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Immune Response of Common Carp (*Cyprinus carpio*) Fed with Herbal Immunostimulants Diets

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Abstract: This experiment was conducted to evaluate the immunostimulatory and growth promoting effects of four different herbal medicinal plants on non-specific immune response and resistance to *Aeromonas hydrophila* challenge of common carp, *C. carpio*. The ethanol extract of 11 plants were screened for their antimicrobial activity against *A. hydrophila*, a bacterial pathogen. Of these, 4 plant extracts, *Ocimum basilicum*, *Cinnamomum zeylanicum*, *Juglans regia* and *Mentha piperita* were selected and mixed thoroughly in equal proportion. The mixed extract was incorporated with the artificial feeds at concentration of 0.0 (A), 250 (B), 500 (C), 750 (D), 1000 (E) and 1250 (F) mg kg^{-1} of dry diet and fed to healthy fish. After 45 days of feeding, a challenge trial was conducted by injection of *A. hydrophila* for 10 days. Every 15 days and also at 10th day post-challenge, immunological, biochemical and haematological parameters of fish were studied. Results indicated that fish fed with herbal immunostimulants diets showed enhanced bactericidal activity, serum lysozyme, respiratory burst activity, WBC, RBC, haemoglobin, total serum protein, albumin and globulin compared to the control group ($p<0.05$). As the value of herbal extracts was increased in diets, the plasma glucose level decreased. The mortality was recorded up to 10 days post-challenge. All experimental groups showed higher survival rate compared to control ($p< 0.05$). The survival percentage was found highest (91.42%) in the group E and lowest (48.58%) in control group. It can be conducted that the plant extracts we used in this study can act as immunostimulants, enhance the non-specific immunity and increase disease resistance of common carp, *C. carpio* to *A. hydrophila* infection.

Key words: *Aeromonas hydrophila*, *Cyprinus carpio*, haematological parameters, herbal immunostimulants, immunological parameters, plant extracts

INTRODUCTION

Seemingly, vaccination is the most promising method of controlling fish disease which enhances the specific immune response of fish (Press and Lillehaug, 1995; Ardo *et al*., 2008). However, effective vaccines for a number of pathogens like the bacterium *Aeromonas hydrophila* have not been developed due to their heterogeneity. Furthermore, a single vaccine is effective against only one type of pathogens (Ardo *et al*., 2008; Leong and Fryer, 1993; Murray *et al*., 2003; Gopalakannan and Arul, 2006) and vaccination of very young fish is also difficult (Kaattari and Piganelli, 1997; Murray *et al*., 2003). Moreover, infectious parasitic, bacterial and fungal diseases in fish are mainly controled by chemotherapeutics and antibiotics. However, recently, the use of antibiotics and chemotherapy have been criticized, because their use have created problems with drug resistance bacteria, toxicity and accumulation both in fish an environment (Farag *et al*., 1989; Citarasu *et al*., 2002; Sagdic and Ozcan., 2003). Immunostimulants seem to represent a useful alternative to vaccination and chemotherapy in the control of fish diseases. They can enhance the non-specific immune response (Sakai, 1999; Verlhac *et al*., 1998; Esteban *et al*., 2000). It is generally accepted that monocytes, granulocytes, neutrophils, macrophages and humoral elements, like lysozyme, agglutinin and metalion binding proteins are the main components of non-specific immune system (Secombes and Fletcher, 1992; Ardo *et al*., 2008; Rao *et al*., 2006; Dalmo *et al*., 1997; Feng *et al*., 2009; Sakai, 1999). In aquaculture, there are many studies reporting a variety of substances including synthetic (Rao *et al*., 2006) bacterial (Goetz *et al*., 2004; Engstad *et al*., 1992), animal and plant products (Hardie *et al*., 1991; Thompson *et al*., 1995; Ardo *et al*., 2008; Rao *et al*., 2006) can be used as immunostimulants to enhance non-specific immune system of cultured fish species. Some plants are rich sources of compounds like volatile oils, saponins, phenolics, tannins, alkaloids,

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polysaccharides and polypeptides. These natural plant products have various activities like antistress, appetizer, tonic, antimicrobials and immunostimulants (Citarasu *et al*., 2002, 2003). Recently in aquaculture, scores of plant extracts have been tested and used with good results in the control of bacterial and viral diseases. Fourteen herbs have been tested against *Aeromonas hydrophila* infection in Tilapia (*Oreochromis niloticus*) and among hese herbs the ethanol extract of *Psidium guajava* has been foundto have the highest antimicrobial activity (Pachanawan *et al*., 2008).

