Effects of dietary *Aloe vera* on some specific and nonspecific immunity in the common carp (*Cyprinus carpio*)

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Abstract

In this study, the immunostimulatory effect of dietary *Aloe vera* crude extract was investigated in *Cyprinus carpio*. Three hundred fish were randomly divided into four groups. The first group was immunized with *Aeromonas hydrophila* bacterin (A.h) and was fed a diet contained 0.5% *Aloe vera*. The second group was immunized with A.h and fed a diet without *Aloe vera*. The third group was not immunized and fed with a diet that contained 0.5% *Aloe vera*. The fourth group remained as the control group and was neither immunized nor fed with *Aloe vera* supplements. Blood samples were taken every 14 d for eight weeks and samples were analyzed for hematological and immunological parameters. White blood count (WBC), red blood count (RBC), packed cell volume (PCV), lysozyme activity, serum bactericidal activity, complement activity, total protein, IgM concentration and specific *A. hydrophila* antibody were assessed. At the end of treatment, 20 fish from each group were challenged with *A. hydrophila*. WBC value, antibody level, lysozyme and bactericidal activity were significantly increased in the serum of fish treated with *Aloe vera* ($p<0.05$). No significant differences were seen in the RBC, PCV or complement activity among the groups. The relative percent survival (RPS) was found to be increased in fish fed with *Aloe vera*. This study indicates that the oral administration of *Aloe vera* is able to enhance some specific and non-specific immune responses in the common carp.

Introduction

Various immunomodulators have been reported to enhance nonspecific immunity in fish. These include killed bacteria and bacterial products (Kodama *et al*., 1998); levamisole (Gopalakannan and Arul, 2006); glucans (Santarem *et al*., 1997); certain vitamins (Hardie *et al*., 1991); and hormones (Kitler *et al*., 1997). These products are generally regarded as harmless and can be used as novel methods of minimizing disease risk and as a good substitution for antibiotics in aquaculture (Sakai, 1999; Gilliver *et al*., 1999; Salisbury *et al*., 2002). There is a growing interest in the use of medicinal herbs as immune stimulants in aquaculture (Raa, 1996) and the immunostimulating effects of herbal medicines in various fish species has been reported (Petit *et al*., 2001). Species in which this enhancement of the immune response has been confirmed, include *Pseudosciaena crocea* (Jian and Wu, 2003); *Cyprinus carpio*, (Jian and Wu, 2004; Sheikhzadeh *et al*., 2009); *Oncorhynchus mykiss* (Dügenci *et al*., 2003; Soltani *et al*., 2009); *Oreochromis mossambicus* (Logambal *et al*., 2000); *Oreochromis niloticus* (Chansue *et al*., 2000); and *Carassius auratus gibelio* (Chen *et al*., 2003).

Aloe barbadensis Miller (*Aloe vera*), is a perennial plant of the lily (Liliaceae) or Aloeaceae family, which is a tropical or subtropical plant characterized by lance-shaped leaves with jagged edges and sharp points (Lawless, 2000). Aloe inner gel is the colorless gel consisting primarily of water and polysaccharides, including pectin, cellulose, hemi cellulose, glucomannan, acemannan and mannose derivatives (Lee *et al*., 2001). Acemannan is considered to be the main functional component of *Aloe vera* and is composed of a long chain of acetylated mannose (Lee *et al*., 2001). The physiological activity of the polysaccaharides in *Aloe vera* has been widely reported. Glucomannan and acemannan from *Aloe*

vera were found to accelerate wound healing, activate macrophages, stimulate the immune system and have antibacterial and antiviral effects in mammals (Choi, 2001; Pugh, 2001; Tan and Vanitha, 2004). Kim (1999) also reported that this plant increased the resistance of rockfish against *Vibrio alginoliticus*. Although the immunomodulatory potential of *Aloe vera* on the human immune system is well established (Tan and Vanitha, 2004), there is to date no report of the effect of *Aloe vera* on the immune system of fish.

In this study, the immunostimulatory effects of dietary Aloe vera were investigated in *Cyprinus carpio* in order to discover its effects on the immune system and the resistance to bacterial infection.

Materials and Methods

Fish

Three hundred juvenile common carp (*Cyprinus carpio*, weighing 108 ± 11.4 g, were obtained from a fish farm in Ahvaz, Khuzestan province, Iran. Fish were transferred to fiberglass tanks and kept for one week to acclimatize. Water quality factors were recorded during the experiment as: temperature, 25 ± 1°C; dissolved oxygen, 8-10 ppm; pH, 7.9 ± 0.3; NO_2, <0.01 ppm and NH_3, <0.1 ppm. The water exchange rate was 10% of the water volume daily.

