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Effect of Plant Extracts Supplemented Diets on Immunity and Resistance to *Aeromonas hydrophila* in Common Carp (*Cyprinus carpio*)

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Abstract: Ten plants were extracted with 70% ethanol and screened for their antimicrobial activities against *Aeromonas hydrophila*, a bacterial pathogen. Using disk diffusion assays, five extracts among these ten (*Inula helenium*, *Tussilago farfara*, *Brassica nigra*, *Echinacea purpurea* and *Chelidoniume majus*) were selected and equal proportions of them were mixed thoroughly with the artificial feeds at concentrations of 0.0 (A), 100 (B), 250 (C), 500 (D), 750 (E) and 1000 (F) mg kg^{-1} of dry diet. The prepared diets were fed to healthy common carp *(Cyprinus carpio)* for 60 days and then challenged with *A. hydrophila*. To evaluate the immune response and resistance against *A. hydrophila* infection of fish, haematological, biochemical and immunological parameters of them were investigated at 20, 40 and 60 days of feeding and also 10th day post-challenge. Results indicated that respiratory burst activity, serum bactericidal activity, lysozyme, serum protein, albumin, globulin, WBC, RBC and haemoglobin content were enhanced ($p<0.05$) in fish fed erbal diets compared to the control group. On 10 days post-challenge, the total survival rates were 38.09% in control group (A) and 61.90, 67.62, 77.14, 85.71 and 80.95% in group B, C, D, E and F, respectively. Among different groups, E generally showed the best performance in the experiment. Further research is needed to isolate and characterize the active compounds from these plants.

Key words: *Aeromonas hydrophila*, *Cyprinus carpio*, haematological parameters, immune response, immunological parameters, plant extracts

INTRODUCTION

In aquaculture, infectious parasitic, bacterial and fungal diseases are mainly controled by chemotherapeutics and antibiotics. However, recently the use of antibiotics and chemotherapy has been criticized because their use has created problems with drug resistance bacteria, toxicity and accumulation both in fish an environment. On the contrary, natural products like plant extracts might have beneficial effects but cause no problems (Farag *et al*., 1989; Citarasu *et al*., 2002; Sagdic and Ozcan, 2003). Some plants are rich sources of compounds like volatile oils, saponins, phenolic compounds, tannins, alkaloids, polypeptides and polysaccharides.

These natural plant products have various activities like antistress, appetizer, antimicrobials and immunostimulants (Citarasu *et al*., 2002, 2003). Many plants extracts have been investigated for antimicrobial activity in fish. Experience demonstratesthat some of these have enormous therapeutic potential. Moreover, they are cheaper and safer while simultaneously are non-toxic, biodegradable and biocompatible. Recently in aquaculture, scores of plant extracts have been tested and used with good results in the control of bacterial and viral diseases. Fortheen herbs have been tested against *Aeromonas hydrophila* infection in tilapia (*Oreochromis niloticus*) and among these herbs the ethanol extract of *Psidium guajava* has been found to have the highest antimicrobial activity (Pachanawan *et al*., 2008). The methanolic extract of *Cynodon dactylon*, *Aele marmelos Tinospora cordifolia*, *Picrorhiza kurooa* and *Eclipta alba* were mixed thoroughly in equal proportion and tested against White Spot Syndrome Virus (WSSV) infection in black tiger shrimp, *Penaeus monodon*. The results indicated that shrimps were fed 800 mg kg^{-1} mixed diet significantly ($p<0.0001$) had more survival (74%) and reduction in the viral load (Citarasu *et al*., 2006). The aqueous *Azadirachta indica* leaf xtract has been tested against *Aeromonas hydrophila* infection in common carp,

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Cyprinus carpio and the results showed that this plant could effectively control *A. hydrophila* infection in *C. carpio* (Harikrishnan *et al*., 2003). *Achyranthes aspera* seed was incorporated in the diet of *Labeo rohita*, roha fingerlings and the results indicated that *A. aspera* seed stimulated immunity and increased resistance to *Aeromonas hydrophila* infection in fish (Rao *et al*., 2006). These findings suggest that herbs and their derivatives such as extracts could be an alternative to the chemotherapeutics in aquaculture. The aim of this study was to determine the effects of several 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 obtained from a commercial fish farm at Bandar Torkman, Golestan, Iran. Before the initiation of the feeding trial, fish were acclimated to experimental conditions for 2 weeks by feeding the basal experimental diet (Table 1) without supplementation of the plant extracts. During this time, they were kept in 600 L containers with recirculated water and fed at 3% body weight once daily.