Citarasu *et al*. (2006) reported the increase in survival and resistance to White Spot Syndrome Virus (WSSV) infection in black tiger shrimp, *Penaeus monodon* feeding immunostimulant herbal supplemented diets. Similarly, Rao *et al*. (2006) demonstrated that dietary supplemention of *Achyranthes aspera* seed stimulated immunity and enhanced resistance to *Aeromonas hydrophila* infection of *Labeo rohita*, rohu fingerlings. Similar results were also observed after feeding Tilapia (*O. niloticus*) with *Psidium guajava* (ethanol extract) incorporated diets. The principal objective of this study was to evaluate the effect of several herbal plant extracts added to diet on immunological, serum biochemical and blood parameters of common carp (*Cyprinus carpio*).

MATERIALS AND METHODS

Fish: The common carp, *C. carpio* were purchased from a commercial fish farm at Bandar Torkman, Golestan, Iran. They were acclimated and kept in 500 L plastic containers with recirculated and aerated water at 22-24 °C for 2 weeks to assess their disease-free health status. During the acclimation period they were fed the basal experimental diet (Table 1) without supplementation of the plant extracts at 3% at body weight once daily.

Preparation and screening of the plant extracts for antibacterial activities: The name of the herbal plants and the parts used are shown in Table 2. Fresh herbal plants were cleaned, dried at 37°C for 3 days and ground well. Dried herbal powders were then soaked in 70% ethanol (1:1 ratio) individually for 48 h (Eloff, 1998; Citarasu *et al*., 2006; Punitha *et al*., 2008). The slurry was then filtered with Wathman No. 1 filter paper and centrifuged for 5 min at 5000 rpm. In order to obtain dried extract, the extraction solvent was removed by using rotary evaporator (IKA® HB10 basic, China) at 40°C. Then, solvent-free extract was dried by using a freeze drier system (Operon: FDB-5503, Korea). Finally, the sherbal extracts were stored at 4°C until use (Arabshahi-Delouee and Urooj, 2007). Detection of antimicrobial activity of herbal plant extract against *A.hydrophila* was conducted using the disc diffusion assay as described. All the tests were replicated three times and zone of inhibition of each extract was measured and noted (Table 1).

Table 1: Composition of basal diet supplemented with herbal immunostimulant

	Diet					
	A (control)	B	C	D	E	F
Ingredients (g kg^{-1} diet)						
Fish meal[a]	175.00	175.000	175.000	175.000	175.0	175.00
Soybean meal	280.00	280.000	280.000	280.000	280.0	280.00
Gluten meal	45.00	45.000	45.000	45.000	45.0	45.00
Casein	45.00	45.000	45.000	45.000	45.0	45.00
Rice bran	175.00	175.000	175.000	175.000	175.0	175.00
Wheat flour	104.00	104.000	104.000	104.000	104.0	104.00
Corn oil	32.00	32.000	32.000	32.000	32.0	32.00
Fish oil[b]	42.00	42.000	42.000	42.000	42.0	42.00
Cellulose[c]	77.00	77.000	77.000	77.000	77.0	77.00
Vitamin premix[d]	12.50	12.500	12.500	12.500	12.5	12.50
Mineral premix[e]	12.50	12.500	12.500	12.500	12.5	12.50
Herbal immunostimulant	0.00	0.250	0.500	0.750	1.0	1.25
Proximate chemical composition (g kg^{-1} diet)						
Dry matter	923.20	916.800	924.200	915.500	908.5	921.10
Crude protein*	345.30	342.200	338.100	339.200	341.4	343.10
Crude fat**	123.50	121.200	124.300	124.100	125.3	121.40
Ash	821.50	827.600	823.100	835.200	831.4	827.30
Moisture	89.10	84.300	82.500	87.100	83.7	85.80

*Calculated crude protein: 34.61%; **Calculated crude fat: 11.95%, [a]Fish meal: Pars kelika Co., Mirood, Iran, [b]Herring oil, [c]Sigma, St. Louis, MO, U.S.A, [d]Vitamin premix contained the following vitamins (each kg^{-1} diet): Vitamin A, 10 000 IU; Vitamin D_3, 2000 IU; Vitamin E, 100 mg; Vitamin K, 20 mg; Vitamin B_1, 400 mg; Vitamin B_2, 40 mg; Vitamin B_6, 20 mg; Vitamin B_{12}, 0.04 mg; Biotin, 0.2 mg; Choline chloride, 1200 mg; Folic acid, 10 mg; Inositol, 200 mg; Niacin, 200 mg; Pantothenic calcium, 100 mg. [e]Contained (g kg^{-1} mix): $MgS0_4.2H_20$, 127.5; KCl, 50.0; NaCl, 60.0; $CaHPO_4$,.$2H_2O$, 727.8; $FeSO_4$. $7H_2O$, 25.0; $ZnSO_4.7H_2O$, 5.5; $CuSO_,.5H_2O$, 0.785; $MnSO_4$ .4H2O, 2.54; $CoSO_4.4H_2O$, 0.478; $Ca(IO_3)$ 2 . $6H_2O$, 0.295; $CrCl_3$, $6H_2O$, 0.128