Experimental food preparation

The diets used in the experiment were prepared by mixing commercial carp food (Chineh Company, Iran), with the crude extract of *Aloe vera* (Baridj essence product) in ratio 5 g of *Aloe vera* per kilogram of food (i.e. 0.5% *Aloe vera*). For better homogenization, one volume of the crude extract of *Aloe vera* was dissolved in 5 volume water and the homogenized solution was then sprayed onto a thin layer of food. The Aloe-free diet was sprayed by the same method with water.

Grouping

Fish were then distributed into 300 L tanks, (75 fish, per tank) equipped with a thermostatic heater (Athman, China), suitable aeration, and external biofilters (Athman, China). Two groups of fish in were intraperitonealy immunized with 100 µl of Aeromonas hydrophila (A.h) at concentration of 9×10^8 cell/ml (Babaet al ., 1993) on days zero and 14 (Immunized treatments). Non- immunized fish were injected with 100 µl of sterile phosphate buffered saline (PBS).

One group from each immunized and non-immunized groups fed with an *Aloe vera* -treated diet, and the others were fed with an *Aloe vera* -free diet. All treatments were fed 5% of their body weight twice a day standard feed based on during the experimental period (six weeks).

Blood sampling and assays

Blood samples (2 ml/fish) were taken from ten fish in each group via caudal vein every 2 week intervals for eight week. Blood sample (500 µl) was taken for hematological analysis on the same day and the remaining blood (1.5 ml) was immediately refrigerated. For 12 h, the sera were separated and stored at -20°C until needed. Then at the end of trial, 20 fish in each group were randomly selected to evaluate the relative percentage survival (RPS).

RPS

A. hydrophila (AH04, Kindly received by Prof. Mehdi Soltani Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Iran) was inoculated in a tryptone soy broth and was incubated at 30°C. After centrifugation at 800 × g for 15 min, the packed cells were washed and prepared in PBS. At the end of treatment, twenty fish in each of the groups were injected intraperitoneally with 0.1 ml/ of $2\times LD_{50}$ suspension of the bacteria 1.6×10^7 cfu/fish in PBS. Daily mortality was recorded for 14 days and the cause of death was ascertained by re-isolating the bacteria from the kidney and liver of dead fish (Misraet al ., 2006). Relative percentage survival (RPS) was calculated as follows:

$$RPS(\%)=\frac{\text{Mortality (\%) of untreated controls - Mortality (\%) of treated}}{\text{Mortality (\%) of untreated controls}}*100$$

Lysozyme activity assay

Serum lysozyme activity was measured as described by Ellis (1990). Briefly, 10 µl of serum was mixed with 200 µl of a Micrococcus lisodeichticus (Sigma) suspension at 0.2 mg ml^{-1} in 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 27°C, and its OD was detected after 1 and 6 min at 530 nm using an ELISA (enzyme-linked immunosorbent assay) plate reader. One unit of lysozyme activity was defined as the amount of enzyme that produced a decrease in absorbance of 0.001 min/ml serum. Lysozyme concentrations were calculated using a standard curve of lysozyme from chicken egg white (Sigma) concentrations.

Serum bactericidal activity

The method used for serum bactericidal activity was followed a modified version of that adopted by Kajita et al. (1990). The serum samples were diluted three times with 0.1% gelatin-veronal buffer (GVB^{++}; pH 7.5, containing 0.5 mM ml^{-1} Mg^{2+} and 0.15 mM ml^{-1} Ca^{2+}). *A. hydrophila* (live washed cells) were suspended in the same buffer to make a concentration of 1×10^6 cfu ml^{-1}. The diluted sera and bacteria were mixed at a ratio of 1:1 and incubated for 90 min at 25°C and continuously agitated. The number of viable bacteria was then calculated by counting the resultant colonies from the incubated mixture on TSA (tryptic soy agar) plates after incubation for 24 h in duplicate.

Total serum protein and globulin

Samples were analyzed for total protein using the method outlined by Lowry et al. (1951). Albumin content was measured using a standard albumin estimation kit (Zistchem Diagnostics, Iran) and the globulin content was estimated by subtracting albumin from total protein

Hematology

Total leukocyte count (TLC) and red blood cell count (RBC) were determined as described by Schaperclaus et al. (1991). The packed cell volume (PCV) was determined by centrifugation at 2000 rpm for 20 min.

