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Immunostimulatory effects of curcumin in fish, *Labeo rohita* (H.)

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Curcumin, an orange-yellow phytochemical, hydrophobic and polyphenolic compound of turmeric (*Curcuma longa* Linn.), has been known to be a potent immunomodulatory agent in mice, horse, human and other higher vertebrates. In the present study, we have found its immunostimulatory properties in fish *Labeo rohita* (H.). Curcumin at low doses (15 and 1.5 μg) significantly ($P<0.05$) increased some non-specific immune parameters such as respiratory burst, myeloperoxidase, haemagglutination, haemolytic and bacterial agglutination activities up to 21 days post administration without any side effects. It has also further increased bacterial agglutination activities in *Aeromonas hydrophila* injected fishes primed with low dose (15 μg) curcumin.

Keywords: Curcumin, *Curcuma longa*, Fish, Immune response, Immuomodulatory, Indian major carp, *Labeo rohita*, Turmeric.
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Introduction

Turmeric (*Curcuma longa* Linn.), a medicinal plant, has been used for thousands of years in Indian Ayurvedic medicine. Components of turmeric are collectively termed as curcuminoids, which mainly include curcumin (diferuloyl methane), demethoxy-curcumin and bisdemethoxy-curcumin. But the major biologically active component of turmeric is curcumin, which is a yellow phytochemical, hydrophobic and polyphenolic compound[1]. Although curcumin has recently gained much attention for its therapeutic potentials in traditional Indian medicine for human uses due to its low toxicity and large biological activities but its pharmacological potential is still under investigation[2-4]. Several research findings indicate that curcumin can act as a potent immuomodulatory agent that can modulate the activation of T cells, B cells, macrophages, neutrophils, natural killer cells, dendritic cells, transcription factors, cell cycle proteins and signal transducing kinases[5]. It also has strong effect on cytokine production, humoral and cell-mediated immunity[6,7]. In this way, curcumin regulates multiple targets, which is needed for treatment of most diseases. Moreover, it is inexpensive, extremely safe even at very high doses and used as an immunomodulator in various animal models including human beings[8-12].

Despite the extensive characterization of its anti-inflammatory and immuomodulatory activities in various animal models, no such study has yet been carried out taking fish as a model organism. Further, diseases are the major stumbling blocks in the development of aquaculture causing heavy losses in the industry. Although antibiotics and chemotherapeutics are used for prophylaxis and treatment in intensive aquaculture, they have been widely criticized for their negative impact[13]. So far, the effects of a number of immunostimulants (such as levamisole, lipopoly-saccharide, glucans, peptidoglycan and muramyl dipeptide) have been extensively studied in a variety of fish species[14]. But knowledge on the use of traditional herbal medicines as immunostimulants is limited, even though such medicines seem to be a rich source of active substances for immunotherapy[15].

As no such information is available till date regarding the use of curcumin in fish health management, the present study was carried out to investigate the immuomodulatory effect of curcumin in fish, *Labeo rohita* (H.).

Materials and Methods

Materials and reagents

Curcumin in the form of CUR- 500 (containing > 95% curcumin) was obtained from UNICO Pharmaceuticals, India. It was dissolved in dimethyl sulphoxide (DMSO) and further diluted in phosphate buffer saline (PBS, *p*H 7.2).

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Experimental design

Indian major carp (*L. rohita*) juveniles of average weight ranging from 30 to 40 g were acclimatized in the wet laboratory of Fish Health Management Division of Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, India, 15 days prior to the start of the experiment. Fishes were divided randomly into 6 groups (Control, negative control, A, B, C and D) with 10 fish in each group and kept in different tanks with a water temperature of 27 to 30ºC. The fish were fed with artificial carp diet with constant aeration and daily one-third water exchange. Fishes were injected intra peritoneally (i. p.) with a dose of 1.5 mg, 150, 15 and 1.5 µg of curcumin in 0.1 ml of PBS per fish in group A, B, C and D, respectively whereas control group was injected with 0.1 ml PBS only. In addition the negative control group was injected with equal percentage of DMSO that is presented in the treated groups. The fishes of all the treated groups including control were bled at an interval of 7, 14, 21 and 42 days of post injection to study various immune parameters. After selecting suitable dose of curcumin that has immunostimulatory effect, another experiment was designed by taking 3 groups (A, B and C) containing ten juveniles of *L. rohita* in each group and maintained in 1000 litre cemented tanks. Each fish in both the groups (B and C) were injected with 15 µg of curcumin in 0.1 ml of PBS whereas group A was injected with PBS only and kept as control. After 7 days, group C was injected with formalin killed *Aeromonas hydrophila* bacterial suspension (Ahv, 10^7 cell/ml), whereas group B was kept as such. The fishes of all the 3 groups were bled at 15 days of post injection to study bacterial agglutination activity.