Preparation and testing of the plant extracts for antibacterial activities: Plants species evaluated in this study are shown in Table 1. Fresh plant parts were washed with deionized water dried at 37°C and ground well. Dried plant 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. The filtrate obtained from ethanol was evaporated to dryness at 40°C in a rotary evaporator (IKA® HB10 basic, China) and the water extract was then freeze-dried by using a freeze drier system (Operon: FDB-5503, Korea). Finally, the dried extracts were stored at 4°C until use (Arabshahi-Delouee and Urooj, 2007). Screening plant extracts for their antibacterial activity against *A.hydrophila* was conducted using the disc diffusion method as described by Bauer *et al*. (1966). All the tests were replicated three times and zone of inhibition of each extract was measured and recorded (Table 2).

Pathogen: *Aeromonas hydrophila* (ATCC 49040) was obtained from the Razi Researches Institute, Karaj, Iran. Bacteria were inoculated into 10 mL of liquid tryptic soy broth (TSB, Sigma) medium and were incubated overnight in a shaker for 12 h at 28°C. The culture broth was then centrifuged at 3000× g for 10 min. The supernatant was then discarded and the bacterial pellet was washed three times with sterile phosphate buffered saline (PBS, pH 7.2) and prepared to 10^8 cfu mL^{-1} as determined using a Neubaur hemocytometer slide (Yadav *et al*., 1992; Harikrishnan *et al*., 2003; Rao *et al*., 2006). This bacterial suspension was used for further experiments.

Preparation of test diets: The ingredients and proximate compositions of the basal and experimental diets are shown in Table 2. Based on the zone of the inhibition, *Inula helenium*, *Tussilago farfara*, *Brassica nigra*, *Echinacea purpurea* and *Chelidoniume majus* were selected for the present study. Equal proportions of the all five plant extracts were mixed thoroughly and

Table 1: Composition of basal diet supplemented with herbal immunostimulant

	Diet					
Parameters	A (control)	B	C	D	E	F
Ingredients (g kg^{-1} diet)						
Fish meal[a]	175.00	175.0	175.000	175.000	175.000	175.0
Soybean meal	280.00	280.0	280.000	280.000	280.000	280.0
Gluten meal	45.00	45.0	45.000	45.000	45.000	45.0
Casein	45.00	45.0	45.000	45.000	45.000	45.0
Rice bran	175.00	175.0	175.000	175.000	175.000	175.0
Wheat flour	104.00	104.0	104.000	104.000	104.000	104.0
Corn oil	32.00	32.0	32.000	32.000	32.000	32.0
Fish oil[b]	42.00	42.0	42.000	42.000	42.000	42.0
Cellulose[c]	77.00	77.0	77.000	77.000	77.000	77.0
Vitamin premix [d]	12.50	12.5	12.5.00	12.500	12.500	12.5
Mineral premix [e]	12.50	12.5	12.5.00	12.500	12.500	12.5
Herbal immunostimulant	0.00	0.1	0.250	0.500	0.750	1.0
Proximate chemical composition (g kg^{-1} diet)						
Dry matter	917.20	908.2	922.100	905.100	918.100	911.3
Crude protein*	335.10	338.3	348.400	342.200	331.200	345.2
Crude fat**	121.30	128.2	126.600	121.100	124.400	123.1
Ash	831.60	827.1	834.500	833.700	821.500	81.6
Moisture	84.20	86.4	82.400	84.500	89.200	83.5

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

Botanical names	Family	Parts used for antibacterial screening	Inhibition zone (mm)*
Inula helenium	Asteraceae	Rhizome and root	25.4±0.15[a]
Tussilago farfara	Asteraceae	Flower	24.5±0.17[a]
Brassica nigra	Brassicaeae	Seed	23.2±0.36[b]
Echinacea purpurea	Asteraceae	Root	20.4±0.36[c]
Chelidoniume majus	Papaveraceae	Whole plant	19.7±0.28[c]
Achilla millefolium	Asteraceae	Leaf and flower	16.8±0.23[d]
Thymus vulgaris	Lamiaceae	Leaf and flower	15.3±0.26[e]
Menth piperita	Lamiaceae	Leaf	13.5±0.11[f]
Salvia officinalis	Lamiaceae	Leaf and flower	13.3±0.10[f]
Olea europaea	Oleaceae	Leaf	8.4±0.05[g]

