical parameters of *Argulus*-infested goldfish *Carassius auratus* (Linn. 1758)

Saurav Kumar · R. P. Raman · Kundan Kumar ·
P. K. Pandey · Neeraj Kumar · B. Mallesh ·
Snatashree Mohanty · Abhay Kumar

Received: 29 May 2012 / Accepted: 12 October 2012 / Published online: 23 October 2012
© Springer Science+Business Media Dordrecht 2012

Abstract Argulosis hampers aquaculture production and alters the host physiology and growth. Azadirachtin is recognized as a potential antiparasitic agent against *Argulus* sp. The present study aimed to investigate the effect of different concentration of azadirachtin solution on haematological and serum biochemical parameters of *Argulus*-infested goldfish *Carassius auratus*. Ninety *Argulus*-infested goldfish were randomly divided into six equal groups. Fish of group 1–5 were treated with azadirachtin solution through bath of 1, 5, 10, 15 and 20 mg L⁻¹ as T1, T2, T3, T4 and T5, respectively, and group 6 was exposed to 2 % DMSO solution without azadirachtin and considered as negative control T0⁻. Along with six treatment groups, a positive control T0⁺ of healthy goldfish free from *Argulus* infestation was also maintained. Parasitic mortality was evaluated after 3 days of consecutive bath treatment. After 7 days of post-treatment, the blood and serum were drawn from each of the treatment groups and haematological and serum biochemical

parameters were evaluated. Total leucocyte count (TLC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), blood glucose, total protein (TP), globulin, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were significantly ($p < 0.05$) high in negative control group when compared with positive control group. It could be concluded that *Argulus* infestation altered marked haematological and serum biochemical parameters. However, in treated groups complete elimination of *Argulus* was found in T4 and T5 groups. Also significant ($p < 0.05$) reduction in haematological and serum biochemical parameters of all the treatment groups were recorded in comparison with negative control group. In addition, T4 and T5 groups showed significantly ($p < 0.05$) high superoxide dismutase (SOD), catalase, total erythrocyte count (TEC) and haemoglobin (Hb). However, higher mean corpuscular haemoglobin concentration (MCHC), blood glucose and lactate dehydrogenase (LDH) levels in T5 group revealed that higher concentration of azadirachtin have notable effects on activity of vital tissues function and physiology of the host. *Argulus* spp. from infested goldfish could be eliminated using bath treatment with solution of azadirachtin having concentration of 15 mg L⁻¹ and that also shifted haematological and serum biochemical parameters towards homeostasis.

Electronic supplementary material The online version of this article (doi:10.1007/s10695-012-9736-8) contains supplementary material, which is available to authorized users.

S. Kumar (✉) · R. P. Raman · K. Kumar ·
P. K. Pandey · N. Kumar · B. Mallesh ·
S. Mohanty · A. Kumar
Central Institute of Fisheries Education, Mumbai 400061,
India
e-mail: sauravsinha535@gmail.com

Keywords Azadirachtin · *Argulus* · Haematology ·
Biochemical · Physiology · *Carassius auratus*

Introduction

The growing demand for ornamental fish and growing interest in aquarium have changed the culture practices. Ornamental fish trade, with an annual growth rate of 8 %, offers a lot of scope for development of ultra-intensification. However, the technology of production is not yet standardized and there is negligence about sanitary aspects, thus facilitating diseases susceptibility in fish. The high stocking density, poor husbandry, abundant occurrence of vectors and a multitude of stress factors promote the infections. While majority of ectoparasites are non-pathogenic and can be tolerated, a few species are implicated as highly pathogenic having a major impact on health status of the host fish. Crustacean ectoparasite *Argulus* spp. (Crustacea: Branchiura) are found distributed throughout the world (Rushton-Mellor 1992). *Argulus* spp. have been described as economically important pathogens of finfish in temperate and tropical regions (Walker et al. 2004). Argulosis outbreak in various culture systems in India and European aquaculture has been reported and to cause mass mortality (Gopalakrishnan 1964; Singhal et al. 1990; Sheila et al. 2002).

Argulus spp. typically attach to the skin of the host and feed on the mucus, epithelial cells and blood. Infested fish show behavioural abnormalities including irritation, discoloration, lethargy and anorexia. It punctures the host's skin, inject a cytolytic toxin through preoral stylet and feed on blood, besides feeding on mucus and epithelial cells (Lamarre and Cochran 1992). Moreover, *Argulus* may act as carrier of various viral and bacterial pathogens. However, the importance of Argulosis in growth suppression and reduction in production is still debatable. Argulosis outbreaks, associated with mass mortalities in some cases, may occur in presence of other pathogens. The most common chemotherapeutics to eliminate the ectoparasites are malathion, cypermethrin, chlorophenol and many other pesticides (Toovey and Lyndon 2000). Other chemicals including teflubenzuron, pyrethrin and dichlorvos have also been evaluated for chemotherapy and were found to play a pivotal role in control of *Argulus* infestation in aquaculture system (Schmahl and Mehlhorn 1985; Treves-Brown 1999). However, the threats of bioaccumulation and residual formation in the host caused by frequent use of these drugs have led to the need of other alterative control methods (Goven et al. 1980; Klinger and Floyd 2002). In view of the above, phytotherapy has

received importance to combat the parasitic disease in aquafarm along with ornamental practices due to its efficacy, cost-effectiveness and eco-friendly properties. Recently, raw extract of garlic (*Allium sativum*) has been reported to kill trichodinids (Madsen et al. 2000), theronts and tomites of *Ichthyophthirius multifiliis* (Buchmann et al. 2003). Ekanem et al. (2004) has reported some potential for killing the free-swimming stages of *I. multifiliis* by using petroleum extracts of *Mucuna pruriens* and *Carica papaya*. Recently, azadirachtin was evaluated as potential antiparasitic agent against *Argulus* spp. in goldfish (Kumar et al. 2012).

Azadirachta indica (A. Jass.) is traditionally known as the “Village pharmacy” or “Village dispensary” in India (Biswas et al. 2002). There are many reports about the antimicrobial, nematocidal, biopesticidal and immunomodulatory activities of *A. indica*. Among 40 different active ingredients from neem, tetranotripernoids or more specifically azadirachtin is an important bioactive compound and highly oxygenated triterpenoids, responsible for antimicrobial and pesticidal activity (Govindachari and Gopalakrishnan 1998). Azadirachtin was used to eliminate *Argulus* and protect the host from ectoparasites but it is also reported to be a strong natural insecticide (Schlüter 1987). Although, neem extract is considered of low toxicity towards non-target aquatic life (Martinez and Souza 2002), water extracts of the bark of neem plant caused respiratory problems in *Tilapia zilli* (Omeregic and Okpanachi 1997), while long exposure to low concentrations of the crude extract of *A. indica* delayed the growth of cichlid fish (Omeregic and Okpanachi 1992). Such results indicate that neem extracts added to water may cause adverse impact on fish. However, the infestation may be associated with haematological and enzymological alteration as well as inflammatory changes (Saurabh and Sahoo 2010).

In almost all the infected fishes, the homeostatic processes are extended beyond the normal limits due to stress (Pickering 1981). Red blood cell (RBC) counts, haemoglobin (Hgb), haematocrit (Ht), leucocytes counts and haematological indices (MCH, MCV and MCHC) are suitable measures of the physiological damages of organs in human clinical medicine as well as in fish. Therefore, blood values have been used to demonstrate the effects of azadirachtin exposure in *Cyprinus carpio* infected with *Aeromonas hydrophila* (Harikrishnan et al. 2003). Estimation of NBT activity and myeloperoxidase levels significantly reflect the

immunological status and leucocyte performance. But, the exposure of many drugs alters the biochemical, haematological and enzyme parameters of vital organ tissues and exerts stress on the fish. Consequently, it is important to recognize the effects of these products on different parameters of fish (Rábago-Castro et al. 2006). The herbal drugs act as antistressor and induce the immunological parameters such as serum lysozyme activity, SOD, NOS and levels of total protein, globulin and albumin (Wu et al. 2007). At the same time, serum biomarker like blood glucose can be viewed as part of a stress response triggered by the presence of biological stressors in water. Glucose is one of the most important sources of energy for animals and it has been reported as an indicator of stress caused by physical factors. However, catalase and SOD are considered as important biomarkers of oxidative stress. Fish are endowed with defensive mechanisms to counteract the impact of reactive oxygen species (ROS) resulting from metabolism of various antioxidant defence enzymes such as SOD, catalase and GST family possessing detoxifying activities (Tjalkens et al. 1998). Thus, the present work aimed to study the effect of azadirachtin solution through bath treatment on haematological and biochemical profiles of *Argulus* spp.-infested goldfish.