Immunoresponse studies

Myeloperoxidase activity

For determination of myeloperoxidase activity, 15 µl of serum was diluted in 135 µl of Hanks balanced salt solution (Ca^{2+}, Mg^{2+} free) and then 50 µl of substrate buffer [20 mM, TMB (3, 30,5,50-tetra methyl benzidine) and 5 mM H_2O_2] was added. The reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid and the optical density (O.D.) was read at 450 nm using UV-VIS spectrophotometer, Thermo Spectronic, UK[16].

Respiratory burst assay

The respiratory burst activity was measured by the reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals[17]. Briefly, 100 µl of heparinised blood from fish of each group was mixed with 100 µl of 0.2% NBT (Sigma, USA) solution and incubated for 30 min at 25°C. After incubation, 50 µl from the above mixture was added with 1 ml of N, N diethylmethyl formamide (Qualigens, India) and then centrifuged at 6,000 X g for 5 min. The O.D. of the supernatant was measured at 540 nm.

Bacterial agglutination activity

The agglutination test was conducted in U shaped microtitre plates. Two-fold serial dilution of 25 µl fish serum was made with an equal volume of PBS in each well, to which formalin-killed *A. hydrophila* (10^7 CFU/ml) suspension was added. The plates were incubated overnight at room temperature. The titre was calculated as the reciprocal of the highest dilution of serum showing complete agglutination of the bacterial cells.

Haemagglutination activity

The haemagglutination activity of serum samples was carried out using a standard method[18]. This assay was done in U-shaped microtitre plates by serial two-fold dilution of 50 µl serum (inactivated at 45ºC for 30 min) with PBS (*p*H, 7.2). Then 50 µl of freshly prepared 1% New Zealand white rabbit red blood cell (RBCs) suspension was added to each well. The plates were kept at room temperature (28-30°C) for 2 h or over night at 4°C, in case if agglutination was not visible within 2 hours. The titre was calculated as the reciprocal of the highest dilution of serum showing complete agglutination of RBCs.

Haemolytic activity

The haemolytic titre of serum was determined in a similar manner as described for HA titre by using fresh sera from all the groups. Titre was expressed as the reciprocal of the highest dilution of serum showing complete haemolysis of the rabbit RBCs[18].

Statistical analysis

The statistical analysis system (SAS) software (version 6.12) was used to analyze all the data[19]. One-way analysis of variance followed by DMRT was done to compare the variations in various immune parameters at significance level of difference at 0.05% level in different injected groups. The mean (± SE) of assayed parameters was calculated for each group of fish.

Results and Discussion

Several studies have shown that curcumin has immunomodulatory activity but its widespread clinical applications have been limited due to poor aqueous solubility and minimal systemic bioavailability[20]. According to Shoba *et al* (1998) orally administered curcumin has poor bioavailability and limits its therapeutic effects due to its rapid metabolism in the liver and intestinal walls[10]. However, reports indicate that curcumin can be injected intraperitoneally without any side effects[21,22]. In the present study also, intraperitonial administration of curcumin showed no toxic effect and systemic abnormalities in fish, even in higher doses during experimental period. In addition the % of DMSO (which is very negligible quantity) in the negative control group showed similar immune response with that of the PBS injected control group with no side effects. Following administration of curcumin at different doses (1.5 mg, 150, 15 and 1.5 µg); immune parameters such as myeloperoxidase, respiratory burst, haemagglutination, haemolytic and bacterial agglutination activities were measured in weekly intervals and presented in Figures 1-5. All these parameters were significantly higher (P<0.05) in groups administered with low dose of curcumin (15 and 1.5 µg) than the other injected groups including control. However, all the above immune parameters in these two injected groups did not vary significantly (P<0.05) in 7th, 14th and 21st days but declined significantly (P<0.05) at 42 days post administration. Whereas the increased bacterial agglutination activities even remained up to 42 days post administration. In the group primed with 15 µg of curcumin and further injected with bacteria, *A. hydrophila,* a further rise in bacterial agglutination activity was observed than the groups injected with curcumin and bacterial antigen separately (Fig. 6). This might be due to antibacterial effect of curcumin against some fish pathogens[23].

