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COMPARATIVE STUDY OF THE EFFECT OF CINNAMALDEHYDE AND CONCANAVALIN A ON HEMATOLOGICAL PARAMETERS AND MARKER ENZYMES IN GOLD FISH (*Carassius auratus)*

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ABSTRACT
Immunostimulants from plant origin are emerging into pharmacological field to serve as a novel drug candidate to - enhance the longevity, productivity, stress combating potential, strength to fight against invading external agents such as pathogens, bacteria, virus etc of fishes., with minimal toxic side effects to serve pharmacologists, aqua culturists, marine biologists. The Hematological parameters, cardiac, hepatic and nephritic marker enzymes suggest the basic immunomodulatory nature as well as non-toxic nature of the compound. In the present study, the stimulatory as well non-toxic nature of Cinnamaldehyde was analysed.

KEYWORDS: Gold fish, Hematological parameters, Immunostimulation, non-toxic.

INTRODUCTION

Fish immunology is receiving increasing attention because fish assume greater importance as models in environmental toxicology and as alternative models in biomedical research. The Blood, kidney and spleen play vital roles in fishes in maintaining the immuno balance in fishes. Trade of ornamental fish and aquarium maintenance is most popular in developed countries and gaining popularity also in many developing countries. The export potential of ornamental fishes from India is of the order of US $ 30 million. (**Ayyappan et al., 2011**). Thus, Fish culture is an important developing industry.

The intense culture practices, poor transport facility and negligence about sanitary aspects facilitate disease susceptibility in fish. The main drawbacks for intensive commercial production of ornamental fish are associated with diseases due to bacterial, viral and parasitic infection. In addition, commercial aquaculture has been negatively hampered by infectious diseases resulting in economic loss (**Lovell, 1996**). However, intensive fish stocking in ponds affect the health of fish.

Consequently, the physiological condition of cultured fish will be affected by environmental conditions. Thus fish farmers have to practice careful husbandry techniques large scale mortalities of fish occur due to these stress followed by parasite, bacterial, fungal and viral infection (Sakai, 1999). To reduce the risk of these diseases, there are various approaches such as quality control of the water of the culture system, control of disease by chemotherapy and immunoprophylaxis (Cossarini-Dunier, 1985).

The innate immune system is of prime importance in the immune defenses of fish. It is commonly divided into 3 compartments: The epithelial/mucosal barrier, the humoral parameters and the cellular component. Immunomodulators are substances that modify the immune system response to treat upon it. They modulate and potentiate the weapons of immune system and make it highly prepared state for any treat it may encounter.

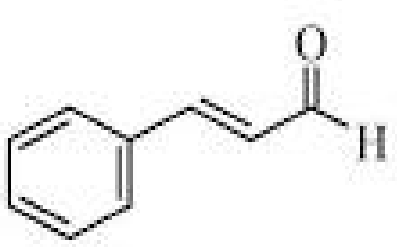
Immunomodulation is the process of modifying an immune response in a positive or negative manner by administration of a drug or compound. Many proteins, amino acids and natural compounds have significant ability to regulate immune response, including Interferons (IFN-γ), Interleukins. (Hill and Sarvetnick., 2002; Bach, 1996) and Steroids (Abo-Zena and Horwitz, 2002). Immunomodulators are biological or synthetic substances which can stimulate, suppress or modulate any of the immune system including both adaptive and innate wings of the immune response. Clinically immunomodulators can be classified into following 3 categories: **Immunoadjuvants, Immunosuppressants and Immunostimulators.** (Arya and Gupta., 2011).

Immunostimulants activate non-specific defense mechanisms thereby protecting the fish against infectious pathogens (Hsu et al., 1997). In fish, several immunostimulants such as levamisole (Kim and Cho.,

1991], chitin (Abeysekera et al., 1999], lactoferrin (Guerra et al., 2003), Nisin (Kalia, 2009), recombinant transferrin (Timothy et al, 2008), modified carbohydrate (Bhutani and Gohil., 2010), β-glucan (Vaidya and Devasagayam, 2007; Horeau and Dasilva., 1999), chitosan (Ayyappan et al., 2011) and various kinds of probiotics (Lovell, 1996; Ninawe, 2006) have been reported till date. These substances play a promising role in aquaculture by enhancing the resistance of cultured fish against diseases. Most of these studies have demonstrated the use of immunostimulants by injection or dietary administration.

India is found to be a country with rich biodiversity and enormous treasure of herbal plants and consequently called as medicinal garden of the world (Bhutani and Gohil, 2010). Many Indigenous system such as Ayurveda, Yoga, Unani, Homeopathy, Naturopathy and Siddha are famous and prevailing in India from decades (Vaidya and Devasagayam, 2007).