Methods and materials

Experimental animals

The animals used for experimental purpose were matured goldfish (*Carassius auratus*) ($n = 105$) with an average weight of 20.75 ± 0.25 g. The fish procured from Aquarium Shop, Kurla, Maharashtra, India were completely free from infestation of ectoparasites and were healthy. The stock was acclimatized under aerated conditions for a period of 10 days and was fed with commercial pelleted goldfish diet.

Experimental design and sampling procedures

Argulus infection was carried out artificially by cohabitation method (Kumar et al. 2012). The experimental animals, that is, healthy goldfish were kept with *Argulus*-infested goldfish. After two weeks of gestation, ten fish were randomly selected and checked for the prevalence and intensity of parasites. Fish were chosen

for the in vivo tests at the prevalence of moderately infested 20–25 *Argulus*/fish (Figs. 11, 12 in supplementary material). *Argulus*-infested goldfish were equally and randomly distributed in six treatment groups in fibre reinforced plastic (FRP) tanks of 150 L capacity, and also a control group (uninfected healthy goldfish) was maintained. In vivo test was performed for three consecutive days for evaluation of antiparasitic activity of azadirachtin against *Argulus* spp. Blood samples were collected after 7 days for evaluation of haematological and serum biochemical profiles. The whole experiment was conducted with complete randomized design with triplicate of each treatment.

Preparation of stock solution and working test solution

The stock solution and working test solution was prepared in organic solvent DMSO as described by (Kumar et al. 2012). The commercially available Azadirachtin EC 25 % (SOM Phytopharma, Hyderabad, India) was used for preparation of stock solution. The different working test solutions were prepared by dissolving stock solution in the bore well water as 1 mg L^{-1} (T1), 5 mg L^{-1} (T2), 10 mg L^{-1} (T3), 15 mg L^{-1} (T4), 20 mg L^{-1} (T5) and the negative control solution ($T0^-$) was made up of water and 2 % DMSO solution with no azadirachtin.

In vivo bioassay

The in vivo test was performed with slight modification (Kumar et al. 2012). Twenty-one fibre tanks ($80 \times 57 \times 42$ cm) of 150 L capacities were arranged with 24 h of aeration facilities in the wet laboratory of Aquatic Environment and Health Management Division, CIFE, Mumbai, India. These tanks were filled with 100 L of bore well water before starting the experiment. The experiment was performed for 7 days. The ninety experimental fish (*Argulus* infested) were randomly divided into five treatment groups and one control (negative) group with stocking density of 5 nos. per tank. A positive control, having five uninfected fish, was also kept. Following the protocol, the bath treatment was given using working test solution $T0^-$, T1, T2, T3, T4 and T5 of 0, 1, 5, 10, 15 and 20 mg L^{-1} azadirachtin, respectively. Negative control $T0^-$, *Argulus*-infested fish, was maintained in tanks containing 2 % DMSO

solution without azadirachtin and positive control T0⁺, normal fish, was maintained without any infestation of *Argulus* and treatment. The all physico-chemical parameters of water such as temperature (25 ± 2 °C), pH (7.2 ± 0.4), dissolved oxygen (5.2 ± 0.5 mg L⁻¹) and ammonia (0.01 ± 0.005 mg L⁻¹) were in the optimum range. The *Argulus*-infested fish were given bath treatment with azadirachtin solution at the above concentrations for three consecutive days. During this period, fish and parasites mortality were recorded. Blood samples were collected from nine fishes of each treatment group for the study of haematological and serum biochemical parameters on 7th day.

Collection of blood and serum

Each fish was anesthetized using clove oil (Merck, Germany) @ 50 µl per litre of water before taking blood from fish. Blood was drawn from Vena caudal by using a tuberculin medical syringe with needle number 24, which was previously rinsed with 2.7 % EDTA solution (Himedia, India). Collected blood was immediately transferred to test tube coated with thin layer of citrate (as an anticoagulant) and mixed well in order to prevent clotting of blood. Serum was collected without using anticoagulant and separated from blood by keeping the tubes in slanting position for around 2 h and then centrifuged at 3,500 rpm for 10 min at 4 °C followed by collection of straw coloured serum with micropipette and stored at -20 °C for further use (Supplementary data).

Haematological studies

The total leucocyte counts (10^3 mm⁻³) were determined by taking 20 µl of blood sample mixed with 3,980 µl of WBC diluting fluid (Himedia, India) and total erythrocyte counts (10^6 mm⁻³) were determined by taking 20 µl of blood sample mixed with 3,980 µl of RBC diluting fluid (Himedia, India) in a clean vial (Schaperclaus et al. 1991). The diluted fluids were observed and cells were counted in Neubauer Hemocytometer (Rohem, India). Packed cell volume was determined by drawing non-dotted blood by capillary action into microhaematocrit tubes. One end of the tube was sealed with synthetic sealant. The sealed tube was centrifuged in a microhaematocrit centrifuge for 5 min at 10,500 rpm. The PCV was measured using

microhaematocrit reader and expressed as percentage (%). The haemoglobin level of blood was analyzed by the cyanomethemoglobin method using Drabkin's Fluid (Qualigens, India). Blood (20 µl) was mixed with 5 ml of Drabkin's working solution. The absorbance was measured using a spectrophotometer (Thermo Electron, Merck) at wavelength of 540 nm. The derived haematological profile of the mean corpuscular volume (MCV; fl), mean corpuscular haemoglobin (MCH; pg) and mean corpuscular haemoglobin concentration (MCHC; %) were calculated according to the equation suggested by Haney et al. (1992).

$$\text{MCV (fl)} = \text{Hct (\%)} \times 10 / \text{RBC (million/mm}^3\text{)}$$

$$\text{MCH (pg)} = \text{Hgb (gm/dl)} \times 10 / \text{RBC (million/mm}^3\text{)}$$

$$\text{MCHC (\%)} = \text{Hgb (gm/dl)} \times 100 / \text{Hct (\%)}$$

Serum biochemical parameters

Serum protein was estimated using the kit (Merck, Germany) according to biuret method (Reinhold 1953). Albumin was estimated by bromocresol green binding method (Doumas et al. 1971). Albumin in a buffered medium binds with bromocresol green (BCG) and produces a green colour whose absorbance is proportional to the albumin concentration. Globulin content was calculated by subtracting albumin values from total serum protein. A/G ratio was calculated by dividing values of albumin by globulin.

SGOT and SGPT activity (U/L) in serum were determined by using SGOT (AST) and SGPT (ALT) diagnostic kit (Merck, Germany), respectively. The diagnostic kits were based on Reitman and Frankel (1957) method.

Enzyme assay

Nitroblue tetrazolium (NBT) test

Nitroblue tetrazolium assay was carried out using the method of Anderson and Siwicki (1995). One hundred microlitres of blood was placed into the wells of 'U' bottom microtitre plates and incubated at 37 °C for 1 h to facilitate adhesion of cells. The supernatant was removed and the loaded wells were washed three times in PBS. After washing, 100 µl of 0.2 % NBT was added and plate was incubated for further 1 h. The

cells were then mixed with 100 % methanol for 2–3 min and again washed thrice with 70 % methanol. The plates were then air dried. One hundred and twenty microlitres 2 N KOH and 140 μ l DMSO (dimethyl sulphoxide) were added into each well to form the formazone blue precipitate. The OD of the turquoise blue coloured solution was then read in microplate reader (Quant, Universal microplate spectrophotometer) at 620 nm.

Serum lysozyme activity

Serum lysozyme activity was measured using colorimetric method (Parry et al. 1965). In a cuvette, 3 ml of *Micrococcus luteus* (Bangalore Geni, India) suspension in phosphate buffer ($A_{450} = 0.5\text{--}0.7$) was taken, and to which 50 μ l of diluted serum sample was added. The content of cuvette was mixed well for 15 s and reading was taken using a spectrophotometer at 450 nm. The reading of lysis of the bacteria was recorded immediately at 15, 30 and 270 s interval. A unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 per minute and lysozyme activity is expressed as U/min.