Bacterial microagglutination titer (MAT)

The agglutination test was conducted in 'U' shaped microtiter plates. Two-fold serial dilution of the 25 ml serum of fish was made with an equal volume of PBS in each well, to which 25 ml of formalin-killed Aeromonas hydrophila (10^7 cells/ml) suspension was added. The plates were incubated overnight at room temperature. The titer was calculated as the reciprocal of the highest dilution (based on $\log_2$) of serum showing complete agglutination of the bacterial cells (Swain et al., 2006).

Alternative complement pathway (ACP) activity

ACP activity was assayed according to the method adopted by Selvaraj et al. (2005). Briefly, 0.5 ml of serially diluted serum in ethylene glycol tetra acetic acid (EGTA)-Mg-gelatin veronol buffer (GVB; Sigma) was placed in a set of test tubes and 0.2 ml of a sheep RBC suspension (2×10^6 cells/ml) was added. This mixture was incubated at 15°C for 90 min. The addition of 2.8 ml of 10 mM EDTA GVB buffer stopped the hemolytic reaction. After centrifugation, the value (percent hemolysis/100) was calculated from the optical density (OD) at 414 nm of the supernatant. The value y/ (1-y) and the reciprocal of the serum dilution were plotted on semi-log graph paper and the ACH_{50} (units ml^{-1}), the reciprocal dilution giving 50% hemolysis (y (1-y) =1), was calculated from the graph.

Statistical analysis

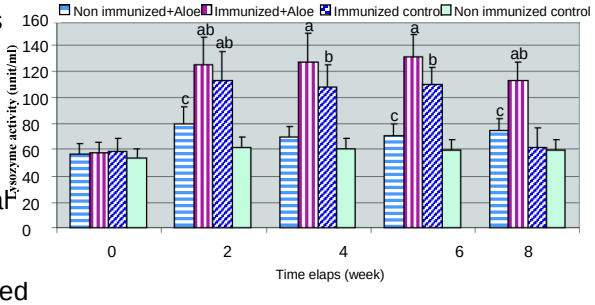
SPSS version 13 software was used for statistical analysis of data. Analysis of Variance (ANOVA) was used for comparison of means among all groups and the student's t-test was used for comparison of data between the groups treated with Aloe vera and the control groups in both immunized and non-immunized arms of the study. A p-value of <0.05 was accepted as significant.

Results

Lysozyme activity

The results of lysozyme activity are showed in Figure 1. Lysozyme activity was enhanced in both the Aloe vera treated groups when compared to the controls, but these enhancements were significant only in week two in the non-immunized treatment group and in weeks two and four in the immunized treatment groups ($p<0.05$).

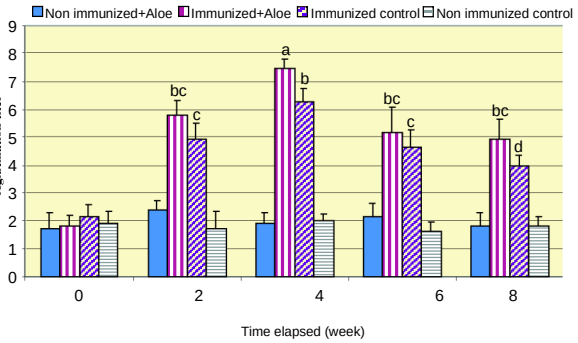
Figure 1: The effect of Aloe vera crude extract on serum lysozyme activity of immunized and non-immunized common carp. Significant differences ($p<0.05$) are marked by different letters



Bacterial agglutination titer

The result of the agglutination titer is showed in Figure 2. Immunization plus Aloe vera treatment showed a significant increase in the anti-A. hydrophila antibody titer ($p<0.05$) during weeks two and four compared with the non-immunized carp that received Aloe vera treatment. No significant difference was seen in the antibody titer between the non-immunized groups.

Figure 2: The effect of Aloe vera crude extract on anti A. hydrophila antibody in immunized and non-immunized fish. Parameters with significant differences ($p<0.05$) are marked by different letters.



Alternative complement pathway

No significant difference was seen in the complement activity between Aloe vera treated and Aloe vera free treatments. This was not only in the immunized but also in the non-immunized groups.

Serum proteins

The levels of total protein and IgM showed significant differences between Aloe treated and Aloe

free groups in immune treatments in weeks two, four and six. Such differences were seen in non-immunized fish just in weeks four and six (Table 1).

Hematology

The hematological parameters after treatment are shown in Table 1. Non-immunized Aloe treated group showed a significantly increased TLC compare to control ($p<0.05$). The RBC and PCV did not show any significant difference among the control and experimental groups (Table 1).

