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Innate immune response and disease resistance in *Carassius auratus* by triherbal solvent extracts

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ABSTRACT

This study reports the effect of aqueous, ethanol and methanol triherbal solvent extract from *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa* on innate immune mechanisms such as phagocytosis activity, respiratory burst activity, alternative complement activity and lysozyme activity and disease resistance in goldfish (*Carassius auratus*) against *Aeromonas hydrophila*. Fish were intraperitoneally injected with different doses of 0, 5, 50 and 100 mg kg^{-1} body weight of each triherbal solvent extracts. The functional immunity in terms of percentage mortality and Relative Percent Survival (RPS) and innate immune response was assessed on week 1, 2 and 4 by challenging with live *A. hydrophila* (1×10^7 cells ml^{-1}). All the chosen innate immune parameters were enhanced in the ethanol and methanol triherbal solvent extract treatment after week 2. However, the aqueous triherbal extract was enhanced only after week 4. The ethanol and methanol triherbal solvent extracts administration preceding the challenge with live *A. hydrophila* decreased the percentage mortality in the experimental groups with the consequence increase in RPS values. The study indicates that all the doses of ethanol or methanol triberbal solvent extracts could be positively influence the immune response and protect the heath status of goldfish against *A. hydrophila* infection.

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1. Introduction

Globally the ornamental fish culture is a powerful income and employment generating industry. India is the third largest fish producer in the world, with an annual production of 6.40 million tones (2003–2004) contributed by 3.46 mt from inland and 2.94 mt from marine resources [1]. Among freshwater fish carps are the most favored for aquaculture; however losses due to infection in the semi-intensive and intensive carp farms are about $7035 million during 1992–1995 (ADB/NACA 1995, www.fao.org/docrep/005/Y3610E/y3610E13.htm). In Asian countries fish culture continues to be ravaged by bacterial diseases such as motile aeromonads septicaemia (MAS), furunculosis and edwardsiellosis. Among these, MAS caused by *Aeromonas hydrophila* is most widespread in freshwater fishes [2]. *A. hydrophila* is a ubiquitous, opportunistic free-living Gram-negative bacterium prevalent in crowded aquatic habitats [3]. MAS infect a number of species producing stress related diseases with the common symptoms of ulcerations, exophthalmia and abdominal distension [4–6].

The use of antibiotics and chemotherapeutics for prophylaxis and treatment in intensive aquaculture has been widely criticized for their negative impacts like accumulation of drugs in tissues, development of drug resistance and immunosuppression [7]. Alternatively, for some diseases vaccines against specific pathogens have been developed with varying degree of success. The wide range of pathogens in fish farming also limits the effectiveness of vaccine [8]. Hence, there is an urgent need to look for ecofriendly disease preventative measures to promote sustainable ornamental fish culture. One of the most promising methods of controlling diseases in aquaculture is strengthening the defence mechanisms of fish through prophylactic administration of natural immunostimulants [9]. Immunostimulants is well known increase resistance to infectious diseases by enhancing nonspecific defence mechanisms. The risk of disease and the level of resistance to infection could be increased by the use of better feeds, vaccines and immunostimulants was alternative to antibiotics and chemotherapeutics agents [10]. The immunostimulants, which have been tested for aquaculture include peptides like glucan [10], levamisole [11], chitosan [12], including a number of plant derived

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immunostimulants [13–15]; these directly activate the innate defence mechanisms acting on receptors and trigger intracellular gene activation that may result in the production of antimicrobial molecules [16]. Recently, these immunostimulants are widely used to improve the impaired immune functions and to stabilize the improved immune status [17]. On the other hand, substances obtained from natural sources being biodegradable and biocompatible have received more attention with regard to fish diseases. A number of plant material/products such as saponin [18], glycyrrhizin [19], aloe [20], *O. sanctum* [13,14], azadirachtin [15], *Viscum album*, *Urtica dioica* and *Zingiber officinale* [21], *Radix astragalin* seu Hedysari and *Radix angelicae sinensis* [22,23], *Astragalus radix* and *Scutellari radix* [24], and *Achyranthes aspera* [25,26] have been reported to enhance the immunity of fish against disease. However there is little information on the application of mixed herbal solvent extract on innate immune mechanisms. The present study was undertaken to examine the effect of different dose of triherbal aqueous, ethanol and methanol solvent leaf extract from *Azadirachta indica*, *O. sanctum* and *Curcuma longa* on the innate immune response and disease resistance in goldfish against *A. hydrophila* infection.