Myeloperoxidase content

Total myeloperoxidase content present in serum was measured according to Quade and Roth (1997) with slight modification of the method used by Sahoo et al. (2005). About 15 μ l of serum was diluted with 135 μ l of Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well plates. Then, 25 μ l of 20 mM 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB) (Hi-media) and 25 μ l of 5 mM H_2O_2 (Qualigens, India) (both substrates of MPO and prepared on the same day) were added. The colour change reaction was stopped after 2 min by adding 50 μ l of 4 M sulphuric acid (H_2SO_4). Plate was centrifuged ($400\times g$) for 10 min, and 150 μ l of the supernatants present in each well was transferred to new 96-well plates. The OD was read at 450 nm in a microplate reader (Quant, Universal microplate spectrophotometer).

Physiological stress biomarkers

Blood glucose level was estimated by the method of Nelson and Somogyi (1945) as described by Oser

(1965) in blood glucose diagnostic kit (Merck, Germany).

LDH activity (U/L) in serum was determined by using LDH diagnostic kit (Merck, Germany). The diagnostic kit was based on Wroblewski and La Due (1955) method.

Enzymes of oxidative stress

SOD (superoxide dismutase)

Superoxide dismutase was estimated following the method described by Misra and Fridovich (1972) based on the oxidation of epinephrine–adrenal chrome transition by the enzyme. Fifty microlitres of the sample was taken in the cuvette and 1.5 ml 0.1 M carbonate–bicarbonate buffer containing 57 mg/dl EDTA (pH-10.2) and 0.5 ml (3 mM) epinephrine was added and mixed well. Change in optical density at 480 nm was read immediately for 3 min in a Shimadzu–UV spectrophotometer. One unit of SOD activity was the amount of protein required to give 50 % inhibition of epinephrine auto oxidation. SOD was expressed as unit activity (amount of protein required to give 50 % inhibition of epinephrine auto oxidation).

Catalase (CAT)

Catalase was estimated following the method described by Takahara et al. (1960). To a reaction mixture of 2.45 ml phosphate buffer (50 mM, pH 7.0), enzyme source was added and the reaction was started by the addition of 1.0 ml of H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 15-s intervals for 3 min. The enzyme blank was run simultaneously with 1.0-ml distilled water instead of H_2O_2 solution. Enzyme activity is expressed as nanomoles H_2O_2 decomposed $\text{min}^{-1} \text{mg}^{-1}$ protein.

Statistical analysis

The data were statistically analyzed using statistical package SPSS version 16 in which data were subjected to one-way ANOVA and Duncan's multiple range tests were used to determine the significant differences between the means. Comparisons were made at 5 % probability level.

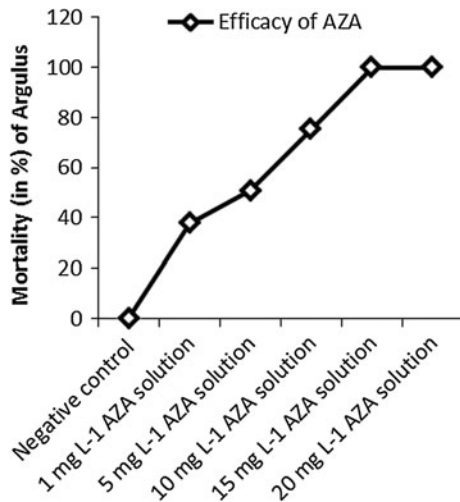


Fig. 1 In vivo test performing to evaluate mortality of *Argulus* spp. at different concentration of azadirachtin solution (AZA) on *C. auratus* for 72 h

Results

In vivo study

In the post-treatment study of in vivo test, the bath treatment with the azadirachtin solution showed a significant dose-dependent decrease in the number of *Argulus* compared with the negative control. At concentration of 1, 5, 10, 15 and 20 mg L⁻¹, the number of *Argulus* of infested goldfish decreased. The 100 % or complete elimination of *Argulus* was found in T4 (15 mg L⁻¹) and T5 (20 mg L⁻¹) groups while significant high mortality (75.33 and 50.83 %) was observed in T3 (10 mg L⁻¹) and T2 (5 mg L⁻¹) groups, respectively, in a 72-h study. The lowest mortality of *Argulus* (37.83 %) was observed in T1 (1 mg L⁻¹) group (Fig. 1).

Haematological studies

Haematological assay of post-treated fish showed significant optimization and recovery of complete blood counts. The values of TLC, TEC, Hb, PCV, MCV, MCH and MCHC are presented in Table 1. *Argulus*-infested negative control group showed the highest values of TLC and MCH and significantly differed ($p < 0.05$) among all the treatment groups. The erythrocyte levels, Hb, PCV and MCV were significantly high ($p < 0.05$) in positive control.

Treated group showed increasing trend of RBC in comparison with infected group (negative control). The haemoglobin level and packed cell volume differed significantly ($p < 0.05$) among all the treatments. The mean corpuscular haemoglobin concentration level differed significantly ($p < 0.05$) among all the treatments and showed the highest value in T3 group (Table 1).

Serum biochemical parameters

The serum albumin and A:G ratio were found to be significantly low ($p < 0.05$) in all the treatment groups in comparison with negative control. However, total protein and globulin level were significantly ($p < 0.05$) high recorded in T3 treatment group (Table 2).

The SGOT and SGPT levels were significantly low ($p < 0.05$) in all the treatment groups in comparison with negative control. The highest SGOT and SGPT values were observed in negative control while the lowest in positive control and T3 group, respectively (Figs. 2, 3).

Enzyme assay

The NBT values were significantly low ($p < 0.05$) in all the treatment groups compared with negative control. The highest value was found in negative control and the lowest value was recorded in positive control (Fig. 4). The lysozyme activity was significantly different ($p < 0.05$) among the treatments and showed higher value in comparison with positive control. The highest activity was found in negative control and lowest recorded in positive control (Fig. 5). Myeloperoxidase level did not differ significantly ($p > 0.05$) among the treatment groups but showed significantly ($p < 0.05$) higher activity in comparison with positive control and the highest value was found in T2 group (Fig. 6).

Physiological stress biomarkers

The blood glucose levels were found significantly ($p < 0.05$) high in T1, T2 and T5 groups along with negative control. The significant ($p < 0.05$) low level of blood glucose was recorded in T4 and positive control groups (Fig. 7). LDH activity was observed to be significantly low ($p < 0.05$) in the treatment groups in comparison with negative control. The highest LDH

Table 1 Haematological profiles (TEC, TLC, Hb, PCV, MCV, MCH and MCHC) of *Argulus*-infested *C. auratus* treated with azadirachtin solution

Treatment	TEC ($10^6/\text{mm}^3$)	TLC ($10^3/\text{mm}^3$)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (%)
Negative control	0.826 ^a ± 0.017	84.73 ^c ± 4.10	7.50 ^a ± 0.25	15.86 ^a ± 0.18	188.02 ^b ± 2.12	86.66 ^c ± 2.61	44.23 ^a ± 1.50
Positive control	1.293 ^c ± 0.029	59.13 ^a ± 4.91	8.03 ^c ± 0.38	20.83 ^c ± 0.18	163.6 ^a ± 1.92	75.46 ^a ± 1.21	45.33 ^a ± 1.55
1 mg L ⁻¹ AZA solution	0.916 ^{ab} ± 0.008	79.26 ^d ± 4.76	7.63 ^a ± 0.23	16.56 ^{ab} ± 0.16	180.7 ^b ± 3.53	83.27 ^c ± 1.58	46.08 ^{ab} ± 1.01
5 mg L ⁻¹ AZA solution	0.920 ^{ab} ± 0.011	77.06 ^c ± 3.17	7.66 ^a ± 0.12	16.66 ^{ab} ± 0.12	181.1 ^{ab} ± 1.63	82.98 ^c ± 1.14	45.80 ^{ab} ± 1.27
10 mg L ⁻¹ AZA solution	0.990 ^b ± 0.015	77.30 ^c ± 4.15	7.83 ^{ab} ± 0.13	16.50 ^{ab} ± 0.15	166.7 ^a ± 2.02	79.17 ^b ± 1.57	47.47 ^c ± 0.62
15 mg L ⁻¹ AZA solution	1.016 ^b ± 0.005	74.73 ^{bc} ± 2.18	7.86 ^b ± 0.26	16.83 ^{ab} ± 0.14	165.2 ^a ± 2.94	77.39 ^{ab} ± 1.31	46.82 ^{bc} ± 0.14
20 mg L ⁻¹ AZA solution	1.020 ^b ± 0.01	65.10 ^b ± 2.08	8.00 ^c ± 0.25	17.00 ^b ± 0.15	166.3 ^a ± 0.87	78.43 ^{ab} ± 0.41	47.14 ^{bc} ± 0.44