Post-challenge protection

The mortality patterns of all groups during the two-week post-challenge period are shown in Figure 3. The highest relative percentage survival (RPS) were recorded in immunized group that also received Aloe vera (75%) and the lowest RPS was observed in the non-immunized non-Aloe vera -treated group (20%). The RPS value was increased in both Aloe vera-treated groups, as compared to the control groups ($p<0.05$).

Discussion

Application of immunostimulators, particularly herbal immunostimulants in the aquaculture industry, can be considered a remarkable advantage because of their safety and the fact that they are considered environmentally friendly (Dügenci et al., 2003; Jian and Wu, 2004). Aloe vera has been found to stimulate the immune responses significantly in both in vitro and in vivo in mammals (Tan and Vanitha, 2004). In the present study, oral administration of Aloe vera increased serum lysozyme activity in both the immunized and non-immunized groups compared to both the immune and normal control groups. It has been observed that immunostimulants, vaccines and probiotics can enhance the plasma lysozyme activity (Swain et al., 2006; Yeo et al., 2007). Lysozymal activity is an important defense mechanism in fish, which causes lysis of bacteria and activation of the complement system and phagocytes by

Figure 3: Effect of Aloe vera extract on cumulative mortality pattern during 14 days post challenge with A. hydrophila. Twenty fish from each group were used for the challenge test.

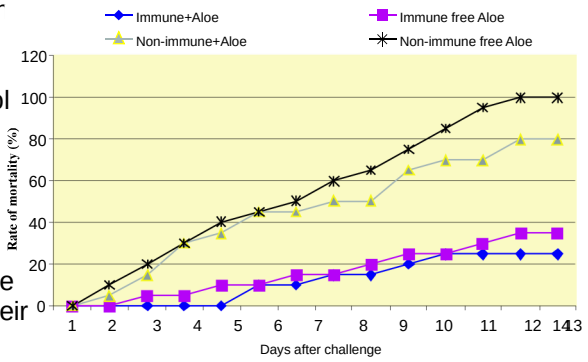


Table 1: The effect of Aloe vera crude extract on some immunological and blood indices of common carp