*Disc diffusion test. Values are expressed as mean±SE. Means receiving same superscript are statistically not significant (p>0.05)

incorporated into five experimental diets containing 100, 250, 500, 750 and 1000 mg of the extract mixture. Control diet was prepared using the same composition of ingredients except the extract mixture. To prepare the diets, initially, dry ingredients were mixed thoroughly and 1% binder was added. Sufficient water along with the oil ingredients were then added to make a paste of each diet. The paste was then cold extruded and pelletized using a hand pelletizer. Finally, the diets were air-dried and stored at -2°C (Abdel-Tawwab *et al*., 2008) in air tight containers until fed.

Experimental design and feeding: Fish (n = 1080) were randomly distributed into 5 experimental groups (B-F) and a control group (A) in triplicate maintained in 18 tanks (500 L capacity) each containing 60 fish. Group A received the control diet, group B, the feed containing 100 mg mixed extract, group C, D, E and F the diet containing 250, 500, 750 and 1000 mg extract mixture, respectively. The fish were fed thrice a day at 8:00, 13:00 and 18:00 at 3% of the body weight until the end of the experiment. The water quality parameters were monitored every day and maintained at optimal levels by regular 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 20 days, 24 h after final feeding, 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 anesthetized with 100 mg tricaein methan sulphate (MS-222) L^{-1}. The blood was then transferred to an Eppendroff tube containing heparin solution, shaken gently and stored in refrigerator at 4°C. For serum, another 10 fish from each tank were anaesthetized and blood samples were collected without heparin and allowed to clot for 2 h at room temperature.

The supernatant serum was separated and collected from the remaining blood after centrifugation (2500× g for 15 min) and then kept in freezer at -80°C until analysis.

Challenge test: On days 60 after feeding, 35 fish from each group were injected intraperitoneally (Schaperclaus *et al*., 1992) with 100 µL of live *A. hydrophila* (1×10^8 cfu mL^{-1}). Mortality of the challenged fish was noted up to 10 days. The surviving fish were then sampled for serum and blood parameters as per the method described earlier.

Hematology and biochemical indices: For the estimation of leucocyte, erythrocyte counts and hemoglobin concentration, the fresh whole blood samples were used. Red (RBC) and White Blood Cell (WBC) counts were done following the method of Schalm *et al.* (1975). Hemoglobin (Hb) content of the blood was estimated by Cyanmethaemoglobin method (Drabkin, 1946; Varley *et al*., 1991). Total protein content was determined according to the method of Lowry *et al.* (1952) and albumin estimation was down following the method of Wotton and Freeman (1982). Globulin was calculated by subtracting albumin level from total protein. For albumin globulin ratio, albumin value was divided by globulin value. Glucose was estimated colorimetrically according to Trinder (1969).

Immunological assays: Bactericidal activity was measured according to the procedure described by Kajita *et al.* (1990). Respiratory Burst (NBT) activity was measured by the Nitroblue Tetrazolium (NBT) assay. For NBT assay, Secombes (1990) method was followed with modification described by Stasiak and Baumann (1996). Lysozyme activity was determined by the method of Parry *et al.* (1965) with slight modification described by Gopalakannan and Arul (2006).

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

RESULTS

Screening of antimicrobial activity of plant extracts against *A. hydrophila*: Plant extracts showed different degrees of inhibition against *A. hydrophila* in disc diffusion assay (Table 2). The maximum inhibitory zone was recorded for *Inula helenium* followed by *Tussilago farfara*, *Brassica nigra*, *Echinacea purpurea* and *Chelidoniume majus*.