Values are expressed as mean ± SE ($n = 9$) in the columns with different superscripts differ significantly ($p < 0.05$)

Table 2 Total protein, albumin, globulin level and A:G ratio of *Argulus* spp.-infested *C. auratus* treated with azadirachtin (AZA) solution

Treatment	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G ratio
Negative control	5.86 ^d ± 0.12	2.58 ^b ± 0.04	3.23 ^{ab} ± 0.11	0.816 ^a ± 0.02
Positive control	4.56 ^a ± 0.14	2.00 ^a ± 0.05	2.83 ^a ± 0.23	0.713 ^a ± 0.01
1 mg L ⁻¹ AZA sol.	4.39 ^a ± 0.08	2.03 ^a ± 0.10	2.85 ^a ± 0.18	0.714 ^a ± 0.01
5 mg L ⁻¹ AZA sol.	5.94 ^{cd} ± 0.08	2.15 ^a ± 0.02	3.50 ^b ± 0.12	0.616 ^a ± 0.01
10 mg L ⁻¹ AZA sol.	6.07 ^d ± 0.03	2.30 ^a ± 0.20	3.63 ^b ± 0.17	0.641 ^a ± 0.02
15 mg L ⁻¹ AZA sol.	5.32 ^b ± 0.12	2.03 ^a ± 0.08	3.28 ^{ab} ± 0.08	0.608 ^a ± 0.01
20 mg L ⁻¹ AZA sol.	5.63 ^{bc} ± 0.19	2.28 ^a ± 0.04	3.23 ^{ab} ± 0.11	0.694 ^a ± 0.01

Mean values in the columns with different superscript differ significantly ($p < 0.05$), data expressed as mean ± SE ($n = 9$)

value was observed in T3 group and the lowest in positive control (Fig. 8).

Enzyme of oxidative stress

SOD and catalase activity differed significantly ($p < 0.05$) among the treatment groups. SOD activity was significantly ($p < 0.05$) high in T5 group and low in negative control (Fig. 9). The highest catalase value was observed in T5 group and the lowest in positive control (Fig. 10).

Discussion

The study showed haemato-serum biochemical profiles of *Argulus*-infested goldfish and post-treatment changes in haematological and serum biochemical

profiles of goldfish, bath treated with azadirachtin solution resulting in a significant enhancement of health condition of treated fish. In vivo study showed that bath treatment with different concentration of azadirachtin solution resulted in significant reduction in *Argulus* infestation on goldfish. The reduction in the parasites in the test groups could be attributed to the effects of the azadirachtin because similar reduction in parasite burden was not observed in the negative control groups, which is in agreement with Kumar et al. (2012). Most of the workers (Ekanem et al. 2004; Wang et al. 2010; Yao et al. 2011) used the herbal extracts through bath or dip treatment as these ectoparasites have little influence by systemic condition of the host. The innate or non-specific immunity plays an important role in protection of the host fish during parasitic infestations, and especially inflammatory response showed predominantly, to recover

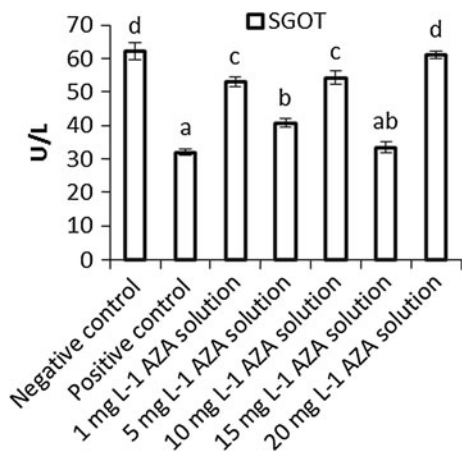


Fig. 2 Serum glutamate oxaloacetate transaminase (SGOT) activity of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with *different superscript* differ significantly ($p < 0.05$) and express as mean \pm SE)

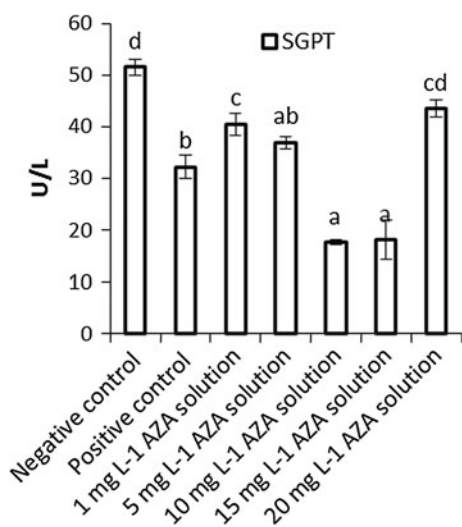


Fig. 3 Serum glutamate pyruvate transaminase (SGPT) activity of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with *different superscript* differ significantly ($p < 0.05$) and express as mean \pm SE)

from infection (Secombes 1996; Sahoo and Saurabh 2008). Shimura et al. (1983) showed an anaemic condition in young *Onchorhynchus masou* infested with 250 *Argulus coregoni* per fish and reported significantly low leucocyte counts, plasma total protein, cholesterol and calcium. Statistically significant changes were not observed in numbers of immature erythrocytes or thrombocytes and in the plasma

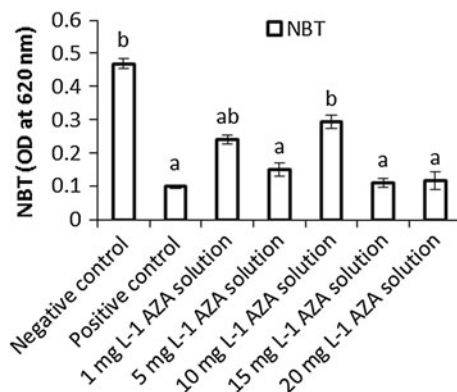


Fig. 4 Nitro blue tetrazolium test (NBT) of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with *different superscript* differ significantly ($p < 0.05$) and express as mean \pm SE)

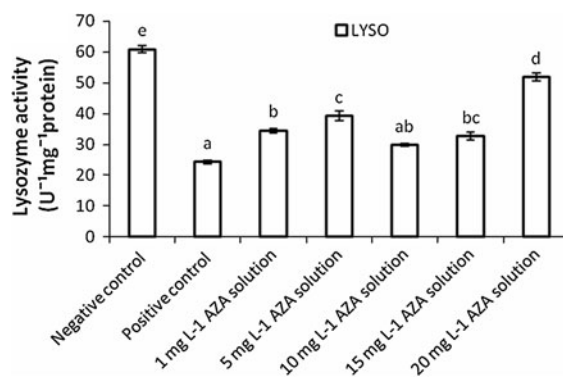


Fig. 5 Lysozyme activity of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with *different superscript* differ significantly ($p < 0.05$) and express as mean \pm SE)

concentration of glucose. In the present study, the post-treatment haematological and serum biochemical assays showed reduction in stress.