Table 2: List of the plants and their inhibitory activity against *A. hydrophila*

Botanical name	Family	Parts used for antibacterial screening	Inhibition zone (mm)*
Ocimum basilicum	Lamiaceae	Leaf	25.7±0.23[a]
Cinnamomum zeylanicum	Lauraceae	Bark	24.3±0.20[b]
Juglans regia	Juglandaceae	Leaf	21.2±0.25[c]
Mentha piperita	Lamiaceae	Leaf	18.3±0.20[d]
Rosmarinus officinalis	Lamiaceae	Leaf	16.7±0.17[e]
Thymus vulgaris	Lamiaceae	Flower and leaf	16.2±0.10[e]
Matricaria chamomilla	Asteraceae	Flower	14.4±0.05[f]
Olea europaea	Oleaceae	Leaf	11.3±0.15[g]
Juglans regia	Juglandaceae	Walnut husk (green part)	11.1±0.20[g]
Capsicum annum	Solanaceae	Fruit	7.4±0.05[h]
Citrus aurantium	Citraceae	Flower	7.1±0.15[h]

*Disc diffusion test. Values are expressed as mean±SE. Means having the same letter in the same column are not significantly different at p>0.05

Bacterial strain: *A. hydrophila* (ATCC 49140) was obtained from the Razi Researches Institute, Karaj, Iran. Bacteria were cultured in nutrient broth (Himedia) for 24 h at 37°C and the culture broth was then centrifuged at 3000×g for 10 min. The supernatant was then removed and the pelleted bacteria were washed three times in sterile phosphate buffered saline was adjusted to 10^{10} cfu mL^{-1} as determined using a Neubaur hemocytometer slide (Yadav *et al*., 1992; Harikrishnan *et al*., 2003; Rao *et al*., 2006). These bacterial suspensions was serially diluted with sterile phosphate buffered saline and used for further experiments.

Test diets: The ingredients and proximate compositions of the basal and experimental diets are shown in Table 1. According to the results of the disc diffusion test and total phenol content, *Ocimum basilicum*, *Cinnamomum zeylanicum*, *Juglans regia* and *Mentha piperita* were selected for the present study (Table 1). The experimental diets were prepared by incorporating equal proportion of the all five ethanol plant extracts and mixed to the feeds in the concentration of 250, 500, 750, 1000 and 1250 mg kg^{-1} of the diets. Control diet (A) was also prepared using the same composition of ingredients, except the plant extract mixture. To prepare the diets, first, ingredients were blended thoroughly with additional water and 1% binder to make a paste of each diet. The pastes were then cold extruded and cut into pellets. The diets were air-dried and stored at -2°C (Sardar *et al*., 2007) in air tight containers until fed.

Experimental design and feeding diet: After acclimatization, fish (n = 1080) were divided randomly into six triplicated (6×3 = 18) groups (A-F) with 60 fish in each group, maintained in 18 tanks (600 L capacity). Group A received the basal diet and acted as control. Group B, C, D, E and F were fed with extract mixture at 250, 500, 750, 1000 and 1250 mg kg^{-1} of feed, respectively. Feed was given thrice a day at 8:00, 13:00 and 18:00 at a rate of 3% body weight day^{-1}. The water quality parameters were monitored regularly and maintained at optimal levels by water exchange (temperature, 26±2.0°C; Dissolved oxygen, 6.5±0.01 mg L^{-1}; salinity, 0.5±0.04 ppt; pH, 6.3±0.2 units; ammonia-nitrogen<0.22).

Sampling: Every 15 days, blood samples were obtained from the caudal vein of randomly chosen 10 fish from each tank by using a 1 mL heparinized syringe after they were starved for 24 h and anesthetized with 100 mg tricaine methane sulphate (MS-222) L^{-1}. The blood was then transferred to an Eppendroff tube containing heparin solution, shaken gently and kept in the refrigerator at 4°C. For serum, another 10 fish from each tank were collected without heparin and allowed to clot for 2 h, at room temperature. Serum was isolated from the remaining blood after centrifugation (3000×g for 5 min) and then stored in freezer at-80°C for further serum biochemical analysis (Maqsood *et al*., 2009; Sardar *et al*., 2007).