Cinnamaldehyde (CM) (C9H8O), (2*E*)-3-phenylprop-2-enal, is the major constituent of *Cinnamomum tamala* (Nees) of Lauraceae family. It is used mainly for flavouring cola-type drinks, with smaller amounts used in bakery products, sauces, confectionery and liquors. Like cinnamon bark oil, its use as a fragrance is limited by its skin sensitizing properties. It is mainly used for flavoring food and widely used in pharmaceutical preparation because of its hypoglycemic and carminative properties**.** Our present study focuses on the immunomodulatory potential of commercially available Cinnamaldehyde, which is present naturally in Cinnamomum tamala.



MATERIALS AND METHODS

Chemicals

Cinnamaldehyde, ConcanavalineA, Benzocaine (anaesthetic) were bought from Sigma Aldrich, Mumbai, India. All other chemicals were of commercial grade.

Experimental Fish and their maintenance

Disease free mature goldfish (Carassius auratus) having average weight (25-30 gms, app) were procured from aquarium in Kolathur, chennai, India. The fish were acclimatized for 10 days in laboratory condition in 100 L Glass tanks at 27 to 30^{0}C under continuous aeration. Fishes were fed with commercial goldfish pelleted diet @ 3% of body weight twice a day.

Experimental Design

The experiment was performed in rectangular plastic tubs (120x45x75 cm, 100 L capacity) covered with perforated lids and the water was drawn from bore well. The acclimatized fishes were randomly distributed into three distinct experimental groups including control. Each group had ten replicates and completely randomized design (CRD) was followed to set up the experiment. Group 1 – Control, Group 2 – Concanavalin A(ConA) (in 0.9% saline) 5mg/Kg b.wt and Group 3 – Cinnamaldehyde (CM) - 5µg/Kg b.wt. The Induction was done intramuscularly for a period of 3 days.

Collection of Blood and separation of Plasma

The fishes were anaesthetized with Benzocaine (20 mg/litre) before collection of blood sample. Blood was drawn by severing the caudal peduncle, by using 1.0 ml hypodermal syringe and 0.45x13mmx26 gauge needles, which was rinsed with heparin sodium (5000 IU/ml) solution before use.

Blood was immediately transferred to the test tube coated with thin layer of EDTA (as an anticoagulant) in order to prevent hemolysis and clotting of blood. The tubes were left undisturbed and plasma separated was collected using micropipette and stored in -20^{0}C until use.

Tissue Homogenate Preparation

10% Homogenate solution (0.1M Tris-Hcl Buffered Saline (TBS) of Heart, kidney and liver were prepared and used for the Enzyme assays.

Biochemical parameters: Hematological Parameters

The Total RBC, WBC (Blaxhall and Daisley, 1973) using Neubaur counting chamber, Differential count (DC) (Wintrobe, 1961), Levels of plasma protein (Lowry, 1951), Total immunoglobulin (Ig) (Anderson and Siwicki, 1995) and Hemoglobin (Hb) (Drabkin and Austin, 1932) were determined.

Marker Enzymes

Heart: Lactate dehydrogenase, (King, 1965a) and Cathepsin D, (Sapolsky et al., 1973), *Liver:* Acid Phosphatase, (king, 1965b) *Liver and Kidney:* Alkaline Phosphatase, (king, 1965b) Aspartate and Alanine Transaminase Activity, (king, 1965c) were assayed.

Statistical analysis

Comparisons were made between a - Control and ConA, b- Control and Cinnamaldehyde, c- ConA and Cinnamaldehyde; Results were expressed as mean ± SE (n=10), *($p<0.05$) ** ($p<0.01$)) *** ($p<0.001$) were considered to be statistically significant. One way ANOVA followed by student's t test using SPSS21 software package.

RESULTS

Wide variations in the hematological and biochemical parameters with significant difference among the groups were observed.

The hematological study to analyse total erythrocyte count (RBC) and total leukocyte count (WBC) in the blood of Control fishes and other treated fishes of C.auratus were represented in Figure 1a and 1b. A significant increase ($p<0.01$) in the levels of RBC was observed in ConA induced fishes when compared to control fishes. The levels of CM treated fishes were also found to be similar with that of ConA induced fishes showing Immunostimulatory potential. The Lymphocyte level (Figure 1c) of CM and ConA treated fishes were increased when compared with control group fishes. Significant decrease in Neutrophil levels was observed in CM and ConA treated fishes compared with control fishes.