The number of erythrocytes was found to be low in *Argulus*-infested group and subsequently increased in the treated groups. In addition, significantly low haemoglobin, haematocrit value and MCHC were observed. *A. coregoni* infestation of trout showed low value of erythrocyte, leucocyte, haemoglobin concentrations and haematocrit (Shimura et al. 1983). A study on the effects of *Argulus* infections on haematological parameters by (Tavares-Dias et al. 1999) found an increase in monocytes and special granulocytic cells, and an increase in the number of thrombocytes in *Piaractus mesopotamicus*. Similarly, significant high leucocyte, MCV and MCH were found in this study

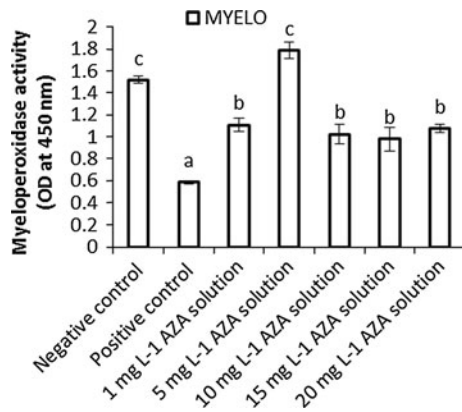


Fig. 6 Myeloperoxidase activity of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with different superscript differ significantly ($p < 0.05$) and express as mean \pm SE)

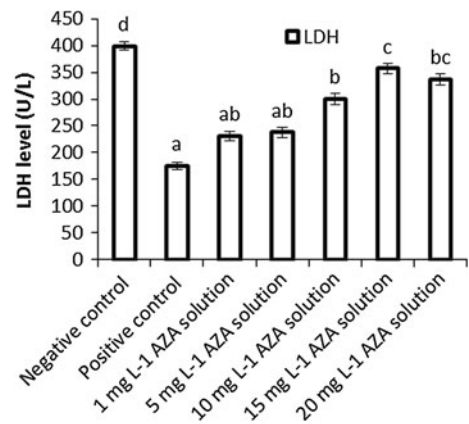


Fig. 8 Lactate dehydrogenase activity (LDH) activity of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with different superscript differ significantly ($p < 0.05$) and express as mean \pm SE)

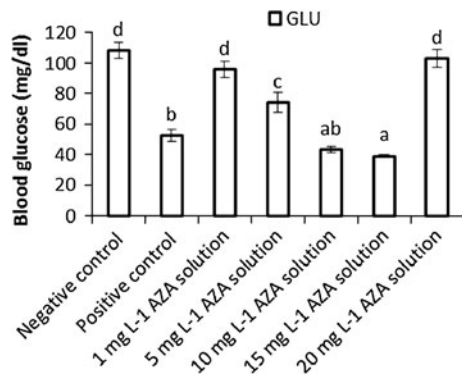


Fig. 7 Blood glucose of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with different superscript differ significantly ($p < 0.05$) and express as mean \pm SE)

representing the secondary infection of bacteria. *Argulus* spp. have often been associated with secondary infections or as a vector of other disease causing agents (Shimura et al. 1983; Rahman 1996). A decrease in MCV may be attributed to shrinking of the erythrocytes, resulting in a microcytic anaemia. The erythrocytes shrink due to insufficient synthesis of haemoglobin (Tort et al. 1988). The fluctuations in MCH and MCHC value, in infected goldfish clearly indicate that the concentration of haemoglobin in the RBCs was much low in the exposed fish compared with control thereby, indicating an anaemic condition. The MCHC is a good indicator of RBC swelling (Wepener et al. 1992). The fluctuation of MCH and MCHC in different treated groups and *Argulus*-infested goldfish suggested that the concentration of

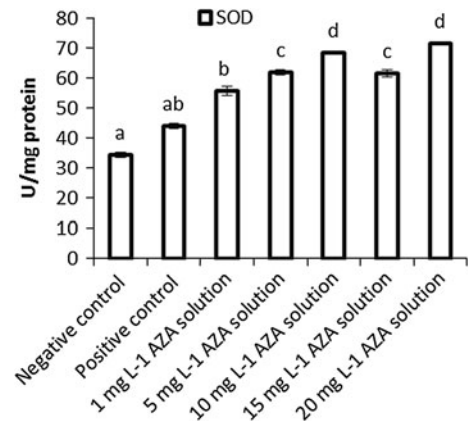


Fig. 9 Superoxide dismutase (SOD) activity of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with different superscript differ significantly ($p < 0.05$) and express as mean \pm SE)

Hb in the RBCs varied due to parasitic infestation and their anaemic condition.

Proteins are the most important component in the serum, and plasma proteins are termed as circulating mobile proteins. The serum proteins are divided into two groups: albumin and globulins. The liver synthesizes albumin and creates an osmotic force that maintain fluid volume within the vascular space. The albumin is an easily available protein reserve and a protein transporter (Anderson et al. 1979). The gamma globulin fraction is the source of almost all the serum biochemical active protein of the blood. Globulins like gamma globulins are essential for maintaining a healthy immune system. Increase in albumin and

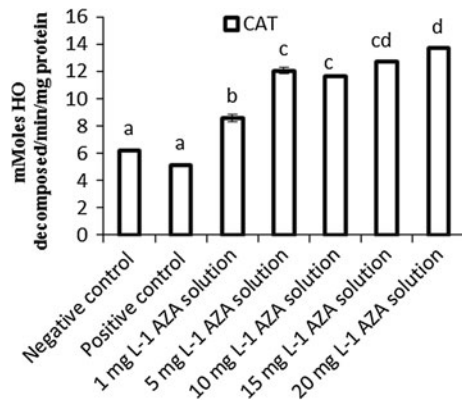


Fig. 10 Catalase activity (CAT) activity of *Argulus*-infested *C. auratus* treated with different concentration of azadirachtin (AZA) solution (values with different superscript differ significantly ($p < 0.05$) and express as mean \pm SE)

globulin levels are considered a strong innate response in fishes (Wiegertjes et al. 1996). Since, gamma fraction makes the largest portion of globulin, it can be inferred that subsequent enhanced globulin level in treatment groups may enhance the immune response and preparatory stage for stress mitigation of goldfish. Similar finding regarding total protein (TP) was reported in olive flounder infected with *Philasterides dicentrarchi* (Harikrishnan et al. 2012). The main reason for increased TP level is stress. For instance, chum salmon infected with erythrocytic necrosis virus had increased TP level due to the destruction of RBCs and resultant release of cell contents into the blood stream (Haney et al. 1992). Harikrishnan et al. (2009) reported no significant alteration in TP level in *A. hydrophila*-infected goldfish. The lower albumin–globulin ratio in the treatment groups may be due to less synthesis of albumin protein and more conversion of globulin to cope the immunological function. The high level of TP and globulin suggested the secondary infection paid to *Argulus* infestation and might be due to immunomodulatory activity of azadirachtin solution.

Aspartate aminotransferase (AST) is also known as serum glutamate oxaloacetate transaminase (SGOT) and alanine amino transferase (ALT) is also known as serum glutamate pyruvate transaminase (SGPT). Both the enzymes are present in the cytosol of the hepatocytes. However, GOT is also found in the mitochondria. SGOT is present primarily in liver and to a lesser extent in kidney and skeletal muscle. SGPT is found in all body tissues especially heart, liver and skeletal

muscle. Both enzymes are specific to liver but SGPT is found in higher concentration in liver than in other organs. Increased activity of SGOT and SGPT in serum, in the absence of acute necrosis or ischaemia of other organs such as myocardium, suggests liver cell damage and leaching of these enzymes in blood. It has been demonstrated that the activity of these enzymes may be enhanced in fish exposed to polycyclic and polychlorinated hydrocarbons (Zhang et al. 1990) and organophosphorus insecticides (Monteiro et al. 2006). Even low-level organic contamination can lead to increased hepatic SGOT activity in fish (Machala et al. 1997). Almeida et al. (2005) reported higher liver glutathione-S-transferase activity in *Prochilodus lineatus* exposed to sediment potentially polluted with organic contaminants from agricultural sources and municipal landfill. Subsequent higher doses of neem extract showed higher SGOT and SGPT levels in the treatment groups which may be related to the metabolism of organic compounds (Winkaler et al. 2007). The present study results showed higher level of SGOT and SGPT in *Argulus*-infested group and post-treatment groups with partial elimination of ectoparasites. The biological stress and higher concentration of organic compounds have a negative impact on liver tissue and this is in conformity with finding of higher SGOT and SGPT levels by Rao et al. (2006) in *L. rohita*, fed with diet containing herb *Achyranthes aspera*. In post in vivo study, high levels of SGOT and SGPT values were found in the *Argulus*-infested negative control group while, low levels were recorded in 15 mg L⁻¹ azadirachtin treatment group, revealing the stress diminution by reduced protein catabolism and hepatocellular damage.