Determination of the LC_{50} value: In order to determine the LC_{50} value, healthy fish (n = 140) were injected intraperitoneally with 100 µL of live *A. hydrophila* at a concentration of 10^4-10^{10} cfu mL^{-1} and mortalities were then observed over a period of 10 days.

Challenge test: After 45 days of feeding, 35 fish from each group were injected intraperioneally (Schaperclaus *et al*., 1992) with 100 µL of live *A. hydrophila* at a concentration of the LC_{50} dose (1×10^8 cfu/fish). Mortality was observed for every 12 h interval up to 10 days). The surviving fish were then sampled for serum and blood factors as per the method described earlier.

Haematological analysis: The fresh whole blood samples were used for the estimation of leucocyte, erythrocyte counts and haemoglobin. Red Blood Cell (RBC) and White Blood Cell (WBC) counts were estimated following the method of Schalm *et al*. (1975). Haemoglobin (Hb) concentration was determined as described by Barros *et al*. (2002).

Biochemical analysis: Total plasma protein content was determined according and albumin was estimated colorimetrically following the method of Wotton and Freeman (1982). For globulin, albumin was subtracted from the total protein. Albumin-globulin ratio was calculated by dividing albumin values by globulin values. Plasma glucose estimation was done colorimetrically according to Trinder (1969).

Determination of immunological parameters: Bactericidal activity in serum samples was analyzed in accordance with the procedure of Kajita *et al*. (1990). For the lysozyme activity assay, the method of Parry *et al*. (1965) was followed with slight modification described by Gopalakannan and Arul (2006). Respiratory burst (NBT) activity was quantified by the nitroblue tetrazolium (NBT) assay. For NBT assay, Secombes (1990)'s method was followed with modification described by Stasiak and Baumann (1996).

Statistical analysis: All data obtained from experiments were analyzed by a one-way Analysis of Variance (ANOVA) using the package. Differences between means were determined and compared by Tukey's test. Significance was also set at 5% level.

RESULTS

Antimicrobial screening of plant extracts against *A. hydrophila*: Table 2 shows diameters of inhibition zones of some plant extract in disc diffusion test. The highest value was obtained for *Ocimum basilicum* (25.7±0.23 mm), *Cinnamomum zeylanicum* (24.3±0.20 mm), *Juglans regia* (21.2±0.25 mm) and *Mentha piperita* (18.3±0.20 mm) while the lowest was for *Capsicum annum* (7.4±0.05 mm) and *Citrus aurantium* (7.1±0.15 mm).

Haematological parameters: WBC count in experimental groups was significantly ($p<0.05$) higher compared with the control group over all the assay periods (except group B and C on day 15 and B on day 55) (Fig. 1).

A gradual significant increase of RBC count ($p<0.05$) was observed in all the groups on day 30, 45 and also after challenge (day 55) (Fig. 2).

Hemoglobin content was significantly ($p<0.05$) higher in group E and F over all the assay period as compared to the control and other experimental groups. In addition, there was no significant difference ($p>0.05$) between group E and F at all assay periods (except on day 55) (Fig. 3).

Biochemical parameters: The total protein content appeared to show an increasing trend in the experimental groups at all the assay periods. Moreover, the maximum and minimum significant ($p<0.05$) total serum protein level was recorded in group D and A (control), respectively. Although, albumin content showed a slight increasing trend in treatment groups at all the assay periods there was no significant variance ($p>0.05$) between group E and F. On different assay days there was a significant difference ($p<0.05$) of globulin level within and between experimental groups as compared to control group except in group B on day 15 and day 30.

The highest globulin content was observed in group E followed by F and D. Albumin globulin ratio in the present study did not show a regular trend. Generally, Albumin globulin ratio in different groups was not significantly different ($p>0.05$) from the control group throughout the periods of experimental study (Table 3).

The glucose level of the test groups is shown in Table 3. The overall results indicated that the glucose level of fish was decreased significantly ($p<0.05$) when they fed on increasing concentration of plant extract. The minimum significant ($p<0.05$) glucose content was recorded in group E and F over all the assay periods.