Haematological analysis: Hemoglobin content was significantly ($p<0.05$) higher in group D, E and F at all the

Table 3: Effects of herbal extracts supplemented diets on haematological parameters of *C. carpio*

Parameters	Groups	Days 20	40	60	70
Haemoglobin (g L^{-1})	A	80±0.66^{c}	78.23±0.74^{c}	85±1.47^{c}	81.20±1.35^{c}
	B	82±0.98^{bc}	91.40±1.35^{b}	95.20±1.43^{abc}	85.10±1.76^{c}
	C	84.30±1.37^{bc}	86.40±1.73^{b}	92.40±2.38^{bc}	87.20±1.09^{bc}
	D	87.40±1.59^{b}	100±2.4^{a}	95.70±2.48^{abc}	93.20±1.61^{ab}
	E	98.10±1.55^{a}	104.30±1.72^{a}	105.60±2.82^{a}	97.30±1.38^{a}
	F	95.23±1.67^{a}	102.20±1.72^{a}	100±0.92^{ab}	88.40±2.45^{bc}
White blood cell (×10 ^{4} mm^{3})	A	2.61±0.011^{e}	2.46±0.017^{e}	2.63±0.030^{e}	2.32±0.020^{d}
	B	2.68±0.020^{bc}	2.74±0.026^{d}	3.14±0.011^{d}	2.57±0.117^{c}
	C	2.7±0.020^{abc}	3.12±0.020^{c}	3.41±0.023^{c}	2.81±0.011^{b}
	D	2.73±0.023^{ab}	3.63±0.020^{b}	3.82±0.020^{a}	2.92±0.017^{ab}
	E	2.81±0.017^{a}	3.78±0.015^{a}	3.81±0.017^{a}	3.1±0.011^{a}
	F	2.75±0.034^{ab}	3.65±0.017^{b}	3.61±0.011^{b}	2.87±0.015^{b}
Red blood cell (×10^{6} mm^{3})	A	1.8±0.085^{a}	1.82±0.0173^{f}	1.92±0.0152^{e}	1.80±0.0115^{e}
	B	1.83±0.0173^{a}	2.11±0.0176^{e}	2.68±0.0173^{d}	1.87±0.0264^{e}
	C	1.83±0.005^{a}	3.26±0.0115^{b}	2.81±0.0115^{c}	2.24±0.0288^{d}
	D	1.92±0.0173^{a}	2.31±0.0^{d}	3.35±0.0230^{b}	2.67±0.026^{c}
	E	1.95±0.0115^{a}	3.42±0.0173^{a}	3.61±0.0208^{a}	3.21±0.0173^{a}
	F	1.96±0.028^{a}	3.24±0.0057^{b}	3.01±0.0176^{c}	2.82±0.0208^{b}

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

Table 4: Effects of herbal extracts supplemented diets on biochemical parameters of *C. carpio*