The NBT test is a simple assay widely used to demonstrate the production of superoxide anion. It is well characterized in macrophages/monocytes and neutrophils (Dalmo et al. 1997). These reactive oxygen species are considered toxic for fish bacterial pathogens (Hardie et al. 1996). In the study, higher respiratory activity was observed in *Argulus* infested but untreated fish compared with control and subsequent decrease in NBT in treated groups. Previous studies have reported inhibition of the respiratory burst of phagocytes after parasitic infestations (Karagouni et al. 2005). On the other hand, increased NBT activity of fish phagocytes has been reported after experimental infection of the Myxosporean, *Enteromyxum scophthalmi* (Sitja-Bobadilla et al. 2006) and

lice-infested female Rohu fish as compared with uninfested control (Saurabh and Sahoo 2010). The increase in oxygen radical production in the blood of infested fish might be due to a gradual increase in response of phagocytic cells in the blood associated with parasite-induced injury and localized inflammation.

The serum lysozyme is used as an indicator of innate immune response in fish (Tort et al. 2003). Fish serum lysozyme is believed to be of leucocyte origin (Lie et al. 1989). It has been found that lysozyme activity increases concomitantly with leucocyte numbers (Fletcher 1973) and antibody titre (Valdimirov 1968). Similarly, negative control showed high lysozyme activity with maximum leucocyte counts in the present study. Lysozyme plays an important role in innate immunity by lysis of bacterial cell wall and thus stimulates the phagocytosis of bacteria (Ellis 1990). *Argulus*-infested goldfish showed high lysosome activity in comparison with healthy uninfested fish. Similar trend of high lysozyme activity was found in infected fish by *Ceratomyxa shasta* (Foott et al. 2004) and *Sphaerospora dicentrarchi* (Munoz et al. 2000). Lysozyme activity might be influenced with leucocytes counts. The serum lysozyme activity was recorded to be significantly high in olive flounder fish challenged with *Philasterides dicentrarchi* (Harikrishnan et al. 2012).

Myeloperoxidase (MPO) is a hemoprotein secreted during activation of neutrophils, which plays an important role in defence of the organism. MPO is stored in primary, azurophilic granules of neutrophils. It is a major component of the broad bactericidal armamentarium of neutrophils. It utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid (Dalmo et al. 1997). Reduced activity may indicate the presence of contaminants of stress (Anderson and Siwicki 1995). In post in vivo study, significant decreased MPO activity in all the treated groups compared with infected one was found and similar trend was reported by (Harikrishnan et al. 2003) in *A. hydrophila*-infected *C. carpio* treated with herbal medicine. Similarly, higher myeloperoxidase activity was reported in infected fish compared with control showing positive correlation between NBT assay and MPO among *Argulus*-infested goldfish, whereas results are in disagreement with the findings of (Cuesta et al. 2006). Nevertheless, a positive correlation between phagocytic activity with monogenean

and *Argulus* parasitic numbers were reported in *Trachinotus marginatus* and *Labeo rohita*, respectively (Chaves et al. 2006; Saurabh and Sahoo 2010).

Glucose measurements show many inconsistencies and should be a complement of stress tests rather than a main indicator. Glucose is one of the most important sources of energy for the animals. It has been reported as an indicator of stress caused by physical factors (Manush et al. 2005). Glucose level also varies among different species and their stage of development (Iwama and Nakanishi 2004). In post in vivo study, negative control (*Argulus*-infested) group showed higher glucose level compared with the treatment groups and experimental test showed subsequent reduction in blood glucose level. The results are in agreement with the elevation of glucose level reported in lice-infested fish (Ruane et al. 2000; Mustafa et al. 2000). The significantly high blood glucose level was found in treatment group of 1 and 5 mg L⁻¹ due to partial elimination of *Argulus* spp., whereas higher dose of 20 mg L⁻¹ also showed increased blood glucose that revealed the physiological stress. The rise in blood glucose may be reflection of a higher rate of metabolism responsible for high glucose turnover due to the metabolic strain of parasitism, as was reported by Wright et al. (1989). Harikrishnan et al. (2009) found increased level of blood glucose in *A. hydrophila*-infected goldfish, fed with azadirachtin. However, olive flounder, fed with standard diet and challenged with *P. dicentrarchi*, showed decreased level of blood glucose when compared with control during period of infection (Harikrishnan et al. 2012).

Lactate dehydrogenase is the terminal enzyme of the glycolysis pathway. LDH converts lactate to pyruvate in the presence of coenzyme NADH that is converted to NAD⁺. Thus, lactate dehydrogenase helps in maintaining the glycolysis cycle by supplying NAD⁺. In the presence of enough oxygen, pyruvate enters in Kreb's cycle, but when there is deficiency of an oxygen in the tissue, pyruvate is converted to lactate. In post in vivo study, bath-treated groups showed significantly different level of LDH compared with control. Generally, LDH activity increases during stress viz. temperature stress (Grigo 1975), starvation stress (Vijayaraghavan and Rao 1986) and confinement stress (Chatterjee 2003). Lower LDH level in treated groups compared with *Argulus*-infested goldfish showed reduction in stress. In the study, the serum

LDH level indicated haemolysis of erythrocytes or higher activity of muscle cells.

Under normal physiological condition, animal cells produce reactive oxygen species (ROS) such as H_2O_2 , OH^- and O_2^- , which may damage cellular components leading to cell death. Catalase activity plays an important role in antioxidative defence of the cell by reducing H_2O_2 . When the rate of ROS generation exceeds that of their removal, oxidative stress occurs. Many environmental pollutants, including pesticides, are capable of inducing oxidative stress in fish (Pandey et al. 2003; Monteiro et al. 2006). This event results in the formation of highly reactive compounds such as free radicals or oxy radicals (O_2^- , H_2O_2 and $\cdot\text{OH}$) that frequently react with cellular macromolecules, leading potentially to enzyme inactivation, lipid per-oxidation, DNA damage and even cell death (Van der Oost et al. 2003). Catalase is the primary cellular enzymatic defence against H_2O_2 , converting it into H_2O and O_2 , and is critical for the process of scavenging free radicals (Dorval et al. 2003). Oxidative stress results when the antioxidant defence is overcome by pre-oxidant forces and reactive oxygen species (ROS) and are not adequately removed (Sies 1996). Living organisms are protected from ROS by several defence mechanisms, including antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, glutathione reductase, etc. In post in vivo study, the treated groups showed high SOD while lower activity was found in *Argulus*-infested group. Catalase activity also showed similar trend while little activity was recorded in negative control group. The high level of SOD and catalase enzymes activity in the treatment groups might have occurred due to trigger of antioxidant enzymes by azadirachtin and resulted in lower oxidative stress in the azadirachtin treated groups.

The study reports the effect of infestation by an ectoparasite *Argulus* spp. on haematology and serum biochemical parameters of gold fish. The ectoparasite could be effectively eliminated using through bath treatment with 15–20 mg L^{-1} of azadirachtin solution. Haematological, serum biochemical and enzymological parameters showed a shift towards homeostasis during the treatment and reflected significant increase in the level of stress enzyme at higher concentration of azadirachtin indicating adverse impact on function of vital tissues and physiology of the host. Since azadirachtin is a powerful natural insecticide, the

judicial uses need further study to evaluate different grade of azadirachtin in different culture situations. Moreover, the effect of modulation of non-specific immune system of the host and ectoparasites infestation should be evaluated.

Acknowledgments Authors are thankful to Dr. W.S. Lakra, Director and Vice chancellor, Central Institute of Fisheries Education, Mumbai, India for providing all the facilities required to carry out the study.