2. Materials and methods

2.1. Fish

Carassius auratus (23 ± 2 g; mean ± SE; $n = 500$) were obtained from a ornamental fish farm in Tiruchirapalli, Tamilnadu, India (Latitude: 10° 48′, North, Longitude: 78° 41′, East). Fish were transported alive in plastic bags containing water enriched with oxygen. They were acclimated for 2 weeks in glass aquaria (100 cm × 60 cm × 40 cm) filled with chlorine free tap water and provided with continuous aeration using electric air pumping compressors; 50% of the water was exchanged weekly twice to remove waste feed and fecal materials. During the experiment water temperature 20 ± 2 °C, pH 6.91 ± 1.44, salinity 0.25 ± 0.05 ppt, dissolved oxygen concentration 6.63 ± 7.78 mg^{-1} and photoperiod 14L:10D were maintained. All the fish were fed with a standard diet (16% fish meal, 16% groundnut oil cake, 16% sesame oil cake, 16% soya flour, 18% rice bran, 18% tapioca powder and 0.25% vitamin and mineral mix (w/v) with a proximate composition of 39% protein, 24% carbohydrate, 11% lipid and 9% ash) *ad libitum* twice a day at 0900 and 1500 h at a rate of 3% of their body weight.

2.1.1. A. hydrophila

A. hydrophila (Type: MTCC 646) obtained from Institute of Microbial Technology, Chandigarh, Government of India was maintained in the laboratory under standard conditions [6]. Subcultures were maintained on tryptic soy agar (TSA: w/v; Himedia, Mumbai) in slopes at 5 °C and routinely tested for pathogenesis [27] by inoculation into goldfish [28]. Stock culture in tryptic soy broth (TSB: w/v; Himedia, Mumbai) was stored at −70 °C in 0.85% NaCl with 20% glycerol (v/v) to provide stable inoculate throughout the experiment. *A. hydrophila* subcultures were prepared according to Harikrishnan et al. [6]. The culture was centrifuged at 1000 g for 10 min at −4 °C. The supernatant were discarded and the bacterial pellet was washed three times and resuspended in phosphate-buffered saline (PBS) at pH 7.4 [6]. The OD of the solution was adjusted to 0.5 at 456 nm which corresponded to 1×10^7 cells ml^{-1}.

2.2. Herbal extract

The leaves, *A. indica*, *O. sanctum* and *C. longa* were collected from the Bharathidasan University campus during May of 2007. The leaves were collected and washed in sterile distilled water. They were shade dried, powdered and stored at −20 °C until further use. The extraction was done by following the methods of Harikrishnan and Balasundaram [29]. Ten grams of each leaf powder to mix the equal quantity of the powders (w/w) were evenly mixed (1:1:1) and was placed into sterilized 100 ml conical flasks separately with 100 ml of solvents (distilled water, ethanol or methanol) added and mixed well. Conical flasks were tightly covered with aluminum foil, kept for 7 d at room temperature, and agitated daily to ensure complete digestion. Later, the above mentioned extracts were filtered through sterile muslin cloth. The filtrate was collected and the solvent was evaporated using rotary vacuum evaporator (Buchi SMP, Switzerland). The residue obtained after evaporation was mixed with sterile distilled water, ethanol and methanol separately at 0, 5, 50 and 100 mg kg^{-1} (w/v) in sterilized screw cap glass container for further use.

2.3. Experimental design and challenge experiment

To study the innate immune mechanisms, goldfish ($n = 250$) were injected intraperitoneally (50 μl) with 0, 5, 50 and 100 mg kg^{-1} body weight of aqueous ($n = 70$), ethanol ($n = 70$) or methanol ($n = 70$) solvent extract using 1-ml tuberculin syringe with 24-gauge needle on day 1. The corresponding control fish received 50 μl of distilled water ($n = 40$). All the groups were maintained separately in replicate tanks ($n = 500$). After herbal treatment, fish were injected (50 μl) with *A. hydrophila* (1×10^7 cells ml^{-1}) on day 7.

2.4. Bleeding and leucocytes separation

Blood samples (6 fish/group) were collected one of the replicate tanks in each treatment from the caudal vein at 1, 2 and 4 weeks after treatment. Heparin was used as an anticoagulant. Individual fish was sampled only once to avoid the influence on the assays due to multiple bleeding and handling stress on the fish. Leucocytes for assay were separated from each blood sample by density-gradient centrifugation [30]. Briefly, 1 ml of histopaque 1.119 (Sigma) containing 100 μl of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes and 1 ml of a mixture of 1.077 density histopaque and hemagglutination buffer and 1 ml of blood was carefully layered on the top. The sample preparations were centrifuged at 700 g for 15 min at 4 °C. After centrifugation, plasma was collected and stored at −20 °C until used for analysis. Separated leucocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). Cells were then washed twice in HBSS and adjusted to 1×10^7 viable cells ml^{-1}.