Parameters	Group	Treatment	Zero day	Week 2	Week 4	Week 6	Week 8
Serum bactericidal activity*	Non immunized	+Aloe	183.3±13.1[a]	172.4±18.1[a]	179.6±16.3[a]	176.5±19.9[a]	176.8±19.8[a]
		free Aloe	172.8±21.0[a]	168.9±19.9[a]	174.5±22.2[a]	180.9±17.1[a]	184.5±36.7[a]
	Immunized	+Aloe	175.8±15.3[a]	137.4±35.6[b]	129.7±27.5[b]	145.2±31.3[ab]	161.3±31.8[ab]
		free Aloe	189.6±19.5[a]	140.1±34.5[b]	131.8±31.6[b]	150.4±30.3[ab]	168.1±23.9[a]
ACH $_{50}$ (unit ml^{-1})	Non immunized	+Aloe	567±94[a]	552±146[a]	539±120[a]	570±125[a]	564±114[a]
		free Aloe	523±135[a]	540±97[a]	514±96[a]	547±84[a]	518±117[a]
	Immunized	+Aloe	528±135[a]	504±143[a]	544±118[a]	533±108[a]	495±132[a]
		free Aloe	544±163[a]	570±125[a]	512±81[a]	564±114[a]	547±69[a]
Total serum protein (g dl^{-1})	Non immunized	+Aloe	3.2±0.5[a]	3.76±0.5[ab]	3.98±0.3[b]	3.72±0.6[ab]	3.6±0.41[aa]
		free Aloe	3.08±0.6[a]	2.86±0.5[a]	3.02±0.6[a]	2.74±0.5[a]	3.46±0.65[aa]
	Immunized	+Aloe	3.36±0.8[a]	4.26±0.6[b]	4.42±0.7[b]	3.86±0.8[aa]	3.7±0.62[aa]
		free Aloe	2.86±0.7[a]	3.5±0.5[a]	3.46±0.8[a]	3.58±0.6[a]	2.7±0.33[a]
Serum globulin (g dl^{-1})	Non immunized	+Aloe	2.3±0.5[a]	2.86±0.5[ab]	3.04±0.2[b]	2.84±0.6[ab]	2.72±0.42[ab]
		free Aloe	2.14±0.7[a]	1.94±0.7[a]	2.12±0.7[a]	1.82±0.6[a]	2.52±0.85[ab]
	Immunized	+Aloe	2.52±0.8[a]	3.4±0.5[b]	3.5±0.8[b]	2.98±0.9[ab]	2.82±0.67[ab]
		free Aloe	2.04±0.8[a]	2.62±0.5[a]	2.56±0.7[a]	2.64±0.8[a]	1.78±0.43[a]
WBC count (/ mm^3)	Non immunized	+Aloe	4095±2549[a]	6560±2656[a]	7740±2109[a]	8045±2089[a]	7400±2863[a]
		free Aloe	5025±2950[a]	5995±2879[a]	5640±2804[b]	4880±2016[b]	5010±1827[b]
	Immunized	+Aloe	4440±2640[a]	5940±2660[a]	7830±2270[a]	8270±1540[a]	7940±4054[a]
		free Aloe	3890±2411[a]	5370±2416[a]	7520±1850[a]	7690±3817[a]	6880±3772[a]
RBC count (×10^{6}cell/mm^3)	Non immunized	+Aloe	1.32±0.35[a]	1.37±0.34[a]	1.22±0.33[a]	1.33±0.28[a]	1.19±0.19[a]
		free Aloe	1.287±0.21[a]	1.334±0.31[a]	1.342±0.34[a]	1.315±0.11[a]	1.167±0.08[a]
	Immunized	+Aloe	1.317±0.29[a]	1.29±0.25[a]	1.289±0.27[a]	1.185±0.14[a]	1.271±0.17[a]
		free Aloe	1.176±0.25[a]	1.339±0.25[a]	1.321±0.28[a]	1.23±0.15[a]	1.362±0.35[a]
PCV (%)	Non immunized	+Aloe	25±8.5[a]	28.1±8.9[a]	26.3±2.2[a]	25.1±7.6[a]	27.7±8.3[a]
		free Aloe	26.5±6.[a] 1	24.2±7.6[a]	25.3±7.5[a]	26.9±5.5[a]	24.8±6.2[a]
	Immunized	+Aloe	26.9±6.8[a]	27.6±5.3[a]	24.7±8.9[a]	25.5±7.5[a]	26.2±3[a]
		free Aloe	24.9±7.2[a]	27.6±4.1[a]	27.2±1.9[a]	27.5±6.4[a]	25±2.8[a]

acting as an opsonin. Elevated lysozyme level was measured in crucian carp (Chen et al., 2003), large yellow croaker (Jian and Wu, 2003) and the common carp (Jian and Wu, 2004) after the fish were fed with various herbal extracts that included Eclipta alba, Radix astragalin seu Hedysari and Radix angelicae sinensis. In this study, administration of Aloe vera in both the immunized and non-immunized groups enhanced the survival rate after a challenge with live A. hydrophila. The highest survival rate (75%) was observed in the immunized group that also received Aloe vera versus zero survival in the non-immunized control group. The present findings are in agreement with the results of Kim et al. (1999), who showed resistance against Vibrio alginolyticus in rockfish fed with an Aloe vera-enriched diet. Similar results have also been reported in tilapia following the oral administration of Rosmarinus officinalis leaf powder (Abutbul et al., 2004), Eclipta alba leaf aqueous extract (Christybapita et al., 2007), extract of Solanum trilobatum (Divyagnaneswari et al., 2007), and Zataria multiflora essential oil (Soltani et al., 2009).

Serum total protein and globulin are considered as good indicators for determining immune system activation (Siwicki et al., 1994). Certain herbal immunostimulants have been reported to increase total protein as well as total globulin in fish (Vasudeva et al., 2004). However, other reports indicate a lack of immunostimulant influence on serum proteins in such populations (Ispir & Mustafa, 2005; Misra et al., 2006). In this study in carp, total serum protein and globulin content were markedly increased after oral administration of Aloe vera compared to controls ($p<0.05$). The increase in serum protein content might be in part due to an increase in the WBC, which is a major source of serum protein production such as lysozyme, complement factors and bactericidal peptides (Misra et al., 2006). This is supported by an enhancement in WBC level in the immunized group that received Aloe vera treatment. Additionally, the serum bactericidal activity in the fish was increased in both the immunized groups compared to the non-immunized groups ($p<0.05$). However, because the administration of Aloe vera resulted in insignificant effect on serum bactericidal activity in both the immunized and non-immunized groups, the presence of anti-A. hydrophila antibody in the immunized fish could be the cause for the increased bactericidal activity. This finding is in contrast to the work of Divyagnaneswari et al. (2007) and Kajita et al. (1990) who studied such an effect in tilapia and rainbow trout respectively. These differences could be explained by the different species of fish, the route of administration, the dosage of immunostimulant used or the water quality.