References

- Almeida JS, Meletti PC, Martinez CBR (2005) Acute effects of sediments taken from an urban stream on physiological and biochemical parameters of the neotropical fish *Prochilodus lineatus*. *Comp Biochem Physiol* 140:356–363
- Anderson DP, Robertson BS, Dixon OW (1979) Plaque-forming cells and humoral antibody in rainbow trout (*Salmo gairdneri*) induced by immersion in a *Yersinia ruckeri* O-antigen preparation. *J Fish Res Board Can* 36(6):636–639
- Anderson DP, Siwicki AK (1995) Basic haematology and serology for fish health programmes. In: Shariff M, Arthur JR, Subasinghe RP (eds) Diseases in Asian Aquaculture II. Fish Health section, Asian Fisheries society, Manila, Philippines, pp 185–202
- Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U (2002) Biological activities and medicinal properties of neem (*Azadirachta indica*). *Curr Sci* 82:1336–1345
- Buchmann K, Jensen PB, Kruse KD (2003) Effects of sodium percarbonate and garlic extract on *Ichthyophthirius multifiliis* theronts and tomocysts: in vitro experiments. *N Am J Aquac* 65:21–24
- Chatterjee N (2003) Biochemical, immunological and molecular probes in stress with application of an antistress formulation on genetically different finfish. Ph.d. thesis submitted to CIFE, Mumbai
- Chaves IS, Luvizzotto-Santos R, Sampaio LAN, Bianchini A, Martinez PE (2006) Immune adaptive response induced by *Bicotylophora trachinoti* (Monogenea: Diclidophoridae) infestation in pompano *Trachinotus marginatus* (perciformes: Carangidae). *Fish Shellfish Immunol* 21:242–250
- Cuesta A, Munoz P, Rodriguez A, Salinas I, Sitja-Bobadilla A, Alvarez-Pellitero P, Esteban MA, Meseguer J (2006) Gilthead seabream (*Sparus auratus* L.) innate defence against the parasite *Enteromyxum leei* (Myxozoa). *Parasitol* 132:95–104
- Dalmo RA, Ingebrihtsen K, Bogwald J (1997) Non-specific defense mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *J Fish Dis* 20:241–273
- Dorval J, Leblond VS, Hontela A (2003) Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) exposed in vitro to endosulfan, an organochloride pesticide. *Aquat Toxicol* 63:229–241

- Doumas BT, Watson W, Biggs HG (1971) Albumin standards and the measurement of serum albumin with bromocresol green. *Gun Chim Acta* 31:87
- Ekanem AP, Obiekezie A, Kloas W, Knopf K (2004) Effects of crude extracts of *Mucuna pruriens* (Fabaceae) and *Carica papaya* (Caricaceae) against the protozoan fish parasite *Ichthyophthirius multifiliis*. *Parasitol Res* 92:361–366
- Ellis AE (1990) Immunity to bacteria in fish. *Fish Shellfish Immunol* 9(4):291–308
- Fletcher TC (1973) Defence mechanisms in fish. In: Malins DC, Sargent JR (eds) *Biochemical and Biophysical perspectives in marine biology*. Academic press, London, pp 189–222
- Foott JS, Harmon R, Stone R (2004) Effect of water temperature on non-specific immune function and ceratomyxosis in juvenile Chinook salmon and steel head from the Klamath River. *Calif Fish game* 90:71–84
- Gopalakrishnan V (1964) Recent developments in the prevention and control of parasites of fishes cultured in Indian waters. *Proc Zool Soc Bengal* 17:95–100
- Goven B, Gilbert J, Gratzek J (1980) Apparent drug resistance to the organophosphate dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate by monogenetic trematodes. *J Wildl Dis* 16(3):343–346
- Govindachari TR, Gopalakrishnan GJ (1998) Insect antifeedant and growth regulating activities of neem seed oil. *Indian Chem Soc* 75:655
- Grigo F (1975) How much is carp (*C. carpio*) stressed by temperature? Blood composition, with a special look at the serum electrolytes. *Zool Anz Fena* 8:215–330
- Haney DC, Hursh DA, Mix MC, Winton JR (1992) Physiological and hematological changes in chum salmon artificially infected with erythrocytic necrosis virus. *J Aquat Anim Health* 4:48–57
- Hardie LJ, Ellis AE, Secombes CJ (1996) In vitro activation of rainbow trout macrophages stimulates inhibition of *Renibacterium salmoninarum* growth concomitant with augmented generation of respiratory burst products. *Dis Aquat Org* 25:175–183
- Harikrishnan R, Rani MN, Balasundaram C (2003) Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. *Aquaculture* 221:41–50
- Harikrishnan R, Balasundaram C, Kim MC, Kim JS, Han YJ, Heo MS (2009) Innate immune response and disease resistance in *Carrasius auratus* by triherbal solvent extracts. *Fish Shellfish Immunol* 27:508–515
- Harikrishnan R, Kim Ju-Sang, Kim Man-Chul, Balasundaram C (2012) Pomegranate enriched diet enhances the hematology, innate immune response, and disease resistance in Olive flounder against *Philasterides dicentrarchi*. *Vet Parasitol*. doi:10.1016/j.vetpar.2011.12.006
- Iwama G, Nikanishi T (2004) *The fish immune system, organism, pathogen and environment*. Academic Press, New York
- Karagouni E, Athanassopoulou F, Tsagozis P, Ralli E, Moustakareas T, Lytra K, Dotsika E (2005) The impact of a successful anti-myxosporean treatment on the phagocyte functions of juvenile and adult *Sparus aurata* L. *Int J Immunopathol Pharmacol* 18:121–132
- Klinger RE, Floyd RF (2002) Introduction of freshwater fish parasites. EDIS-Electronic Data Information Source-UF/IFAS Extension. University of Florida. <http://edis.ifas.ufl.edu/FA033>
- Kumar S, Raman RP, Kumar K, Panday PK, Kumar N, Mohanty S, Kumar A (2012) In vitro and in vivo antiparasitic activity of Azadirachtin against *Argulus* spp. in *Carassius auratus* (Linn. 1758). *Parasitol Res* 110:1795–1800
- LaMarre E, Cochran PA (1992) Lack of host species selection by the exotic parasitic crustacean, *Argulus japonicus*. *J Freshw Ecol* 7:77–80
- Lie O, Evensen O, Sarensen A, Fraysadal E (1989) Study on lysozyme activity in some fish species. *Dis Aquat Org* 6:1–5
- Machala M, Petřivalský M, Nezveda K, Ulrich R, Dušek L, Piačka V, Svobodová Z (1997) Responses of carp hepatopancreatic 7-ethoxyresorufin-O-deethylase and glutathione-dependent enzymes to organic pollutants: a field study. *Environ Toxicol Chem* 16:1410–1416
- Madsen HCK, Buchmann K, Møllergaard S (2000) Treatment of trichodiniasis in eel (*Anguilla anguilla*) regarded in recirculation systems in Denmark: alternatives to formaldehyde. *Aquaculture* 186:221–231
- Manush SM, Pal AK, Das T, Mukherjee SC (2005) Dietary high protein & vitamin C influences in mitigating stress due to chelate claw ablation in *Macrobrachium rosenbergii* males. *Comp Biochem Physiol* 142A:10–18
- Martinez CBR, Souza MM (2002) Acute effects of nitrite on ion regulation in two neotropical fish species. *Comp Biochem Physiol A* 133:151–160
- Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. *J Biol Chem* 247:3170–3175
- Monteiro DA, Almeida JA, Rantin FT, Kalinin AL (2006) Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). *Comp Biochem Physiol* 143C:141–149
- Munoz P, Sitja-Bobadilla A, Alvarez-Pellitero P (2000) Cellular and humoral immune response of European sea bass (*Dicentrarchus labrax* L.) (Teleostei: Serranidae) immunized with *Sphaerospora dicentrarchi* (Myxosporea: Bivalvulida). *Parasitology* 120:465–477
- Mustafa A, Mac Williams C, Fernandez N, Matchett K, Conboy GA, Burka JF (2000) Effects of sea lice (*Lepeophtheirus salmonis* Kroyer, 1837) infestation on macrophage function in Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immunol* 10:47–59
- Nelson NA, Somogyi M (1945) Cited by Oser BL, 1965. In: Hawk's physiological chemistry, 14th edn. McGraw Hill Publication, New York, pp 113
- Omeregíe E, Okpanachi MA (1992) Growth of *Tilapia zilli* exposed to sublethal concentrations of crude extracts of *Azadirachta indica*. *Acta Hydrobiol* 34:281–286
- Omeregíe E, Okpanachi MA (1997) Acute toxicity of water extracts of bark of the Neem plant, *Azadirachta indica* (Lodd) to the cichlid *Tilapia zilli* (Gervais). *Acta Hydrobiol* 39:47–51
- Pandey S, Parvez S, Sayeed I, Haque R, Bin-Hafeez B, Raisuddin S (2003) Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (Bl. and Schn.). *Sci Total Environ* 309:105–111
- Parry RM, Chandau RC, Shahani RM (1965) A rapid and sensitive assay of muramidase. *Proc Soc Exp Biol Med* 119:384–386