2.5. Phagocytosis activity

Phagocytic activity of blood leucocytes was determined spectrophotometrically by the method of Seeley et al. [31]. This assay involves the measurement of congo red-stained yeast cells that have been phagocytised by cells. To perform the assay, 1000 μl of the leucocyte suspension was mixed with 2000 μl of the congo red-stained and autoclaved yeast cell suspension (providing a yeast cell:leucocyte ratio of 20:1). The mixtures were incubated at room temperature for 60 min. Following incubation, 1 ml ice-cold HBSS was added and 1 ml of histopaque (1.077) was injected into the bottom of each sample tube. The samples were centrifuged at 850 g for 5 min to separate leucocytes from free yeast cells. Leucocytes were harvested and washed twice in HBSS. The cells were then resuspended in 1 ml trypsin–EDTA solution (5.0 g l^{-1} trypsin and 2.0 g l^{-1} EDTA, Sigma) and incubated at 37 °C overnight.

The absorbance of the samples was measured at 510 nm using trypsin–EDTA as a blank.

2.6. Respiratory burst activity

Respiratory burst activity of isolated leucocytes was quantified by reduction of ferricytochrome C [32]. Briefly, 100 µl of leucocyte suspension and an equal volume of cytochrome C (2 mg l^{-1} in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Cayman) at 1 mg ml^{-1} were placed in triplicate in microtitre plates. In order to test specificity, another 100 µl of leucocyte suspensions and solutions of cytochrome C containing PMA and superoxide dismutase (SOD, Cayman) at 300 U ml^{-1} were prepared in duplicate in microtitre plates. Samples were then mixed and incubated at room temperature for 15 min. Extinctions were measured at 550 nm against a cytochrome C blank in a multiscan spectrophotometer. Readings were converted to nmoles O_2^- by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA given alone for each fish, and converting O.D. to nmoles O_2^- by multiplying by 15.87. Final results were expressed as nmoles O_2^- produced per 10^5 blood leucocytes.

2.7. Natural haemolytic complement activity

The activity of the natural haemolytic complement of plasma was assayed using sheep red blood cells (SRBC, Biomedics) as targets [33]. Equal volumes of SRBC suspension (6%) in phenol red free Hank's buffer (HBSS) containing Mg^{2+} and EGTA were mixed with serially diluted plasma to give final plasma concentrations ranging from 10% to 0.078%. After incubation for 90 min at 22 °C, the samples were centrifuged at 400 g for 5 min at 4 °C to avoid unlysed erythrocytes. The haemoglobin content of the supernatants was assessed by measuring the absorbance at 550 nm in a microplate reader. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 µl of distilled water or HBSS to 100 µl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting $Y/(1 - Y)$ against the volume of plasma added (ml) on a $\log_{10} - \log_{10}$ scaled graph. The volume of plasma producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental group.

2.8. Lysozyme activity

Plasma lysozyme activity was measured spectrophotometrically according to the method of Ellis [34]. A 0.02% (w/v) suspension of *Micrococcus lysodeikticus* made up in 0.05 M phosphate buffer (pH 6.2) was used as substrate. Lyophilised hen egg white lysozyme was used as a standard. A new standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25 °C. The results were expressed as mg ml^{-1} equivalent of hen egg white lysozyme activity.

2.9. Disease resistance

A. hydrophila (MTCC 646) was inoculated in to TSB at 28 °C. The culture was centrifuged at 800 g for 15 min at 4 °C. The packed cells were washed and the required dose was prepared in PBS. Groups of 10 fish each in triplicates were administered with 50 µl of 0, 5, 50 and 100 mg kg^{-1} body weight of aqueous, ethanol and methanol triherbal solvent extract on day 1. The control fish were received 50 µl of distilled water. An untreated and a PBS injected control were also maintained. After the administration of triherbal solvent extracts fish were challenged with *A. hydrophila* (1×10^7 cells ml^{-1}) intraperitoneally (50 µl) on day 7. Earlier the challenge dose was standardized to give 80% mortality in the untreated group. Mortality was recorded on week 1, 2 and 4. The clinical symptoms were noted including hemorrhagic septicemia, distended abdomen and lesions on the ventral surface of the body. The cause of death was confirmed by re-isolating the organism from liver or kidney of 15% of dead fish using *Aeromonas* isolation medium (Himedia, Mumbai, India). RPS was calculated by the following formula of Ellis [35],

$$RPS = 1 - \frac{\text{(Percent mortality in treated group)}}{\text{Percent mortality in control group}} \times 100$$

2.10. Statistics

The data (mean ± standard error, SE) were analyzed by one way analysis of variance (ANOVA) followed by Tukey's test was used to compare the means between individual treatments in SPSS at $P < 0.05$ levels.