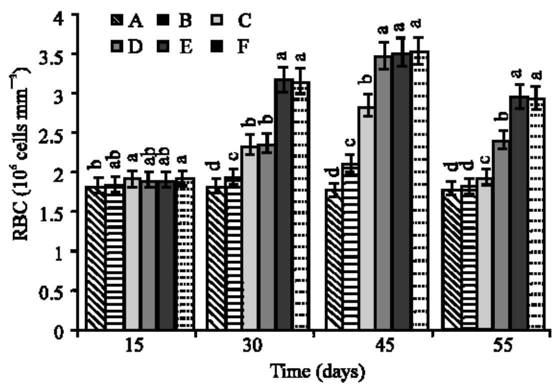

Fig. 2: RBC count (10^6 cells mm^{-3}) of common carp, *C. carpio*, fed on herbal immunostimulants supplemented diets (B-F) and control diet (A). Means with the same letters are not significantly different ($p>0.05$). Data are expressed as mean±SE

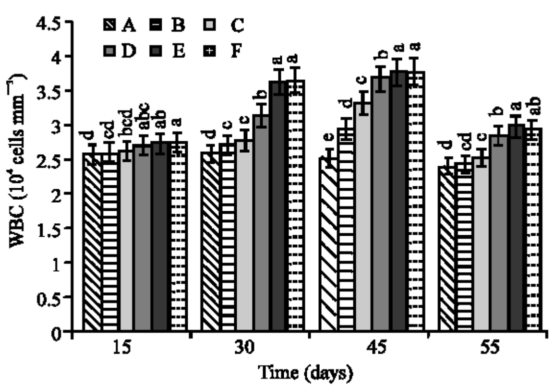

Fig. 1: WBC count (10^4 cells mm^{-3}) of common carp, *C. carpio*, fed on herbal immunostimulants supplemented diets (B-F) and control diet (A). Means with the same letters are not significantly different ($p>0.05$). Data are expressed as mean±SE

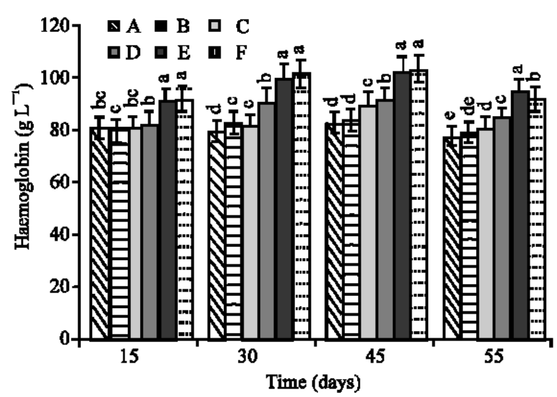

Fig. 3: Haemoglobin concentration (g L^{-1}) of common carp, *C. carpio*, fed on herbal immunostimulants supplemented diets (B-F) and control diet (A). Means with the same letters are not significantly different ($p>0.05$). Data are expressed as mean±SE

Table 3: Effects of herbal immunostimulants supplemented diets on biochemical parameters of common carp, *C. carpio*