Parameters	Groups	Days 20	40	60	70
Total protein g L^{-1})	A	21.63±0.29^{d}	20.4±0.43^{d}	23.8±0.05^{d}	16.7± 0.79^{d}
	B	23.5±0.40^{cd}	24.6±0.05^{c}	28.7±0.63^{bc}	21.16± 0.66^{c}
	C	25.4±0.10^{bc}	28.1±0.55^{b}	28.4±0.65^{c}	24.8±0.36^{b}
	D	25.4±0.55^{bc}	27.3±1.19^{bc}	30.3±0.30^{b}	25.2±0.60^{b}
	E	33.1±0.20^{a}	33.1±0.20^{a}	32.5±0.65^{a}	28.3±0.25^{a}
	F	27.7±0.05^{a}	29.3±0.30^{b}	28.5±0.30^{c}	26.2±0.60^{ab}
Albumin (g L^{-1})	A	9.33±0.120	8.93±0.202^{d}	10.43±0.145^{c}	7.56±0.338^{c}
	B	10.50±0.173^{c}	11.66±0.088^{c}	12.60±0.288^{b}	8.66±0.233^{c}
	C	11.56±0.088^{b}	12.96±0.272^{b}	12.43±0.491^{b}	11.63±0.176^{b}
	D	11.80±0.151^{b}	12.80±0.472^{b}	13.13±0.375^{b}	11.76±0.317
	E	13.03±0.284^{a}	15.53±0.145^{a}	14.83±0.352^{a}	12.96±0.218^{a}
	F	11.76±0.088^{b}	13.23±0.088^{b}	13±0.321^{b}	11.10±0.152^{b}
Globulin (g L^{-1})	A	12.30±0.20^{c}	11.46±0.23^{d}	13.36±0.12^{d}	9.06±0.56^{c}
	B	13±0.23^{bc}	12.93±0.08^{cd}	16.10±0.34^{bc}	12.30±0.47^{b}
	C	13.83±0.03^{b}	15.13±0.24^{b}	15.96±0.20^{bc}	13.16±0.29^{ab}
	D	13.56±0.43^{bc}	14.50±0.72^{bc}	17.16±0.26^{ab}	13.43±0.29^{ab}
	E	14.26±0.31^{b}	17.56±0.08^{a}	17.66±0.46^{a}	15.33±0.28^{a}
	F	15.93±0.13^{a}	16.06±0.23^{ab}	15.50±0.26^{c}	15.10±0.66^{a}
A:G ratio	A	0.758±0.008^{cd}	0.778±0.002^{d}	0.784±0.021^{a}	0.838±0.045^{ab}
	B	0.807±0.001^{bc}	0.901±0.021^{a}	0.780±0.003^{a}	0.721±0.018^{b}
	C	0.835±0.006^{b}	0.856±0.005^{bc}	0.778±0.024^{a}	0.884±0.023^{a}
	D	0.871±0.025^{ab}	0.883±0.011^{ab}	0.765±0.030^{a}	0.870±0.009^{ab}
	E	0.913±0.00057^{a}	0.883±0.006^{ab}	0.840±0.025^{a}	0.879±0.013^{a}
	F	0.738±0.011^{d}	0.823±0.008^{c}	0.839±0.030^{a}	0.738±0.040^{ab}
Glucose (mg L^{-1})	A	1245±6.65^{a}	1253±3.51^{a}	1224±3.51^{a}	1176±3.46^{a}
	B	1242±8.62^{a}	1108±4.61^{c}	1215±3.78^{a}	1018±4.72^{b}
	C	1212±6.92^{a}	1059±5.33^{d}	1104±4.58^{b}	1022±6.02^{b}
	D	1187±6.02^{c}	1203±5.13^{b}	1003±5.56^{c}	867±5.13^{d}
	E	1115±4.58^{d}	1002±5.50^{e}	862±4.16^{d}	784±4.35^{e}
	F	1112±5^{d}	988±4.93^{e}	863±4^{d}	934±4.16^{c}

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

assay period as compared to the control and other experimental groups. Moreover, The difference among D, Eand F was not significant (p>0.05) (except group D on day 20 and group F on day 70) (Table 3). At all the assay periods, a gradual significant increase of WBC count (p<0.05) was observed which reached a maximum in group E and then decreased in group F (Table 3). The RBC count was enhanced in all the experimental groups. The highest significant RBC level (p<0.05) was observed in group E on day 40, 60 and post-challenge period (day 70). In addition, there was no significant impact (p>0.05) of different doses on day 20 (Table 3).

Biochemical parameters: The total protein level increased in all experimental groups compared with the control group (Table 4). The maximum level of total protein was recorded in group E for all the assay duration (except in group F for day 20). Similarly, albumin content was significantly ($p<0.05$) higher in group E as compared to other groups on different assay days. The globulin level showed a slight increasing trend in the treatment groups but there was no significant difference ($p>0.05$) between E and F on different assay periods. Albumin globulinratio in the present study did not show a regular trend. Moreover, Albumin globulin ratio in different groups was not significantly different ($p>0.05$) from the control group throughout the periods of experimental study (Table 4). The level of glucose was shown in Table 4. Compared to the control group, a gradual significant decrease ($p<0.05$) in the glucose value was observed for all days except in group B and C for day 20 and B for day 60.

Immunological assay: The results of the serum bactericidal activity are shown in Fig. 1. Bactericidal 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 20). In addition, the highest level of bactericidal activity was observed in group E and F. Though the bactericidal activity in E and F was highest, the difference between E and F was not significant ($p>0.05$). The respiratory Burst (NBT) activity showed an increasing trend from A (control group) to E and it was then started to decrease in group Fat all the assay periods and after challenge (Fig. 2). In all the treatments, a gradual significant increase of lysozyme activity ($p<0.05$) was observed which reached a maximum in group E at all assay periods including post-challenge (Fig. 3).