3. Results

3.1. Phagocytosis activity

The phagocytosis activity in head-kidney leucocytes was significantly enhanced after the administration of all the triherbal solvent extracts and the doses after week 4. Fish inject with all the doses of ethanol triherbal extract alone showed enhanced phagocytic activity on second week compared to the control. However, no significant phagocytosis activity were found the other groups at week 1 and 2 (Fig. 1).

3.2. Respiratory burst activity

The effect of different solvent extracts and the doses on respiratory burst activities of isolated phagocytic cells is shown in Fig. 2. The respiratory burst activity significantly higher at week 2 of 50 and 100 mg kg^{-1} doses of ethanol triherbal extract, but not at week 1 compared with the control group. However, in our experiment, 50 and 100 mg kg^{-1} doses of ethanol and methanol triherbal extracts significantly enhanced the respiratory burst activity of goldfish on week 4.

3.3. Natural haemolytic complement activity

The effect of all the triherbal solvent extracts on plasma natural haemolytic complement activity (ACH_{50}) is shown in Fig. 3. Serum complement activity, measured by the mean number of ACH_{50} units ml^{-1} serum, was increased by all the solvent extract after week 4. When fish were treated with aqueous, ethanolic and methanol triherbal solvent extracts for second week, the complement activity increased to a statistically significant degree ($P < 0.05$), although not in a dose dependent manner. There were no significant changes in this activity during the first week of treatments.

3.4. Plasma lysozyme activity

The lysozyme activity was significantly enhanced by the administration of all the solvent extract and the doses on week 2 and 4 ($P < 0.05$). However, there was no activity was found all the solvent extracts and the doses on the first week of treatments (Fig. 4). Therefore, in the present study, it was observed that the lysozyme activity was well enhanced on treatment with ethanol or methanol triherbal solvent extract on week 2 and 4.

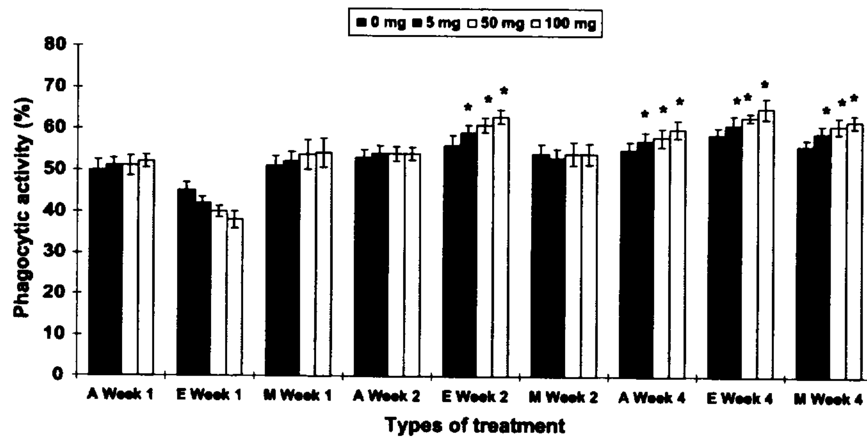

Fig. 1. Phagocytic activity of isolated phagocytic cells from blood of fish in control and aqueous (A), ethanol (E) and methanol (M) leaf extracts and doses treated goldfish (*Carassius auratus*) against *A. hydrophila* on week 1, 2 and 4. Data are expressed as the mean ± SEM ($n = 6$). Statistical differences ($P < 0.05$) from the control group are indicated by asterisks.