Parameters	Groups	Days 15	30	45	55
Total protein	A	19.80±0.17^{d}	20.30±0.30^{e}	19.60±0.15^{e}	17.20±0.10^{d}
	B	21.30±0.11^{c}	22.40±0.20^{d}	24.30±0.15^{d}	20.20±0.10^{c}
	C	22.20±0.26^{b}	24.80±0.15^{c}	27.80±0.20^{c}	21.90±0.26^{b}
	D	21.70±0.11^{bc}	27.30±0.11^{b}	29.20±0.23^{b}	22.30±0.10^{b}
	E	0.10±26.70^{a}	31.20±0.17^{a}	31.80±0.20^{a}	25.30±0.32^{a}
	F	25.96±021^{a}	30.10±0.35^{a}	29.70±0.11^{b}	24.70±0.36^{a}
Globulin	A	10.90±0.264^{e}	12±0.305^{c}	10.20±0^{d}	9.40±0.346^{c}
	B	11.60±0.152^{de}	11.80±0.32^{c}	14.60±0.057^{c}	12.10±0.200^{b}
	C	12.66±0.333^{c}	12.40±0.173^{c}	15.90±0.251^{b}	13.60±0.321^{ab}
	D	11.90±0.208^{cd}	15±0.152^{b}	15.40±0.351^{bc}	12.10±0.173^{b}
	E	14.60±0.057^{a}	18.10±0^{a}	17.50±0.208^{a}	14± 0.300^{a}
	F	13.66±0.272^{b}	17.30±0.46^{a}	15.60±0.208^{bc}	13.60±0.472^{ab}
Albumin	A	8.90±0.36^{b}	8.30±0.11^{d}	9.40±0.15^{c}	7.80±0.26^{c}
	B	9.70±0.25^{b}	10.60±0.11^{c}	9.70±0.10^{c}	8.10±0.17^{c}
	C	9.53±0.21^{b}	12.40±0.057^{ab}	11.90±0.30^{b}	8.30±0.057^{c}
	D	9.80±0.15^{b}	12.30±0.10^{b}	13.80±0.20^{a}	10.20±0.10^{b}
	E	12.10±0.11^{a}	13.10±0.17^{a}	14.30±0.11^{a}	11.30±0.11^{a}
	F	12.30±0.057^{a}	12.80±0.26^{ab}	14.10±0.26^{a}	11.10±0.25
A:G	A	0.818±0.05^{ab}	0.629±0.021^{d}	0.921±0.014^{a}	0.834±0.059^{a}
	B	0.837±0.032^{ab}	0.90±0.033^{ab}	0.664±0.004^{c}	0.670±0.024^{ab}
	C	0.754±0.033^{b}	1.0±0.016^{a}	0.749±0.028^{bc}	0.611±0.018^{b}
	D	0.824±0.026^{ab}	0.820±0.013^{bc}	0.897±0.031^{a}	0.834±0.019^{a}
	E	0.828±0.010^{ab}	0.723±0.009^{cd}	0.817±0.013^{ab}	0.807±0.019^{a}
	F	0.900±0.021^{a}	0.741±0.032^{cd}	0.904±0.028^{a}	0.818±0.043^{a}
Glucose	A	1235±6.65^{a}	1243±4.35^{a}	1233±3.60^{a}	1187±3.60^{b}
	B	1231±6.65^{a}	1134±4.61^{b}	1214±3.05^{ab}	1217±3.78^{a}
	C	1233±7.23^{a}	1137±6.55^{b}	1197±3.51^{b}	1154±3.46^{c}
	D	1201±5.13^{b}	1003±6.24^{c}	945±5.29^{c}	864±3.46^{d}
	E	1131±4.51^{c}	987±7.09^{d}	873±4.51^{d}	844±3.46^{e}
	F	1143±6.65^{c}	993±5.29^{cd}	864±3.60^{d}	842±4.16^{e}

Values are expressed as mean±SE. Means having the same letter in the same column are not significantly different at p>0.05

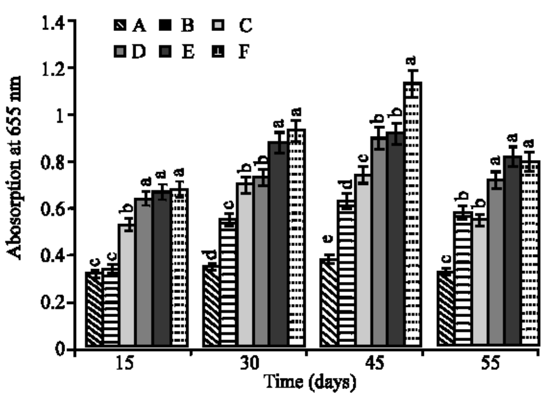

Fig. 4: Respiratory burst activity of common carp, *C. carpio*, fed on herbal immunostimulants supplemented diets (B-F) and control diet (A). Means with the same letters are not significantly different (p>0.05). Data are expressed as mean±SE

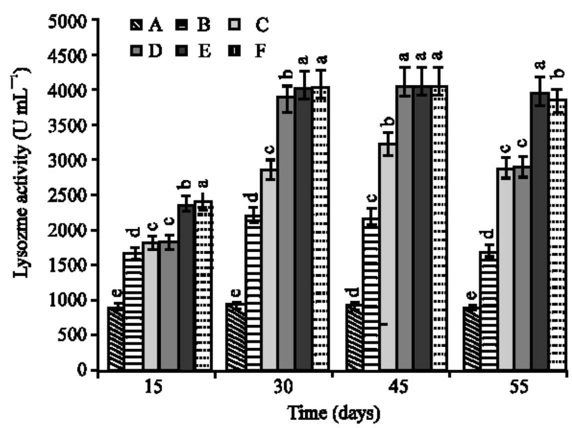

Fig. 5: Lysozyme activity of common carp, *C. carpio*, fed on herbal immunostimulants supplemented diets (B-F) and control diet (A). Data are expressed as mean±SE. Mean values bearing same superscript are not statistically significant (p>0.05)

Immunological parameters: The respiratory burst (NBT) activity in the five experimental groups was significantly ($p<0.05$) higher than control group at all the assay periods including post-challenge (except group B on day 15) and the highest value was observed in group E and F. Though the respiratory burst (NBT) activity in group E and F was highest, the difference between E and F was not significant ($p>0.05$) (except on day 45) (Fig. 4).

The results of the serum lysozyme activity are shown in Fig. 5. Lysozyme activity in the five experimental groups was significantly ($p<0.05$) higher than control group at all the assay periods and post-challenge.