Challenge study: The mortalities of the challenged fish were observed from the first day post-challenge. After day 8 there was no mortality up to day 10. The survival percentage was found highest (85.71%) in the group E followed by group F (80.95%) and lowest (38.09%) in control group (Fig. 4).

DISCUSSION

It is generally accepted that monocytes, granulocytes, neutrophils, macrophages and humoral elements, like lysozyme, agglutinin and metalion binding proteins are the main components of the non-specific immune system (Secombes and Fletcher, 1992; Ardo *et al*., 2008; Dalmo *et al*., 1997; Sakai, 1999). Within aquaculture, there are many studies reporting herbal medicine extracts can be used as immunostimulants to enhance non-specific immune system

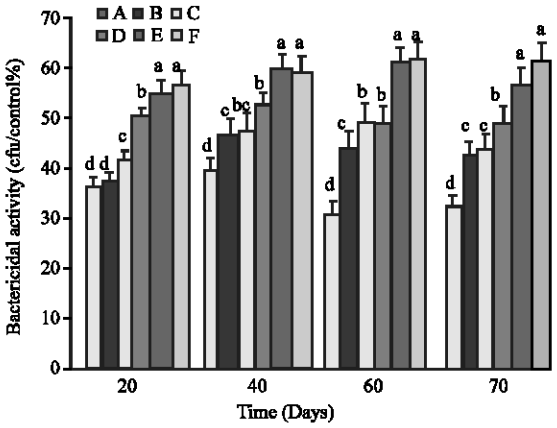

Fig. 1: Bactericidal activity (cfu/control) of common carp, *C. carpio* fed on herbal diet (B-F) and control diet (A). Data are expressed as mean±SE. Mean values bearing same superscriptare not statistically significant ($p>0.05$)

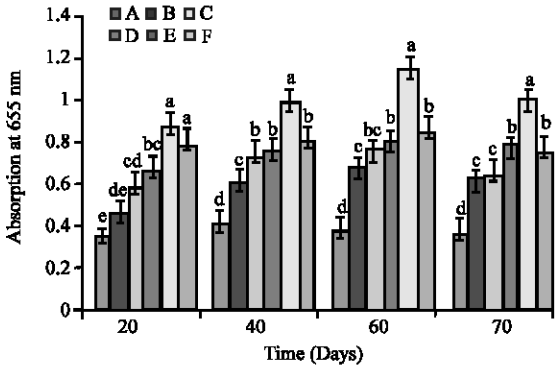

Fig. 2: Respiratory burst (NBT) activity of common carp, *C. carpio*, fed on herbal diets. Means with the same letters are not significantly different ($p>0.05$). Data are expressed as mean±SE

of cultured fish species (Sakai, 1999; Shao *et al*., 2004; Tan and Vanitha, 2004; Rao *et al*., 2006; Sahu *et al*., 2007; Ardo *et al*., 2008). 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, 1996; Ardo *et al*., 2008). It is considered that these oxygen forms are toxic for bacterial pathogens (Hardie *et al*., 1996; Itou *et al*., 1996; 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 (Siwicki and Studnicka, 2006; Sahu *et al*.,

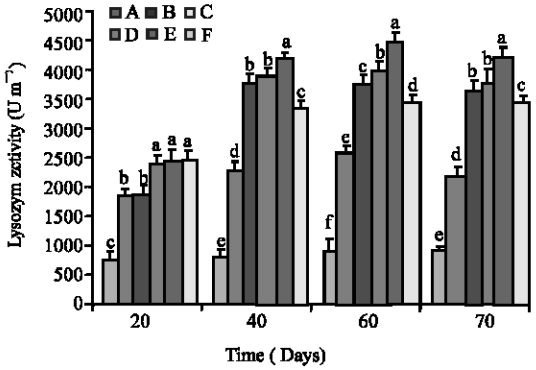

Fig. 3: Changes in plasma lysozyme activities in control group (A) and in groups fed diets containing different concentration of herbal extracts (B-F) in common carp, *C. carpio*. Data are expressed as mean±SE. Values receiving same superscript are statistically not significant (p>0.05)