The fact that administration of immunostimulants in immunized fish leading to a rise in antibody titer proved in many fish species including: cat fish, (Clarias batrachus; Kumari and Sahoo, 2006), salmon (Salmo solar; Aakre et al., 1994), Indian major carp (Catla catla; Vasudeva et al., 2004); and the common carp (Cyprinus carpio; Selvaraj et al., 2005). In this study, the incorporation of Aloe vera into the diet enhanced the serum antibody level against A.h in the immunized group at four and eight weeks post-immunization ($p<0.05$). There was also an insignificant difference between the fish that received Aloe vera supplementation and the normal controls. Therefore, it appears that the use of Aloe vera in fish may act as an adjuvant to enhance specific immunity.

There was no significant difference in the alternative complement activity (ACH50) between the Aloe vera-treated or Aloe vera-free groups. Although complement activity has been found to increase following administration of immunostimulants such as levamisole (Kajita, 1990) and chitosan (Gopalakannan et al., 2006) similar studies have found that oral administration of other immunostimulants including ß-glucan do not induce a change in the alternative complement pathway in carp (Selvaraj et al., 2005) and turbot (Bauer et al., 1996). The results of this study, mmunostimulatory effects of administration of Aloe vera in common carp, are in agreement with the findings of latter work in which various immunostimulants used in other fish species.

A significant increase was observed in the WBC count in the non-immunized fish that received Aloe vera treatment in comparison to the control group. However, Aloe vera did not induce any significant change in the WBC count of the immunized groups. Similar results have been found in other studies (Jeney et al., 1993; Siwicki, 1994; Ispir and Mustafa 2005; Sheikhzadeh et al., 2009). Therefore Aloe vera can be considered capable of improving non-specific immunity in carp by enhancing the population of immunocompetent cells, as observed in this study.

In this study, there were no significant differences in either the PCV and RBC values of Aloe vera-treated or control fish. It therefore seems that the administration of Aloe at 0.5% per feed does not have a negative impact on hematopoiesis in these carp.

In conclusion, the present results have demonstrated that the oral administration of Aloe vera in common carp can enhance some of the specific and non-specific immune responses. This appears to be achieved primarily by increasing lysozyme activity, serum bactericidal power and the total protein and IgM levels. Furthermore, the data reported in this study shows that a 0.5% Aloe vera supplementation per feed can increase the resistance to Aeromonas hydrophila septicemia. However, the precise mechanism of this immunostimulatory effect remains unclear.

