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Full Length Research Paper

Immunomodulatory and growth response of *L. rohita* to dietary fortification of clove and/cardamom extract

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Present study was conducted to evaluate the immuno-modulatory and growth response of clove and/or cardamom extracts in the diet of *L. rohita.* The solvent extracts (ethyl acetate) of clove and cardamom were selected based on the highest antioxidant properties and antimicrobial activities exhibited by these two extracts out of eleven commonly used spices screened for the same. Both the spice extracts were mixed separately or in combination at the level of 0.5 and 1.0%. Thus, six experimental diets were Cl-0.5%, Cl-1.0%, Cd-0.5%, Cd-1.0%, C.C-0.5% and C.C-1.0%. Highest growth rate (P<0.05) was recorded in the Cl-0.5 group. Hepatosomatic index (HSI), gastro somatic index (GSI) and protein efficiency ratio (PER) was also highest (P<0.05) in Cl-0.5 group. Lowest mortality was recorded in the Cl-0.5 group after challenged with *Aeromonas hydrophila*. It can be concluded that clove extract at 0.5% in the diet promotes growth, enhances antioxidant activity and protects the immunity of *L.rohita* challenged against *A.hydrophila*. Further studies are needed to find out the effective use of clove and its extract with special reference to the timing, dosage, and method of administration.

Keywords: Antimicrobial activity, antioxidant property, growth performance, immunity, *L rohita*, spice extract,

INTRODUCTION

Intensification of aquaculture is always under the threat of disease occurrence, where stress is the primary causative agent. Several antibiotics, vaccines and chemotherapeutic agents as well as some immunostimulants are being used in many fish farms and hatcheries to prevent these diseases. Antibiotics are frequently used to control fish diseases causes by bacteria, but there is an increasing risk of developing antibiotic resistant strains of bacteria. As an alternative, feeding many plant extracts through diet protects the fish against chronic oxidative stress-related diseases (Sakai, 1999). Many plants contain different compounds in their natural extracts, generally rich in antioxidants (El Saleh *et al*. 2004). Enormous reports are available regarding the immunomodulatory role of different plant extracts in animal (Al Jishi and Abuo Hozaifa 2003).

Like plant extracts, spices used in cooking purpose are rich source of antioxidants (Suhaj 2006). Wojdyło *et al* (2007) measured the phenolic contents (Folin–Ciocalteu) and total equivalent antioxidant capacities of 32 spices, where major phenolic acids identified in these spices included caffeic, p-coumaric, ferulic, and neochlorogenic acid. Predominant flavonoids were quercetin, luteolin, apigenin, kaempferol and isorhamnetin. Shan et al. (2005)

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and (Shan et al. 2007) also found that, out of 46 spice extracts evaluated, many exhibited antibacterial activity against the food borne pathogens. The antibacterial activities of these extracts were closely associated with their phenolic contents.

Dorucu *et al* (2009) investigated the effect of black cumin seeds, *Nigella sativa*, on some immuno-hematological parameters and defense mechanisms of rainbow trout, *Oncorhynchus mykiss* and suggested that black cumin seed could be recommended to be used for farmed fish to reduce mortalities caused by pathogenic microorganisms. Metwally (2009) reported that addition of garlic in any form in fish diet can promote growth rate, increase the antioxidant activity and decrease mortality rate in fish.

Eugenia caryophyllus (Clove) belongs to family Myrtaceae, has a number of medicinal properties and its systemic as well as local use has been advocated in traditional medicine. Clove is reported to possess antioxidant (Shobana and Naidu 2000), anti-bacterial (Cai and Wu 1996), anti-pyretic (Fenj and Lipton 1987), anti-candidal (Chami *et al.* 2004), local anesthetic (Ghelardini et al. 2001), and aphrodisiac activity (Tajuddin *et al.* 2003). Since clove has a number of medicinal properties and is a potent antioxidant, the present study was undertaken to evaluate its anti-stress effect in *Labeo rohita*.

Similarly cardamom (*Amomum subulatum*) is the seed of a tropical fruit in the ginger family. In ayurvedic medicine it is used to remove fat and as a cure for urinary and skin complaints. The seeds are aromatic, sweet, cooling, carminative (cures flatulence), digestive stimulant and tonic. Cardamom finds usage in indigestion, anorexia, burning sensation, debility and asthma (Kikuzaki *et al.* 2001). Several phytochemicals, phenolic compounds and trace elements can be isolated from alcoholic extract of clove and cardamom, possessing the anti-inflammatory and antioxidant activity which may be used to counteract the oxidative cellular damage (Steinmetz, Potter 1996; Ness, Powles 1997). In the last years the modern western world has been learning what many Asians and Native Americans have known for centuries, namely that plant extracts and spices can play a significant role in health and nutrition (Bye and Linares 1999).