The bactericidal activity values gradually increased from control group (A) to group F as the percentage of

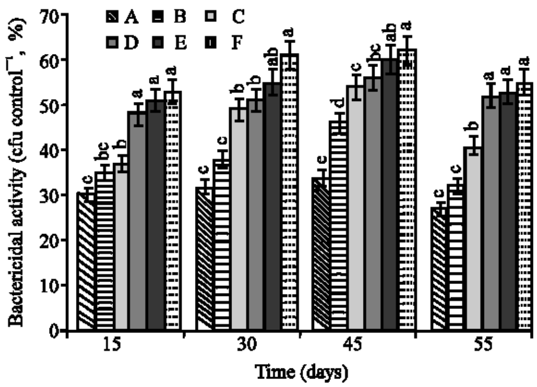

Fig. 6: Bactericidal activity (cfu/control) of common carp, *C. carpio*, fed on herbal immunostimulants supplemented diets (B-F) and control diet (A). Data are expressed as mean±SE. Values receiving same superscript are statistically not significant ($p>0.05$)

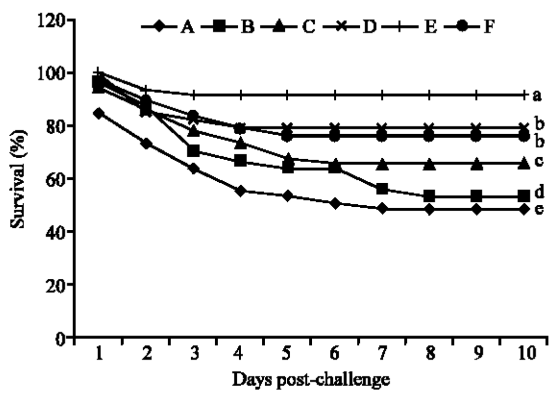

Fig. 7: Survival rate of common carp, *C. carpio*, fed on herbal immunostimulants supplemented diets (B-F) and control diet (A) after challenged with *A. hydrophila*. Statistical differences ($p<0.05$) between groups are indicated by different letters (a-e)

herbal extract mixture increased in the diet. The highest bactericidal activity was recorded in group F on day 45 followed by group E on day 45 (Fig. 6).

LC_{50} value: At a concentration of 10^4, 10^6, 10^8 and 10^{10}, of *A. hydrophila*, the mortality was <10, 10%, about 50 and 90%, respectively. Hence, 10^8 cfu mL^{-1}, the LC_{50} value was chosen for further experiments.

Challenge test with A.hydrophila: After challenge of the fish with *A. hydrophila*, mortality was recorded for 10 days. There was no mortality of fish for the first 12 h. Moreover, after day 8 there was no mortality up to day 10. All experimental groups showed higher survival rate compared to the control. The lowest survival rate was recorded in the control group (48.57%) and highest rate was observed in the group E (91.42%) (Fig. 7).

Statistical analysis: All data obtained from experiments were analyzed by a one-way analysis of variance (ANOVA) using the package. Differences between means were determined and compared by Tukey's test. Significance was also set at 5% level.

DISCUSSION

As the alternative to chemotherapy, application of natural products, like plant extracts, in aquaculture is new and developing venture which needs further research in fish (Citarasu *et al*., 2002; Jian and Wu, 2003; Sivaram *et al*., 2004). Phagocytosis and killing activity by phagocytic cells is an important defense mechanism against pathogenic bacteria (Neumann *et al*., 2001; Rao *et al*., 2006). Fish phagocytes are able to produce superoxide anion (O_2^-) during a process called respiratory burst (Neumann *et al*., 2001; Sahu *et al*., 2007; Secombes and Fletcher, 1992; Secombes, 1990; Ardo *et al*., 2008). It is considered that these oxygen forms are toxic for bacterial pathogens (Hardie *et al*., 1991; Sahu *et al*., 2007).

The respiratory burst (NBT) activity can be quantified by the Nitroblue Tetrazolium (NBT) assay, which measures the quantity of intracellular superoxide radicals produced by leukocytes (Sahu *et al*., 2007; Ardo *et al*., 2008). Herbal based immunostimulants can enhance the respiratory burst activity of fish phagocits. For instance, Rao *et al*. (2006) reported that Superoxide anion production by the blood leucocytes was enhanced in *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed. Ardo *et al*. (2008) also reported that feeding Nile tilapia (*Oreochromis niloticus*) with two herbal extracts (*Astragalus membranaceus* and *Lonicera japonica*) alone or in combination significantly enhanced phagocytic and respiratory burst activity of blood phagocitic cells. Similarly, the plant extracts we used in this study could enhance respiratory burst (NBT) activity in treatment groups compared to control group.