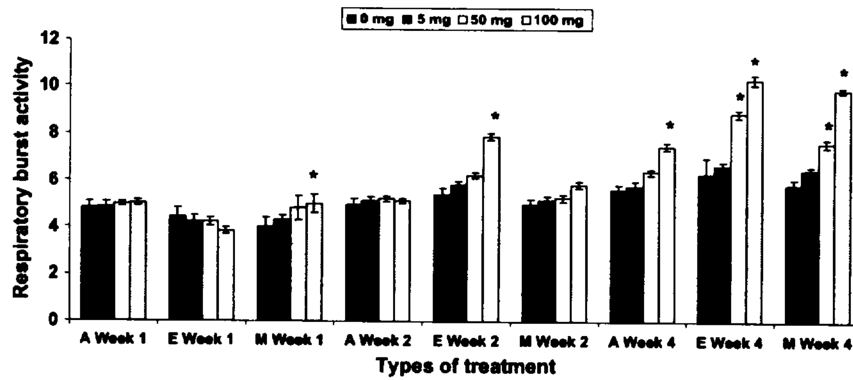

Fig. 2. Respiratory burst activity of phagocytic cells isolated from blood of fish in control group and in groups treated with different dosages of triberbal solvent extracts. Legends are the same as in Fig. 1.

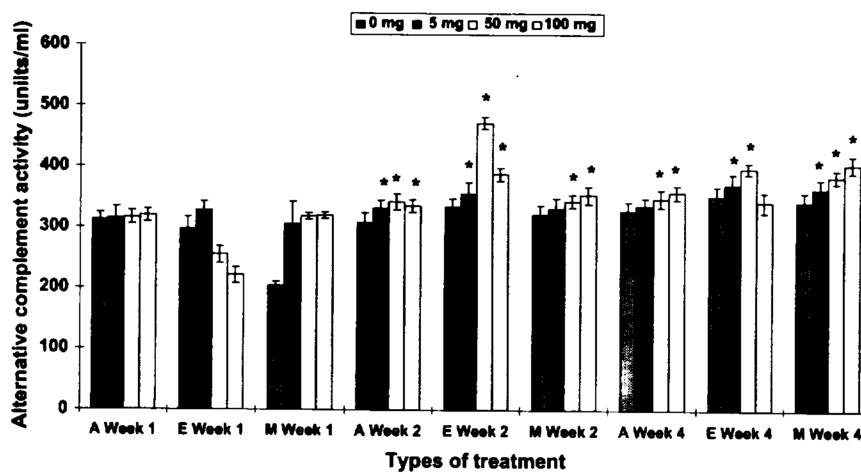

Fig. 3. Plasma natural haemolytic complement activity in control group and in groups treated with different dosages of triberbal solvent extracts. Legends are the same as in Fig. 1.

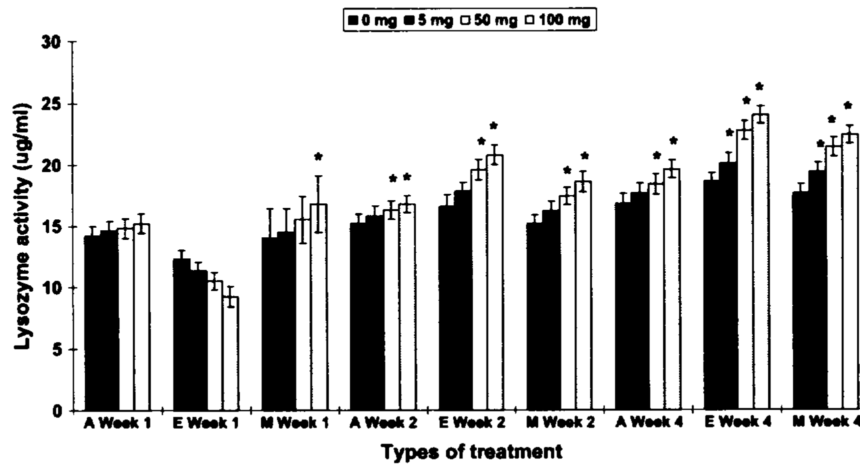

Fig. 4. Plasma lysozyme activity in control group and in groups treated with different dosages of triherbal solvent extracts. Legends are the same as in Fig. 1.

3.5. Disease resistance

After 6 days of aqueous triherbal extract treatment, fish were challenged with *A. hydrophila* and cumulative mortality was registered during 30 days (Fig. 5). The cumulative mortality over the experimental period in the control group (0 mg kg^{-1}) was 85%. All the other dosages of treated groups showed reduced mortality compared to the control. Cumulative mortalities in fish treated with 5 and 50 mg kg^{-1} of aqueous extract reached 40% and 25%, respectively. Fish injected 100 mg kg^{-1} of aqueous extracts had the higher mortality of 50% compared to the control. The percentage mortality was significantly reduced at 40% and 30% in fish after injecting with 5 and 50 mg kg^{-1} triherbal ethanol solvent extract (Fig. 6). The percentage mortality was untreated control group (0 mg kg^{-1}) was 90%. The highest dose of 100 mg kg^{-1} ethanol solvent extract produced the high mortality of 50%. The percentage mortality was significantly decreased in the triherbal methanol solvent extract treated fish when challenged with live *A. hydrophila* (Fig. 7). Among the groups administered with single dose of methanol solvent extract, the doses of 5 and 50 mg kg^{-1} has given the maximum protection with a percentage mortality of 45% and 30%, followed by 50% mortality in group treated with 100 mg kg^{-1} extract. The untreated group (0 mg kg^{-1}) was found high mortality of 90%. The enhancement of nonspecific immune parameters by ethanol and methanol triherbal solvent leaf preparation is possibly an important factor in reducing the percentage mortality and thereby protecting the fish against live *A. hydrophila* challenge.