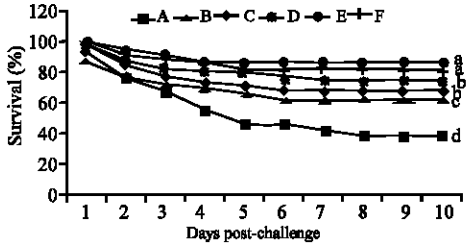

Fig. 4: Survival rate of *C. carpio* fed on herbal extracts supplemented diets (B-F) and control diet (A) after challenged with *A. hydrophila*. Statistical differences (p<0.05) between groups are indicated by different letters (a-d)

2007; Ardo *et al*., 2008). Gopalakannan and Arul (2006) reported that dietary supplementation of chitosan, chitin and levamisole significantly (p≤0.001) enhanced the NBT reduction in *C. carpio*. Herbal based immunostimulants can also enhance the respiratory burst activity of fish phagocits. *Magnifera indica* (mango) kernel as a feed additive enhanced the superoxide anion production in *Labeo rohita* fingerlings (Sahu *et al*., 2007). 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. Superoxide anion production by the blood leucocytes was also enhanced in *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed (Rao *et al*., 2006). Similarly, in the present study, a significant increase was observed in respiratory Burst (NBT) activity in all experimental groups after feeding the fish with plant extract.

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-acetylmuramic 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). Several reports are available in which immunostimulants can enhance the lysozyme activity (Ardo *et al*., 2008; Hanif *et al*., 2005; Chen *et al*., 2003; Puangkaew *et al*., 2004). The level of serum lysozyme was 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). Similarly, plasma lysozyme activity was increased in crucian carp by feeding four Chinese herbs (*Rheum officinale andrographis paniculata, Isatis indigotica, Lonicera japonica*) (Chen *et al*., 2003). In the experiment, fish fed with herbal extract supplemented diets showed a significant increase of lysozyme activity compared with control group. In the present study compared with control group, serum bactericidal activity was significantly increased in all experimental groups. This revealed that the immunostimulant herbals incorporated diets helped to increase the humoral elements in the serum. This is in agreement with the report of Sivaram *et al*. (2004) 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).

The plant extracts, used in this study could enhance total plasma protein, albuminand globulin values in treatment groups compared to control group. Similar resultswere reported after feeding the rohu fingerlings (*Labeo rohita*) with *Achyranthes aspera* seed (Rao *et al*., 2006). Also, Sahu *et al*. (2007) 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, measurable total protein, albuminand globulin levels suggest that high concentrations are likely to be a result of the enhancement of the non-specific immune response of fishes.

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). An inverse relationship between glucose level and value of plant extract mixture

in the diet was observed. As the value of extract mixture increased in the diet, the level of glucose decreased. This might be due to the capability of plant extracts to reduce the effects of stressors.

This is in agreement with the reports of Sahu *et al.* (2007) and Citarasu *et al.* (2006) that glucose levels were reduced in the aquatic animals fed on herbal immunostimulant diets. Hemoglobin, WBC and RBC counts in the present investigation were significantly higher in extract mixture supplemented groups as compared to the control.

The finding of the present study are similar to those of Sahu *et al.* (2007) who reported that WBC and RBC counts were higher in *Labe rohita* fingerlings fed *Magnifera indica* kernel when compared to control.

These observations are also in agreement with the findings of Gopalakannan and Arul (2006) who reported that there was an increase in the WBC count after feeding the common carp with immunostimulants like chitin. In the present study after challenge with *A. hydrophila*, all experimental groups showed higher survival rate compared to the control. This might be due to the enhancement of the non-specific immune system of the fish by plant extracts.

Similar results were reported after feeding *Labeo rohita* fingerlings with *Magnifera indica* kernel and challenging with *A. hydrophila* (Sahu *et al*., 2007). Also Pachanawan *et al.* (2008) reported that survival rate after challenging the fish with *A. hydrophila* was enhanced in tilapia (*Oreochromis niloticus*) fed diets containing either dry leaf powder of *Psidium guajava* or ethanol extract of *P. guajava* leaf. Ardo *et al.* (2008) also reported that feeding tilapi (Oreochromis niloticus) with two Chinese medicine herbs reduced mortality when fish were experimentally infected with *A. hyrophila.*

CONCLUSION

In conclusion, the plant extracts used in this study considerably could enhance the non-specific immunity and increase disease resistance of common carp, *C. carpio*. In addition, the underlying molecular mechanism beside the isolation and characterization of the active compounds from these plants require more study.

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