In the present study, compared with control group, serum lysozyme was significantly increased in all experimental groups. The present observation was in corroboration with the findings of Chen *et al*. (2003) who reported that plasma lysozyme activity was increased in crucian carp by feeding four Chinese herbs (*Rheum officinale and rographis paniculata*, *Isatis indigotica*, *Lonicera japonica*). The level of serum lysozyme was also enhanced In *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed (Rao *et al*., 2006). Elevated lysozyme was also observed in Japanese eel (*Anguilla japonica*) after feeding with Korean mistletoe extract (KM-110; *Viscum album* Coloratum) (Choi *et al*., 2008). It is generally accepted that Lysozyme is a humoral component of the non-specific defense mechanism that has the ability to prevent the growth of infectious microorganism by splitting β-1, 4 glycosidic bonds between N-acety lmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls (Alexander and Ingram, 1992; Gopalakannan and Arul (2006); Grinde, 1989; Choi *et al*., 2008).

The plant extracts we used in this study could enhance serum bactericidal activity in all experimental groups. In agreement with the present findings, Sivaram *et al*. (2004) reported that serum bactericidal activity was enhanced in juvenile greasy groupers (*Epinphelus tauvina*) fed antibacterial active princioles of *Ocimum sanctum* and *Withania somnifera*. Similarly, grouper (*E. tauvina*) juveniles fed with diets containing different doses of extract mixture of some herbs showed a significant increase in their serum bactericidal activity (Punitha *et al*., 2008). This revealed that the immunostimulant herbals incorporated diets helped to increase the humoral elements in the serum.

The results of this study showed that feeding *C. carpio* with supplemented diets containing herbal plant extracts enhanced total plasma protein, albumin and globulin values in treatment groups. Similar to present observations were obtained by Rao *et al*. (2006) after feeding the rohu fingerlings (*Labeo rohita*) with *Achyranthes aspera* seed. Similar observations were also obtained by Sahu *et al*. (2007) who reported that serum protein, albumin and globulin levels in *L.rohita* fingerlings fed with *Magnifera indica* kernel were higher than control. Since serum proteins include various humoral elements of the non-specific immune system, high concentrations of total serum protein, albumin and globulin might be due to the enhancement of non-specific immune response of fishes.

The results of the present study demonstrated that as the value of herbal plant extracts increased in the diet, the value of plasma glucose decreased. This is probably due to the capability of plant extracts to reduce the effects of stressors. It has been shown that glucose level increases in the infected or stressed animals to ward off the infection or stress (Citarasu *et al*., 2006). Similar to present observation was found in *Labeo rohita* fingerlings (Sahu *et al*., 2007) and black tiger shrimp, *Penaeus monodon* (Citarasu *et al*., 2006) that glucose levels were reduced after feeding with herbal immunostimulant diets.

According to our results, herbal diets could increase Hemoglobin content, WBC and RBC counts of fish in experimental groups compared to control group. In agreement with the present findings, Sahu *et al*. (2007) reported that WBC and RBC counts were higher in *Labe rohita* fingerlings fed *Magnifera indica* kernel when compared to control. Gopalakannan and Arul (2006) also reported that there was an increase in the WBC count after feeding the common carp with immun ostimulants like chitin.

Results indicated that dietary plant extract supplementation could significantly ($p<0.05$) enhance the resistance of *C. carpio* against *Aeromonas hydrophila* infection. This might be due to the enhancement of the non-specific immune system of fish by herbal plant extracts. In agreement with the present findings, Sahu *et al*. (2007) reported that survival rate after challengeing the fish with *A. hydrophila* was enhanced in *Labeo rohita* fed diets containing *Magnifera indica* kernel. Similar results were also reported after feeding tilapia (*Oreochromis niloticus*) with two Chinese medicine herbs and challenging with *A. hydrophila* (Ardo *et al*., 2008). Pachanawan *et al*. (2008) also reported that survival rate after challenging the fish with *A. hydrophila* was increased in tilapia (*Oreochromis niloticus*) fed diets containing either dry leaf powder of *Psidium guajava* or ethanol extract of *P. guajava* leaf.

CONCLUSION

In this study it can be concluded that the plant extracts used in this study could increase the non-specific immunity and significantly decrease mortality when common carp experimentally infected with *A. hydrophila*, a bacterial pathogen. Moreover, further studies are needed to determine the molecular mechanisms beside the isolation and characterization of the active compounds from the plants.

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