4. Discussion

Enhancement of the immune system seems to be the most promising method of preventing fish diseases. The nonspecific immune system of fish is considered to be the first line of defence against invading pathogens, and is more important for fish than for mammals [36]. The major components of the innate immune system (nonspecific) are macrophages, monocytes, granulocytes and humoral elements, like lysozyme or complement system [37,38]. Immunostimulants and adjuvants used in fish vaccines are of interest, as they offer an alternative to the drugs, chemicals and antibiotics currently used in fish culture to control disease. Medicinal herbs as immunostimulants in aquaculture have been received more attention last two decades not only for their immune stimulating functions but also for their growth promoting effects and little or no side effects. It has been shown that herbal based immunostimulants are capable of enhancing immune responses and/or reducing losses from viruses, bacteria and/or parasitic infections in carp [27,39,40]. The nonspecific immune response is often reported as a function of macrophage activity such as phagocytosis and chemotaxis. Immunostimulants can be applied via injection, bathing or oral administration, the latter seems to be the most practicable [24,41–43]. To our knowledge, no studies focus on the optimal dose, sustainable time period and efficacy of triherbal solvent extracts in the economically important goldfish, *C. auratus*. Therefore, the present work attempted to address this lack of information by describing the general action of

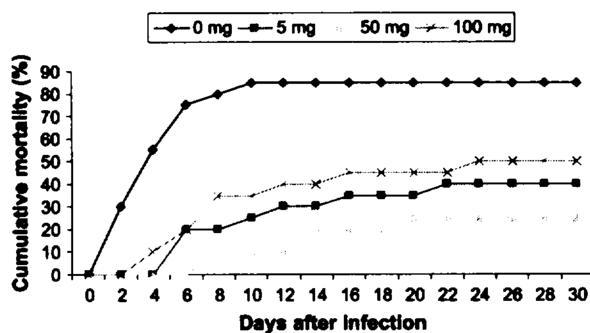

Fig. 5. Effect of different dosage aqueous triherbal leaf extract administered as single dose on the percentage of cumulative mortality (%) in goldfish (*Carassius auratus*).

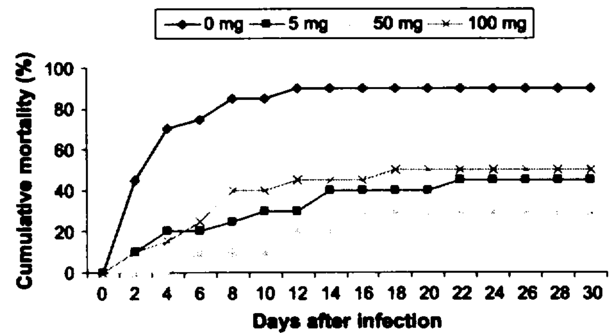

Fig. 6. Effect of different dosage ethanol triherbal leaf extract administered as single dose on the percentage of cumulative mortality (%) in goldfish (*Carassius auratus*).

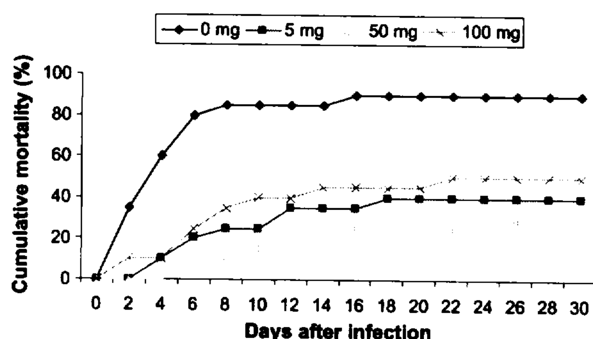

Fig. 7. Effect of different dosage methanol triherbal leaf extract administered as single dose on the percentage of cumulative mortality (%) in goldfish (*Carassius auratus*).

triherbal solvent extracts on the innate and acquired immune system of goldfish. Based on a previous study carried out in Chinese sucker [45] and also taking the above points into consideration, we used levels of 0, 5, 50 and 100 mg kg^{-1} triherbal solvent extracts intraperitoneal administration and measured the immune response and disease resistance of goldfish on 1, 2 and 4 weeks. This is because the administration through injection enabled the immunostimulant to be quickly absorbed and functional, while in the oral administration, the immunostimulant is slowly absorbed by the fish.