Phagocytic and neutrophil activities can act as indicators of the non-specific immune response[24] and can be measured by NBT and myeloperoxidase activities. In this study, both NBT and myeloperoxidase activities were higher at low dose (15 and 1.5 µg) of curcumin indicating that both the activities are also dose dependent. According to Franck *et al* (2008) curcuminoids have dose dependent inhibitory effects on reactive oxygen species production and myeloperoxidase release by activated neutrophils[25]. Few studies reported the effect of curcuminoids directly on the myeloperoxidase activity[25]. However, our results showed that, at higher doses, the activities of curcumin remained unaltered.

Phagocytosis is the first step of macrophage to kill invading microorganisms. Curcumin has been found to differentially activate macrophages and increases its phagocytic activities in rat and mice[26-28]. The prolonged curcumin-administrations (i.p.) did not impair the cytotoxic function of natural killer cells, the generation of reactive oxygen species and nitric oxide and cytokine production[22].

The haemolytic and haemagglutinating activities are also used to estimate innate immune response, which is mediated, by natural agglutinin and complement proteins. Figures 3 and 4 shows a dose of 15 and 1.5 µg of curcumin significantly increasing both haemagglutination and haemolytic activities. Sangvanich *et al* (2007) have also found the strongest haemagglutinating activity against rabbit erythrocytes by using crude extract of *Curcuma* species[29].

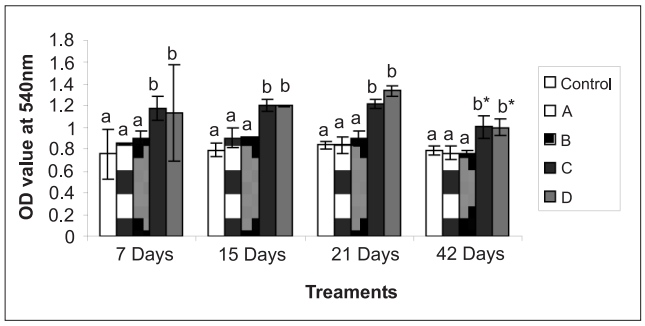

Fig. 1—The respiratory burst activity of *Labeo rohita* in different treated groups
Values are mean OD values ± S.E. Mean values bearing same superscript are not statistically significant (P>0.05).

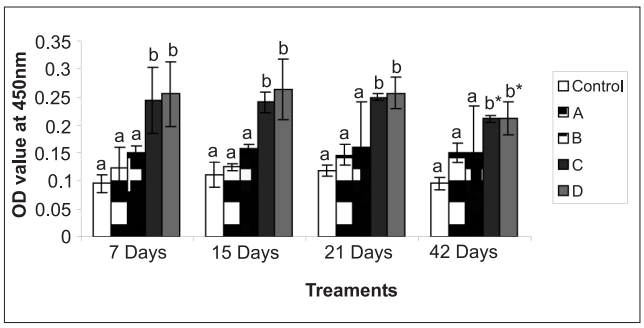

Fig. 2—The myeloperoxidase activity of *Labeo rohita* in different treated groups
Values are mean OD values ± S.E. Mean values bearing same superscript are not statistically significant (P>0.05).