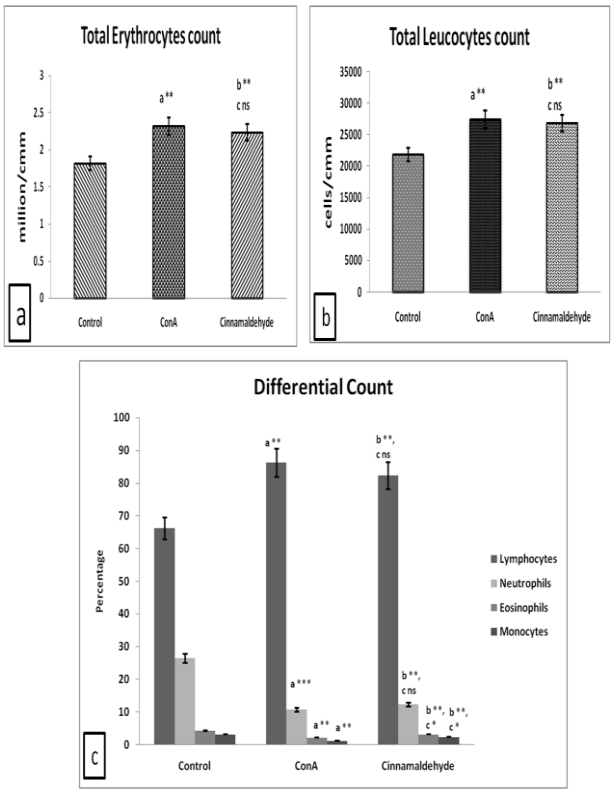


Figure 1: a) Total Erythrocytes count; b) Total Leucocytes count; c) Differential count of Leucocytes in percentage in control and Treated fishes.

The hemoglobin (Figure 2a) levels of ConA and CM treated fishes, showed significant ($p<0.001$ and $p<0.01$) elevated levels when compared to control fishes. The Plasma protein levels of CM administered fishes presented in Figure 2b were significantly ($p<0.05$) higher compared to control fishes. In ConA treated group, total protein levels were significantly ($p<0.01$) higher than control group. Significant ($p<0.01$) difference was clearly found between CM and ConA treated group fishes. Immunoglobulins (Ig) are the major humoral component of the specific immune response. In the present investigation the Total Immunoglobulin levels of ConA induced group illustrated in Figure 2b were enhanced in comparison with control group. The Total Immunoglobulin levels of CM treated fishes were also increased significantly on par with ConA treated group narrating the immunostimulatory potential.

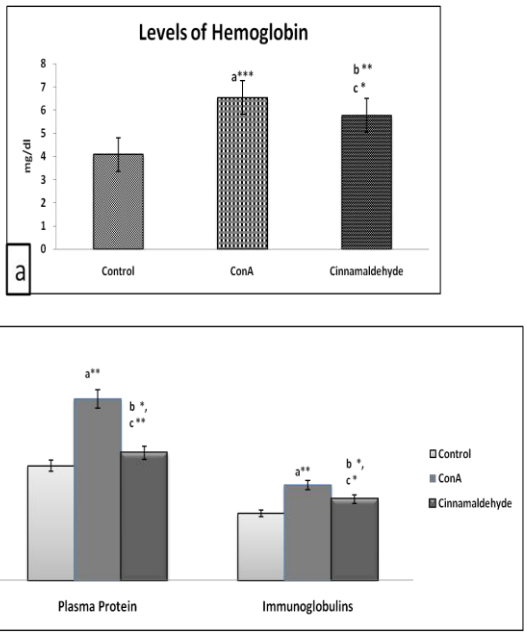


Figure 2: a) Total Hemoglobin b) Levels of Plasma protein and immunoglobulins in control and Treated fishes.

The Lactate Dehydrogenase (LDH) and Cathepsin D levels of CM administered fishes presented in Figure 3 were in near normal limits, whereas in ConA treated fishes, levels were increased significantly ($p<0.01$) when compared with the control.