The present results showed that experimental fish injected with 5, 50 and 100 mg kg^{-1} doses of the ethanol triherbal solvent extracts significantly enhanced phagocytic activity on week 2. However, all the triherbal solvent extracts and doses were enhanced the phagocytic activity after fourth week. Phagocytic cells are the most important cellular components of the innate immune system of fish [44]. Their phagocytic activity is a primitive defence mechanism [45] and an important characteristic of the nonspecific immune system [31]. Herbal medicine extracts can also enhance phagocytosis in various fish species [21,46]. In our experiments, all the extracts with doses were able to significantly enhance the phagocytic activity of leukocytes isolated from goldfish only after fourth week. However, fish treated with all the doses of ethanol triherbal extract showed enhanced phagocytic activity on the second week compared to the control. On the other hand, no effect was found when fish were treated with all the extracts and doses on first week. This result suggests that the ethanol extract only enhanced phagocytic activity from second week. The present results agree with the previous observations on dosage of Scutellaria extract [24]. Therefore, all the doses of ethanol triherbal was enhanced the phagocytic activity on the second week, whereas aqueous and methanol triherbal extracts were enhanced after fourth week.

We observed a significant increase in the respiratory burst activity in all the extracts and doses of ethanol triherbal on the fourth week. On the other hand, no effect was found when fish were injected with all the doses and extracts on the first and second week of treatment. A significant enhancement of respiratory burst activity of ethanol triherbal on the second and fourth week had a positive effect on the intracellular superoxide anion production of isolated goldfish leukocytes. Phagocytes produce toxic oxygen forms during a process called respiratory burst [47]. Since superoxide anion is the first product to be released from the respiratory burst, the measurement of O_2^- has been accepted as a precise way of measuring respiratory burst [48]. We observed a significant increase in the respiratory burst activities in the 100 mg kg^{-1} dose group on the second week after the start of treatment. The present results showed that experimental fish treated with 5, 50 and 100 mg kg^{-1} doses of all the extracts significantly enhanced respiratory burst after fourth week. The present results agreement with the previous report in *Labeo rohita* fed with 0.5% *A. aspera* seed incorporated diet showed significant enhancement of superoxide anion production [26]. Jang et al. [19] reported that *in vitro* treatment with glycyrrhizine isolated from *Glycyrrhiza glabra* enhanced the respiratory burst activity of macrophages and the proliferative responses of lymphocytes from rainbow trout. Ethanol triherbal extract only can also increase the intracellular respiratory burst activity of goldfish phagocytic cells on second week. This effect was observed in large yellow croaker and common carp after feeding them with a diet containing a mixture of *Astragalus membranaceus* and *A. sinensis* extracts [22,23]. We observed a significant increase in the respiratory burst activities in the 100 mg kg^{-1} dose of ethanol triherbal group on the second week. Therefore the ethanol 100 mg kg^{-1} doses triherbal solvent extracts had a positive effect on the extracellular superoxide anion production of isolated leucocytes.

The present study indicates that all the extracts and doses could increase the alternate complement activity after 2 or 4 weeks of administration (Fig. 3). This is in contrast to the findings of Cook et al. [49] where oral application of EcoActiva™, a commercial b-glucan preparation did not result in any change in alternate complement activity. However, significant enhancement of serum complement activity was noticed after 10 weeks of feeding with 500 mg levamisole kg^{-1} diet in marine teleost gilthead seabream (*Sparus aurata* L.) [50]. The bactericidal activity of complement has been well recognized as one of the key killing mechanisms of clearing bacteria in fish [51]. However, no significant effect was seen on the first week of the alternative complement pathway. This is in line with the findings of Cuesta et al. [52] where intraperitoneal administration of propolis on gilthead seabream (*S. aurata* L.) did not result in a significant change in alternative complement activity while oral administration only provoked very low variations in this activity.