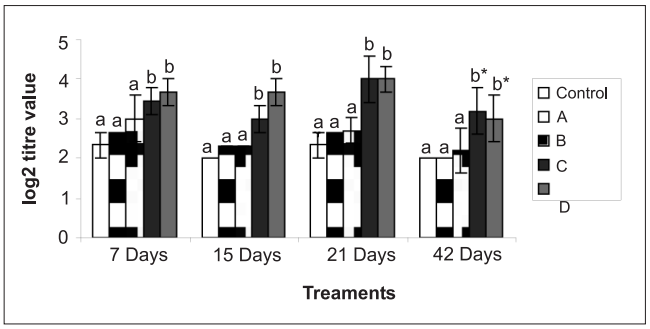

Fig. 3—The haemagglutinating activity of *Labeo rohita* in different treated groups
Values are mean $\log_2$ titer values ± S.E. Mean values bearing same superscript are not statistically significant (P>0.05).

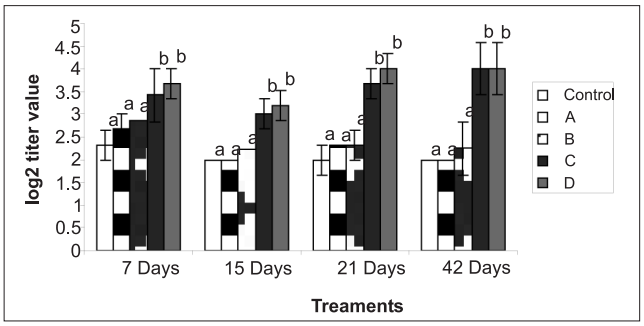

Fig. 5—The bacterial agglutination activity of *Labeo rohita* in different treated groups
Values are mean $\log_2$ titer values ± S.E. Mean values bearing same superscript are not statistically significant (P>0.05).

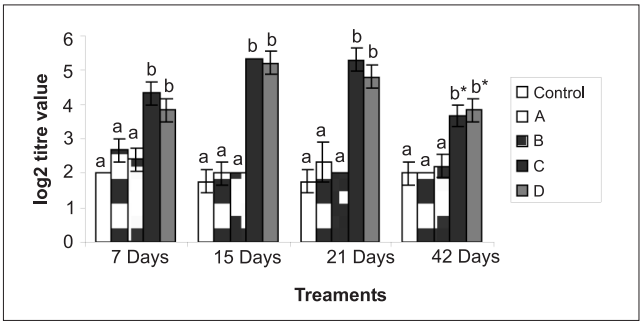

Fig. 4 — The Haemolysin titre of *Labeo rohita* in different treated groups
Values are mean $\log_2$ titer values ± S.E. Mean values bearing same superscript are not statistically significant (P>0.05).

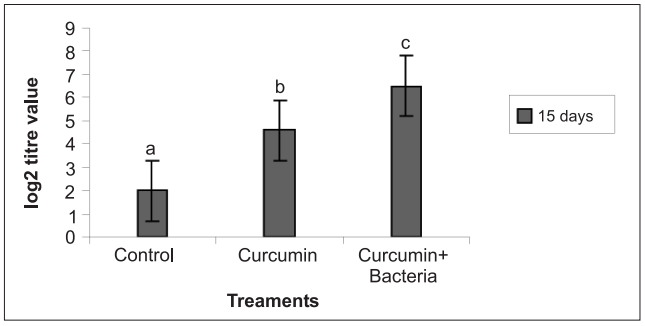

Fig. 6—The bacterial agglutination activity of *Labeo rohita* in different treated groups
Values are mean $\log_2$ titer values ± S.E). Mean values bearing different superscript are statistically significant (P>0.05).

Various *in vivo* studies also shown that low doses of curcumin enhance antibody production and lymphocyte proliferations while higher doses have opposite effect in mice and other higher vertebrates[28,30,31]. In contrast, the dietary intake of curcumin in rats showed significantly elevated IgG levels a higher dose than the minimum dose as observed by South *et al*[32].

Conclusion

The results obtained from the present study showed that, curcumin can significantly stimulate some of these immune parameters without any side effects and acts as an immunostimulant at low doses. However, its use and efficacy in fish health management needs to be further evaluated by using various pathogens.

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