- Pickering AD (1981) Introduction: the concept of biological stress. In: Pickering AD (ed) Stress and fish. Academic Press, London
- Quade MJ, Roth JA (1997) A rapid, direct assay to measure degranulation of bovine neutrophils primary granules. *Vet Immunol Immunopathol* 58:239–248
- Rábago-Castro JL, Sanchez JG, Pérez-Castañeda R, González-González A (2006) Effects of the prophylactic use of Romet[®]-30 and copper sulphate on growth, condition and feeding indices in Channel catfish (*Ictalurus punctatus*). *Aquaculture* 253:343–349
- Rahman MM (1996) Effects of a freshwater fish parasite, *Argulus foliaceus* Linn. infection on common carp, *Cyprinus carpio* (Linn.). *Bangladesh J Zool* 24:57–63
- Rao YV, Das BK, Pradhan J, Chakraborti R (2006) Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol* 20:263–273
- Reinhold JG (1953) Standard methods of clinical chemistry 1:88
- Reitman S, Frankel S (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic transaminases. *Am J Clin Pathol* 28:56–63
- Ruane NM, Nolan DT, Rotlant J, Costellone J, Wendellar Bonga SE (2000) Experimental exposure of rainbow trout *O. mykiss* (Walbaum) to the infective stages of the sea louse *Lepeophtheirus salmonia* (Kroyer) influences the physiological response to an acute stressor. *Fish Shellfish Immunol* 10:451–463
- Rushton-Mellor SK (1992) Discovery of the fish louse, *Argulus japonicus* Thiele (Crustacea: Branchiura). *Britain Aquac Fish Manag* 23:269–271
- Sahoo PK, Mukherjee SC, Ayyappan S (2005) Concept of immunostimulation in finfish and shellfish. *Workshop on Aquaculture Medicine, Natl 71*
- Sahoo PK, Saurabh S (2008) Immune response to parasitic pathogens in fish. *Course manual, Winter school on recent advances in fish and shellfish immunology and its application, CIFA, Bhubaneswar, India, pp 70–74*
- Saurabh S, Sahoo PK (2010) Non-specific immune responses of the Indian major carp *Labeo rohita* (Hamilton) to infestation by the freshwater fish louse *Argulus siamensis* (Wilson). *Indian J Fish* 57:45–53
- Schaperclaus W, Kulow H, Schreckenbach K (1991) Haematological and serological technique. In: Kothekar VS (ed) *Fish disease, vol 1, 2nd edn*. Oxonian Press, New Delhi, pp 71–108
- Schlüter U (1987) In Schmutterer H, Ascher KRS (eds) *Natural pesticides from the neem tree and other tropical plants*. GTZ Press, Eschborn, pp 331–348
- Schmahl G, Mehlhorn H (1985) Treatment of fish parasites. 1. Praziquantel effective against Monogenea (*Dactylogyrus vastator*, *Dactylogyrus extensus*, *Diplozoon paradoxum*). *Z Parasitenk* 71:727–737
- Secombes CJ (1996) The non-specific immune system. In: Iwama G, Nakanishi T (eds) *The fish immune system: organism, pathogens and environment*. Academic press, San Diego, pp 63–103
- Sheila F, Sivakumar AA, Chandran R (2002) Infestation and prevalence of copepod parasite, *Argulus indicus* on some freshwater fishes. *Nat Environ Pollut Technol* 1:201–206
- Shimura S, Inoue K, Kasai K, Saito H (1983) Studies on effects of parasitism of *Argulus coregon* (Crustacea: Branchiura) on Furunculosis of *Oncorhynchus masou* (Salmonidae). *Fish Pathol Tokyo* 18:37–40
- Sies H (1996) Biochemistry of oxidative stress. *Agew Chem Int Ed* 25:1058–1071
- Singhal RN, Jeet S, Davies RW (1990) The effects of argulosis-saprolegniasis on the growth and production of *Cyprinus carpio*. *Hydrobiologia* 202:27–31
- Sitja-Bobadilla A, Redondo MJ, Bermudez R, Palenzuela O, Ferreira I, Rianza A, Quiroga I, Nieto JM, Alvarez-Pellitero P (2006) Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporae: Myxozoa). *Fish Shellfish Immunol* 21:485–500
- Takahara S, Hamilton BH, Nell JV, Kobra TY, Oguna Y, Nishimura ET (1960) Hypocatalesemia a new genesis carrier state. *J Clin Investig* 29:610–619
- Tavares-Dias M, Martins ML, Kronka SN (1999) Evaluation of the haematological parameters in *Piaractus mesopotamicus* Holmberg (Osteichthyes, Characidae) with *Argulus* sp (Crustacea: Branchiura) infestation and treatment with organophosphate. *Rev Bras Zool* 16(2):553–555
- Tjalkens RB, Valerio LG Jr, Awasthi YC, Petersen DR (1998) Association of Glutathione S transferase isoenzyme-specific induction and lipid peroxidation in two inbred strains of mice subjected to chronic dietary iron overload. *Toxicol Appl pharmacol* 151:174–181
- Toovey JPG, Lyndon AR (2000) Effects of hydrogen peroxide, dichlorvos and cypermethrin on subsequent fecundity of sea lice, *Lepeophtheirus salmonis*, under fish farm conditions. *Bull Eur Ass Fish Pathol* 20:224
- Tort L, Torres P, Hidalgo J (1988) The effects of sublethal concentrations cadmium on haematological parameters in the dogfish *Scyliorhinus canicula*. *J Fish Biol* 32:277–282
- Tort L, Balasch JC, Mackenzie S (2003) Fish immune system. A cross roads between innate and adaptive responses. *Immunología* 3:277–286
- Treves-Brown KM (1999) Availability of medicines for fish. *Fish Vet J* 4:40–55
- Valdimirov VL (1968) Immunity of fish. *Bull Off Int Epizoot* 69:1365–1372
- Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol* 13:57–149
- Vijayaraghavan S, Rao JVR (1986) Starvation stress effects on tissue lactate and lactate dehydrogenase activity in *Anabas scandens* (cuvier). *Comp Physiol Ecol* 11(4):233–236
- Walker PD, Flik G, Bonga SEW (2004) The biology of parasites from the genus *Argulus* and a review of the interactions with its host. In: Wiegertjes GF, Flik G (eds) *Host-parasite interactions*, GARLAND Science/BIOS Science Publications, pp 107–129
- Wang GX, Wang JF, Yuan JL, Shen YH, Zheng W, Li L (2010) Activity of sanguinarine from *Macleaya cordata* to *Dactylogyrus* and six pathogenic bacteria in aquaculture. *Acta Bot Boreal Occident Sin* 27:1650–1655
- Wepener V, Van Vuren JHH, Du Preez HH (1992) The effect of hexavalent chromium at different pH values on the haematology of *Tilapia sparmani* (Cichlidae). *Comp Biochem Physiol* 101:375–381

- Wiegertjes GF, Stet RJM, Parmeatier HK, Van Muiswinkel WB (1996) Immunogenetics of disease resistance in fish; a comparable approach developmental and comparative Immunology. *J Exp Boil* 20:365–381
- Winkaler EU, Santos TRM, Machado-Neto JG, Martinez CBR (2007) Acute lethal and sublethal effects of neem leaf extracts on the neotropical freshwater fish *Prochilodus lineatus*. *Comp Biochem Physiol* 145:236–244
- Wright PA, Perry SF, Moon TW (1989) Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J Exp Biol* 147:169–188
- Wroblewski F, La Due JS (1955) Lactate dehydrogenase activity in blood. *Proc Soc Exp Boil Med* 90:210–213
- Wu G, Yuan C, Shen M, Tang J, Gong Y, Li D, Sun F, Huang C, Han X (2007) Immunological and biochemical parameters in carp (*Cyprinus carpio*) after Qompsell feed ingredients for long-term administration. *Aquac Res* 38(3):246–255
- Yao J-y, Li X, Shen J, Pan X-y, Hao G, Xu Y, Ying W, Ru H, Liu X-l (2011) Isolation of bioactive components from *Cheilodinium majus* L. with activity against *Trichodina* sp. *Aquaculture* 318:235–238
- Zhang YS, Andersson T, Förlin L (1990) Induction of hepatic xenobiotic biotransformation enzymes in rainbow trout by β -naphthoflavone. Timecourse studies. *Comp Biochem Physiol B* 95:24–253