In the present study, both ethanol and methanol solvent extracts of triherbal leaves were found to have significant stimulatory effects on the second week of the lysozyme activity tested and disease resistance in *C. auratus*. Lysozyme is an important component in the immune system of fish. In the present study, it was observed that the lysozyme activity was well enhanced on treatment with all the solvent extracts and doses of triherbal on fourth week. Similar results of lysozyme activity was observed on 20, 25 and 30 days after feeding Jian carp [23] and large yellow croaker, *Pseudosciaena crocea* [22] with Traditional Chinese medicine (TCM) formulated from Astragalus root and Angelica root at a ratio 5:1 (w/w). *Oreochromis niloticus* fed with 0.1 and 0.5% *Astragalus radix* root for 1 week [24] and *L. rohita* fed with 0.5% of *A. aspera* seed were shown to enhance lysozyme activity [27]. The activity of serum lysozyme increased significantly in *Oncorhynchus mykiss* fed with a marine alga, *Dunaliella salina* at 100 and 200 mg kg^{-1} for 9 weeks. [53]. However, in the present study no significant effect was seen on the alternative complement pathway activity on the first week. In the present study, a significant increase in the lysozyme activities was observed in the 5, 50 and 100 mg kg^{-1} doses of the ethanol and methanol triherbal solvent extracts on the second and the fourth weeks. However, no significant effect of first week was seen on the lysozyme activity at any doses and extracts.

After challenge with *A. hydrophila*, all treated groups showed a reduced mortality compared to the control group. The best survival rate was observed in the group treated with ethanol or methanol triherbal extract. Ethanol triherbal extracted treated group reduced the mortality even more in the group treated with methanol triherbal. Mortality following challenge with *A. hydrophila* was decreased in the group of fish treated with triherbal

solvent extracts. Compared with untreated controls, ethanol and methanol triherbal extracts treatment increased the survival rate of the fish infected with *A. hydrophila*, by a minimum of 30% in the 50 mg kg^{-1} group, to a maximum of 45% in the 5 mg kg^{-1} group. In the control groups, ethanol and methanol 90% mortalities occurred within 30 days, whereas in the aqueous extract treated group, mortalities occurred 85%. The enhancement of nonspecific immune parameters by triherbal leaf preparation is possibly an important factor in reducing the percentage mortality and thereby protecting the fish against live *A. hydrophila* challenge. Earlier studies in this line also revealed that dietary supplementation of *O. sanctum* [13] and *Nyctanthes arbortristis* (unpublished data) leaves and intraperitoneal injection of water soluble and hexane soluble fraction of *Eclipta alba* and *N. arbortristis* leaves (unpublished data) enhanced the disease resistance against *A. hydrophila* in *Oreochromis mossambicus*. The present finding is in agreement with the results of Abutbul et al. [54] in tilapia fed with a diet containing ethyl acetate extract of *Rosmarinus officinalis* leaf powder and Fujiki et al. in carp administered with the fraction II of *Undaria pinnatifida*. They also found that the administration of this fraction 6 and 3 days prior to intraperitoneal challenge with *Eclipta tarda* significantly increased the survival rate [55]. The disease resistance against *A. hydrophila* was enhanced in *L. rohita* fed with 0.5% of Achyranthes [27]. It is important to estimate the increased protection in the ethanol and methanol triherbal extract treated fish to determine the efficacy of an immunostimulant. In this study after challenge with *A. hydrophila*, all dosage groups showed a reduced mortality compared to the control group. These results indicate that ethanol and methanol extracts have some ability to activate the immune system of goldfish.

There was no positive correlation between the effect of immunostimulant and dosage, in addition, high dosage might not enhance or inhibit the immune response [22,23]. To summarize, the results of our study showed that *A. indica*, *O. sanctum* and *C. longa* extracts in combination (mixing ratio of 1:1:1, w/w) could significantly enhance respiratory burst and phagocytic activity of blood phagocytes, plasma lysozyme activity and alternative complement activity. Comparatively, 50 mg kg^{-1} doses of ethanol or methanol had more obvious, stable and sustained effects on these nonspecific immune parameters. In addition, the lowest mortality was observed in the group treated with 50 mg kg^{-1} doses of ethanol extracts in the disease resistance against *A. hydrophila*. In conclusion, the administration of ethanol or methanol solvent extract enhanced some of the nonspecific immune parameters and disease resistance against *A. hydrophila* in goldfish on second week. But the ethanol solvent extract seems to be a better immunostimulant, which can have a promising role in aquaculture to prevent diseases and disease outbreaks. Also further investigation on the immunostimulatory effect of these plant preparations when administered along with feed in the field for disease prevention in aquaculture is warranted.

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