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References

1. Aakre, R.; Wergeland, H.I.; Aasjord, P.M. and Endersen, C. (1994) Enhanced antibody response in Atlantic salmon (Salmo salar L.) to Aeromonas salmonicida cell wall antigens using a bacterin containing β-1,3-M-Glucan as adjuvant. Fish and Shellfish Immunology 4: 47-61.
2. Abutbul, S.; Golan-Goldhirsh, A.; Barazani, O. and Zilberg, D. (2004) Use of Rosmarinus officinalis as a treatment against Streptococcus iniae in tilapia (Oreochromis sp.). Aquaculture 238: 97-105.
3. Anderson, D.P.; Jeney, G. (1992) Immunostimulants added to injected Aeromonas salmonicida bacterin enhance the defense mechanisms and protection in rainbow trout (Oncorhynchus mykiss). Vet. Immunol. Immunopathol 34: 379-389.
4. Baulny, M.O.D.; Quentel, C.; Fournier, V.; Lamour, F. and Gouvello, R.L. (1996) Effect of long-term oral administration of b glucan as an immunostimulant or an adjuvant on some non-specifc parameters of the immune response of turbot Scophthalmus maximus. Dis. Aquat. Org. 26: 139-147.
5. Brunt, J.; Newaj-Fizul, A. and Austin, B. (2007) The development of probiotics for the control of multiple bacterial diseases of rainbow trout, Oncorhynchus mykiss (Walbaum). J. Fish Dis. 30: 573-579.
6. Chansue, N.; Ponpornpisit, A.; Endo, M.; Sakai, M. and Yoshida, S. (2000) Improved immunity of tilapia Oreochromis niloticus by C-UP III, a herb medicine. Fish Pathology. 35: 89-90.
7. Chen, X.; Wu, Z. and Yin, J. (2003) Effects of four species of herbs on immune function of Carassius auratus gibelio. J. Fish Sci. of China. 10: 36-40.
8. Choi, S.W.; Son, B.W.; Son, Y.S.; Park, Y.I.; Lee, S.K. and Chung, M. H. (2001) The wound-healing effect of a glycoprotein fraction isolated from aloe vera . Brit. J. Derm. 145: 535-545.
9. Christybapita, D.; Divyagnaneswari, M. and Dinakaran, M.R. (2007) Oral administration of Eclipta alba leaf aqueous extract enhances the non-specific immune responses and disease resistance of Oreochromis mossambicus. Fish Shellfish Imm. 23: 840-852.
10. Divyagnaneswari, M.; Christybapita, D.R. (2007) Enhancement of nonspecific immunity and disease resistance in Oreochromis mossambicus by Solanum trilobatum leaf fractions. Fish Shellfish Imm. 23: 249-259.
11. Dügenci, S. K.; Arda, N. and Candan, A. (2003) Some medicinal plants as immunostimulant for fish. Journal of Ethnopharmacology. 88: 99-106.
12. Ellis, A.E. (1990) Lysozyme assay. In: Stolen, J.S., Fletcher, D.P., Anderson, B.S., Robertson, B.S. (Eds.), Techniques in Fish Immunology. SOS Publication, Fair Haven, NJ., USA. pp: 101-103.
13. Gilliver, M.A.; Bennett, M.; Begon, M.; Hazel, S.M. and Hart, C.A. (1999) Antibiotic resistance found in wild rodents. Nature. 401: 233-234.
14. Gopalakannan, A.; Arul, V. (2006) Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of Cyprinus carpio and control of Aeromonas hydrophila infection in ponds. Aquaculture. 255: 179-187.
15. Gudmundsdóttir, B.K.; Magnadóttir, B. (1997) Protection of Atlantic salmon (Salmo salar L.) against an experimental infection of Aeromonas salmonicida sub sp. achromogenes. Fish Shellfish Imm. 7: 55-69.
16. Hardie, L.J.; Fletcher, T.C. and Secombes, C.J. (1991) The effect of dietary vitamin C on the immune response of the Atlantic salmon. Aquaculture. 95: 201-214.
17. Ispir, U.; Mustafa, D.M. (2005) A Study on the Effects of Levamisole on the Immune System of Rainbow Trout (Oncorhynchus mykiss, Walbaum). Turk. J. Vet. Anim. Sci. 29: 1169-1176.
18. Jeney, G.; Anderson, D.P. (1993) Glucan injection or bath exposures given alone or in combination with bacterin enhance the non-speci.c defense mechanisms in rainbow trout (Oncorhynchus mykiss). Aquaculture. 116: 315-329.
19. Jian, J.; Wu, Z. (2003) Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, Pseudosciaena crocea (Richardson). Aquaculture. 218: 1-9.
20. Jian, J.; Wu, Z. (2004) Influences of traditional Chinese medicine on nonspecific immunity of Jian carp (Cyprinus carpio var. Jian). Fish and Shellfish Immunology. 16: 185-191.
21. Kajita, Y.; Sakai, M.; Atsuta, S. and Kobayash, M. (1990) The immunomodulatory effects of levamisole on rainbow trout, Oncorhynchus mykiss. Fish Pathol. 25: 93-98.
22. Kim, H.K.; Hwang, W.J. and Bai, S.C. (1999) Resistance to Vibrio alginolyticus in juvenile rockfish Sebastes schlegelifed diets containing different doses of aloe, Aquaculture. 180: 13-21.
23. Kitlen, J.W.; Hejbol, E.K.; Zinck, T.; Varming, K.; Byatt, J.C. and McLean, E. (1997) Growth performance and respiratory burst activity in rainbow trout treated with growth hormone and vaccine. Fish Shellfish Immunol. 7: 297-304.
24. Kodaina, H.; Hirota, Y.; Mukaoto, M.; Baba, T. and Azuma, I. (1998) Activation of rainbow trout Oncorhynchus mykiss phagocytes by muramyl dipeptide. Dev. Comp. Immunol. 17: 129-140.
25. Kumari, J.; Sahoo, P. K. (2006) Dietary β-1, 3 glucan potentiates innate immunity and disease resistance of Asian catfish, Clarias batrachus (L.) J. Fish Dis 29: 95-101.
26. Lawless, J.; Allan, J. (2000) Aloe vera -natural wonder cure. London: Harper Collins Publishers.
27. Lee, J.K.; Lee, M.K.; Yun, Y.P.; Kim, Y.; Kim, J.S. and Kim Y.S. (2001). Acemannan purified from aloe vera