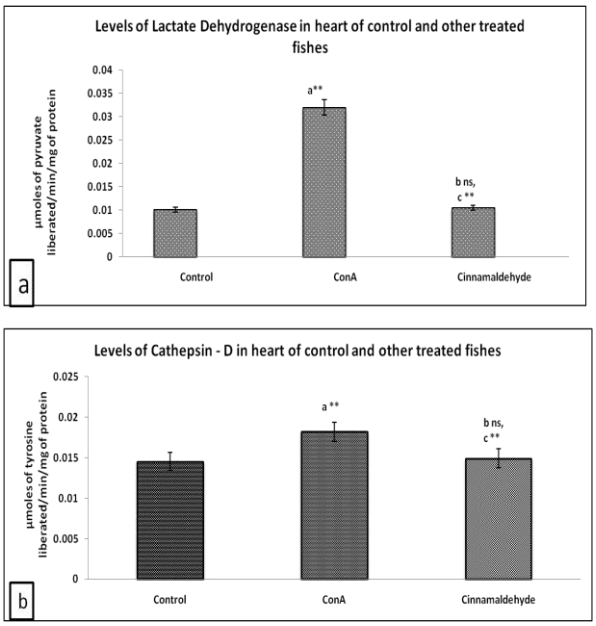


Figure 3: Levels of a) Lactate dehydrogenase; b) Cathepsin- D levels in heart of control and other Treated fishes.

The Alkaline and Acid Phosphatases levels of CM administered fishes presented in Figure 4a and 4b were in near normal limits with slight variations ($p<0.05$) compared with control. In ConA treated fishes, ACP and ALP levels were increased significantly ($p<0.01$) compared with the control. Significant ($p<0.05$) difference was observed between CM and ConA treated group fishes.

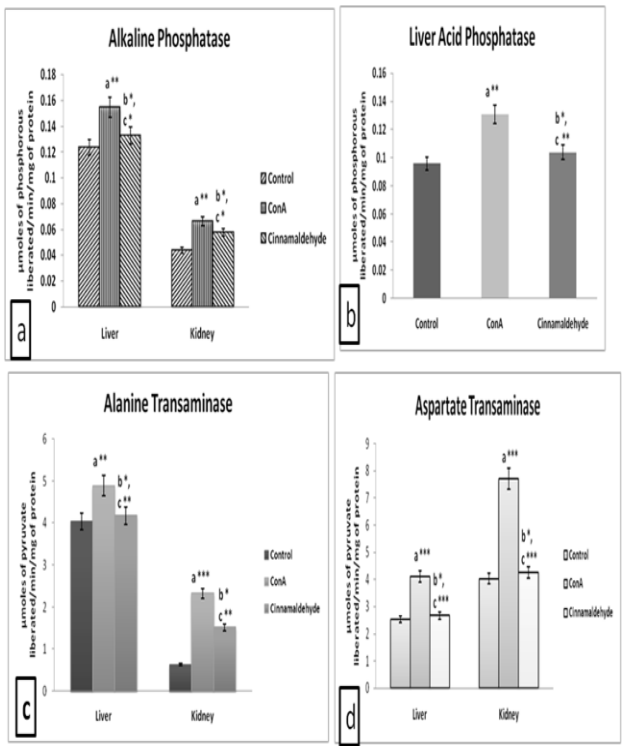


Figure 4: a) Alkaline Phosphatase levels in Liver and kidney; b) Acid Phosphatase levels in Liver; c) Alanine Transaminase levels in Liver and kidney; d) Aspartate Transaminase levels in Liver and kidney in control and other treated fishes.

Transaminases play an important role in protein and amino acid metabolism. Alanine transaminase (ALT) and Aspartate transaminase (AST) are indicators of liver and kidney damage. Assay of ALT and AST represented in Figure 4c and 4d, revealed slight variation ($p<0.05$) in CM administered fishes compared to control fishes. Increased activities of AST & ALT in tissues were observed in ConA treated fishes ($p<0.01$ and $p<0.001$) compared to control fishes. Liver infiltration, liver lesions are some of the conditions, where increased AST levels can be seen.

DISCUSSION

The innate immune system of fish is the first line and primitive defense against invading pathogens. The major components of the immune system are macrophages, monocytes, granulocytes and humoral elements such as lysozyme, immunoglobulins and the complement system. Immunoglobulins are the major humoral component of the specific immune response.

The present study summarizes the immunostimulatory role of CM in terms of RBC, WBC, DC, hemoglobin, Plasma protein and Immunoglobulins and non toxic and protective effect in terms of enzyme assays including marker enzymes of liver and kidney by maintaining their normal levels thereby preventing any toxic or lethal side effects comparable with standard ConA.

The total protein level (Soroush Ghodratizadeh et al., 2011) and immunoglobulin levels (Perera and Asoka Pathiratne., 2008) in the blood of immunostimulant levamisole treated Indian carp (*Catla catla*) were significantly higher than that of the respective control fish. Similarly the current findings also narrates significant increase in Total protein and immunoglobulin levels of Con A and slight increase in CM treated groups when compared to control group, demonstrating the stimulatory role of CM as of ConA.