induces phenotypic and functional maturation of immature dendritic cells. Int. Immunopharm. 1: 1275-1284.
28. Logambal, S.M.; Venkatalakshmi, S. and Michael, R.D. (2000) Immunostimulatory effect of leaf extract of Ocimum sanctum Linn. In Oreochromis mossambicus (Peters). Hydrobiologia. 430: 113-120.
29. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.
30. Misra, C.K.; Das, B.K.; Mukherjee, S.C. and Meher, P.K. (2006) The immunomodulatory effects of tuftsin on the non-specific immune system of Indian Major carp, Labeo rohita. Fish Shellfish Imm. 20: 728-738.
31. Pugh, N.; Ross, S.A.; ElSohly, M.A. and Pasco, D.S. (2001) Characterization of aloeride, a new high-molecular-weight polysaccharide from aloe vera with potent immunostimulatory activity. J. Agricultural Food Chem. 49: 1030-1034.
32. Raa, J. (1996) The use of immuno-stimulatory substances in fish and shellfish farming. Rev. Fish Sci. 4: 229-288.
33. Raa, J.; Roerstad, G.; Engstad, R. and Robertsen, B. (1992) The use of immunostimulants to increase resistance of aquatic organisms to microbial infections. Dis. Asian Aquacult. 1: 39-50.
34. Sakai, M. (1999) Current research status of fish immunostimulants. Aquaculture. 172: 63-92.
35. Salisbury, J.G.; Nicholls, T.J.; Lammerding, A.M.; Turnidge, J. and Nunn, M.J. (2002) A risk analysis framework for the long-term management of antibiotic resistance in food-producing animals. Int. J. Antimicrob. Agents. 20: 153-164.
36. Santarem, M.M.; Novoa, B. and Figueras, A. (1997) Effects of ß-glucans on the non-specific immune responses of turbot Scophthalmus maximus L. Fish Shellfish Immunol. 7: 429-437.
37. Schaperclaus, W.; Kulow, H. and Schreckenbach, K. (1991) Hematological and serological technique. In: Kothekar, V.S. (Ed.), Fish Disease, vol. 1. Gulab Primani, Oxonian Press, New Delhi, India. pp: 71-108.
38. Selvaraj, V.; Sampath, K. and Sekar, V. (2005) Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (Cyprinus carpio) infected with Aeromonas hydrophila. Fish Shellfish Imm. 19: 293-306.
39. Sheikhzadeh, N.; Soltani, M.; Mousavi, H. A. E. (2009) Effects of Eucalyptus globules labill essential oil on some immunological variables of common carp (Cyprinus carpio). J. Vet. Res. 64: 47-54.
40. Siwicki, A.K.; Anderson, D.P. and Rumsey, G.L. (1994) Dietary intake of Immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Vet. Immunol. Immunopathol. 41: 125-139.
41. Soltani, M.; Esfandiary, M. and Khaeenia, S. (2009) Effects of Zataria multiflora essential oil on rainbow trout (Oncorhynchus mykiss) egg hatchability and survival of larvae compared with hydrogen peroxide and malachite green. J. Vet. Res. 64: 127-134.
42. Swain, P.S.; Dash, P.K.; Sahoo, P.; Routray, S.K.; Sahoo, S. D.; Gupta, P.K.; Meher, N. and Sarangi, A. (2006) Non-specific immune parameters of brood Indian major carp Labeo rohita and their seasonal variations. Fish and Shellfish Immunology. 22: 38-43.
43. Tan, B.K.; Vanitha, J. (2004) Immunomodulatory and antimicrobial effects of some traditional Chinese medicinal herbs: a review. Cur. Med. Chem. 11: 1423-1430.
44. Vasudeva, Y.R.; Romesh, M.; Singh, A. and Chakrabarti, R. (2004) Potentiation of antibody production in Indian major carp Labeo rohita, rohu, by Achyranthes aspera as a herbal feed ingredient. Aquaculture. 238: 67-73.
45. Watanuki, H.; Ota, K.; Malina, A.C.; Tassakka, A. and Sakai, M. (2006) Immunostimulant effects of dietary Spirulina platensis on carp, Cyprinus carpio. Aquaculture. 258: 157-163.
46. Yuan, C.; Li, D.; Chen, W. and Sun, F. (2007) Administration of a herbal immunoregulation mixture enhances some immune parameters in carp (Cyprinus carpio). Fish Physiol. Biochem. 10: 1007-1120.