WBCs are involved in the regulation of immunological function and their numbers increase as a protective response in fish to stress (Nussey et al., 2002; Pimpao et al., 2007). Winkaler *et al.* (2007) and Saravanan et al., (2011) have reported that WBC levels in neem leaf extract treated Carp fish, Cirrhinus mrigala increased from the control level, as a consequence of gill damage and due to toxic effects induced by it. In the present study, the significant increase in WBC indicates the overcoming potential of fishes due to induced stress and toxic effect by ConA and CM.

Increased total leukocyte cell count in monosex Nile tilapia, *Oreochromis niloticus* exposed to acute concentration of deltamethrin may be due to stimulated lymphopoiesis and enhanced release of lymphocytes from lymphomyeloid tissue (El-Sayed *et al.*, 2007) similar to our findings in ConA and CM treated fishes.

In toxicological studies, the alterations in the enzymatic activities directly reflect the metabolic disturbances and cell damage in specific organs (Casillas *et al.* 1983). There has been enhanced levels of LDH, ACP and ALP in liver than muscle and brain in effect to the stress induced by Cypermethrin as well as λ-cyhalothrin pyrethroids in *C.punctatus* indicated the stress based tissue impairment and metabolic changes (towards formation of lactate)., the stress indications of toxicants in fishes and autolysis of cells after theirs death.

Any damage in organs can directly be observed with ACP, ALP levels (Kumar and Sharma, 2012). Khan and Sharma, (2012), observed increased levels of ACP and ALP in liver and kidney tissues during sub lethal concentrations of chlorpyrifos. Pigs (Manzano *et al.* 2007) and Fishes (Cyprinus carpio) showed increased concentrations of AST, ALT with exposure to KCN due to hepatic (hepatic necrosis and loss of hepatocytes) and renal damages (focular degeneration) caused by them (Fahimeh Sadati, et al., 2013). Increased levels may be

significantly noted during Liver metastasis, fatty liver, biliary cirrhosis, acute hepatitis, chronic hepatitis, liver cirrhosis and renal diseases such as nephrosis, renal tubular acidosis, renal tubular defects etc., Similar increase in ACP, ALP, AST and ALT levels of ConA treated fishes narrates the toxic effects in addition to the immunostimulatory role and no significant change in the activity of transaminases and phosphatases of CM induced fishes comparable with control emphasizes it as a nontoxic immunostimulatory agent.

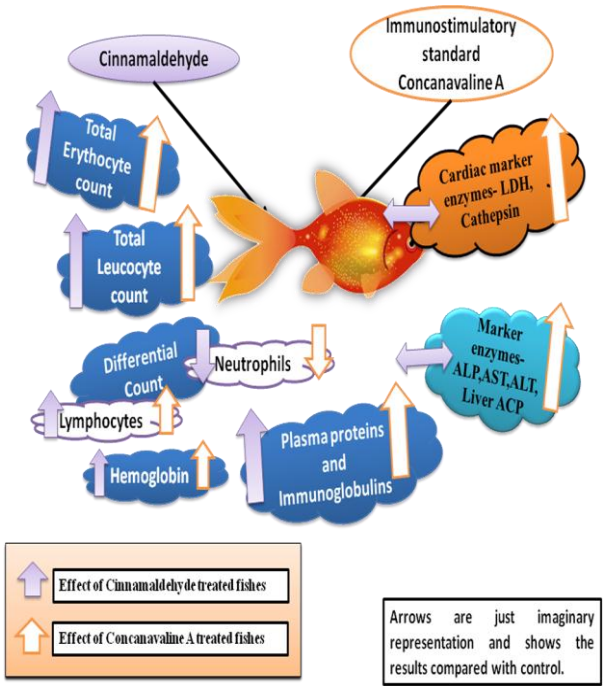


Figure 5: Summary of the effect of Cinnamaldehyde and Immunostimulatory standard ConcanavalineA on biochemical parameters in Carassius auratus.

CONCLUSION

Enhancing the life span of aquaculture fishes through immunostimulant mechanism by overcoming the infections and toxic agents is an urgent need by aqua culturists and marine biologists. Laying down the target through herbal formulation is an even better attempt to fit for the best future. An immunostimulatory compound serving to combat the levels of stress caused by external agents by balancing the enzymic machinery can be proposed as a best candidate in drug discovery. Therefore, along with stimulatory credential, basic biochemical parameters being not affected, Cinnamaldehyde can be proposed as an immunostimulant from the current research work (Figure 5). Further insight into the molecular mechanism of target is the future goal of study to be tapped down.

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