In this context, many have focused on the use of medicinal plant products as potential therapeutic measures for modulating the immune response and, specifically, on the use of herbs/spices to prevent and control fish diseases. In the study reported here, *Labeo rohita* were fed with fish feed containing clove extract, cardamom extract or the combination of the two extracts. Key factors of growth performance, antioxidant ability and immunological parameters were determined during the experiment and subsequently the fish were challenged with *Aeromonas hydrophila* to determine disease resistance. There are no reports regarding use of clove/cardamom extracts in fish feed elsewhere.

MATERIAL AND METHODS

Fish and Culture Conditions

Labeo rohita (average weight 15.05±2.1g) were obtained from Mahaad Fish Farm, Maharashtra, India and transported to wet lab of Central Institute of Fisheries Education, Mumbai, India and acclimatized for 15 days in 500-L fiber glass tanks. During the experimental period, 50% of water was renewed daily to maintain the water quality. Feeding was done twice daily to satiation. Mean water temperature, pH and dissolved oxygen were around 25±2.0^{0}C, 7.6 and 6.6±0.01 ppm, respectively during the experimental period.

Spice Extracts

The extracts were prepared according to the method previously described (Virdi *et al.* 2003) with some modification. The spices were ground into powder in a laboratory grinder and sieved into fine powder to be used for extraction. About 10g of finely powdered clove and cardamom was weighed separately and extracted with ethyl acetate in a soxhlet's apparatus for at least 24 hrs at 70^{0}C. The solvent with extract was filtered with Wathman no.1 filter paper and centrifuged at 5000rpm for 5 min to obtain clear supernatant. In order to get pure extract, the solvent was removed by using a rotary evaporator (IKA HB10 basic, Labortechnik, Saufen, Germany) at 70°C. Solvent fee extract was finally stored at 4^{0}C until use.

Experimental Design and Diets

Seven experimental diets were formulated (Table 1), so as to contain respective concentrations of clove and cardamom extracts separately and in combination as follows

C – Control (No extract)
Cl-0.5 - Clove extract 0.5%
Cl-1.0 - Clove extract 1.0%
Cd-0.5- Cardamom extract 0.5%
Cd-1.0- Cardamom extract 1.0%
C.C-0.5- Clove+Cardamom extract 0.5% (1:1)
C.C-1.0-Clove+Cardamom extract 1.0% (1:1)

All the ingredients were pressed through a pelletizer (1 mm dia). The pellets were dried at room temperature for overnight and stored at -20^{0}C until use. Feed were given to satiation twice a day throughout the eight weeks of feeding trial. Three hundred fifteen fish (n=315) were equally divided into seven treatment in triplicate (7x3=21), where 15 fish were stocked in (50 L capacity) tanks.

Table 1: Composition of the experimental diets (g/100 grams)

Ingredients	Control	Cl (0.5)	Cl (1.0)	Cd (0.5)	Cd (1.0)	C.C (0.5)	C.C (1.0)
Casein	29.7	29.7	29.7	29.7	29.7	29.7	29.7
Gelatin	10	10	10	10	10	10	10
Dextrin	18.3	18.3	18.3	18.3	18.3	18.3	18.3
Starch soluble	21.5	21.5	21.5	21.5	21.5	21.5	21.5
Cellulose	10	9.5	9	9.5	9	9	9
Cod liver oil	3	3	3	3	3	3	3
Sunflower oil	3	3	3	3	3	3	3
Vit & min mix	2	2	2	2	2	2	2
CMC	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Betaine	0.9	0.9	0.9	0.9	0.9	0.9	0.9
BHT	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Spice extract	-	0.5	1.0	0.5	1.0	0.5	1.0
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Composition of vitamin mineral mix (PREEMIX PLUS) (quantity/2.5kg), Vitamin A, 55,00,000 IU; Vitamin D3, 11,00,000 IU; Vitamin B2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6 mcg; Calcium Pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 L-lysine, 10 g; DL- Methionine, 10 g; Selenium, 50 ppm

Growth Performance

Sampling was done every 15 days. At the end of experimental trial of 60 days, average weight of fishes in each tank was taken and sampled for tissue and blood analysis. Two to three fishes from each replicates were sampled and stored frozen (-20^0 C) for analysis of whole body composition. Six fishes from each tank were used for blood, tissue and viscera for estimation/ calculation of enzyme activities, hepatosomatic index (HSI) and gastro somatic index (GSI). Growth performance was calculated as follows:

Weight gain (WG %) = 100 x (final weight – initial body weight)/ initial body weight

Specific growth rate (SGR) = 100 x In (final weight - initial body weight)/duration of experiment

Feed conversion ratio (FCR) =Feed consumed (g, dry weight)/weight gain (g)

Protein efficiency ratio (PER) =weight gain (g)/protein intake (g)

Hepato-somatic index (HSI) = 100 x (liver weight, g)/ (whole body weight, g)

Gastro-somatic index (GSI) = 100 x (visceral weight, g)/ (whole body weight, g)

Challenge study

Lyophilized *Aeromonas hydrophila* (ATCC 49140) was taken from IMTECH (Institute of Microbial Technology, Chandigarh, India). Lyphillized *Aeromonas hydrophila* was inoculated into 10ml of liquid tryptic soy broth (TSB, Sigma) medium and was grown overnight at 28^{0}C. Culture was centrifuged at 1000g for 10 min. Supernatant was removed and the pelleted bacteria were washed twice in sterile phosphate buffered saline (PBS) solution. A challenge study was done for 10 days after 60days of experimental period in which remaining fishes in each tank were injected intraperitoneally with 100µl of live *Aeromonas hydrophila* (3x10^6 CFU) suspended in PBS. Mortality of the challenged fish was observed and noted for every 12 h interval up to 10th day. The tissue of dead fish was also taken to ascertain whether the cause of mortality was due to *Aeromonas hydrophila*.

Sampling

Fish were sampled on 10th day after challenge and blood (100 µl) was drawn from the caudal vein using anticoagulant and kept in refrigerator. Blood samples were also collected for serum analysis in two ml ependroff tubes without anticoagulant and allowed to clot overnight. The tubes were then centrifuged at 2500 rpm for 15 min and the serum was collected in ependroff tubes and stored at -40^0 C.

Determination of Superoxide Anion (NBT)

The intracellular respiratory burst activity was measured (Secombes 1990) with the following minor modifications. Peripheral blood leucocytes (1x10^6 cells per well in triplicate) were incubated with 25µl nitroblue tetrazolium (NBT, 1mg/ml) in 75 µl culture medium for 2 h at 28^{0}C. The supernatant was removed and the cells were fixed with 100% [v/v] methanol for 5 min. Each well was washed twice with 125 µl of 70% methanol. The fixed cells were allowed to air dry. The reduced NBT was dissolved using 125 µl of 2 N potassium hydroxide (KOH) and 150 µl of DMSO per well and the optical density was measured at 650nm.

Table 2: Growth parameters of different experimental groups at the end of the experiment.

Treatment	Wt.gain (%)	SGR	FCR	PER	HSI	GSI
Control	$36.76^{a}\pm0.057$	$0.84^{a}\pm0.01$	$2.02^{cd}\pm0.01$	$1.32^{ab}\pm0.01$	$1.86^{a}\pm0.30$	$4.24^{a}\pm0.27$
Cl (0.5%)	$40.95^{f}\pm0.044$	$0.90^{b}\pm0.01$	$1.83^{a}\pm0.01$	$1.71^{d}\pm0.01$	$2.97^{b}\pm0.27$	$5.89^{b}\pm0.15$
Cl (1.0%)	$39.27^{c}\pm0.118$	$0.87^{c}\pm0.01$	$1.96^{b}\pm0.11$	$1.68^{c}\pm0.03$	$2.88^{ab}\pm0.06$	$5.58^{ab}\pm0.30$
Cd (0.5%)	$38.81^{b}\pm0.290$	$0.86^{c}\pm0.03$	$2.01^{d}\pm0.02$	$1.44^{a}\pm0.12$	$1.90^{ab}\pm0.41$	$5.20^{ab}\pm0.13$
Cd (1.0%)	$39.54^{c}\pm0.017$	$0.87^{c}\pm0.01$	$1.99^{bc}\pm0.01$	$1.53^{bc}\pm0.03$	$2.88^{ab}\pm0.48$	$5.38^{ab}\pm0.81$
CC (0.5%)	$28.91^{d}\pm0.161$	$0.89^{d}\pm0.01$	$1.98^{b}\pm0.01$	$1.59^{bc}\pm0\ .03$	$2.11^{ab}\pm0.28$	$4.72^{ab}\pm0.34$
CC (1.0%)	$37.12^{e}\pm0.017$	$0.89^{d}\pm0.01$	$2.01^{cd}\pm0.01$	$1.34^{a}\pm0.03$	$2.56^{ab}\pm0.23$	$4.68^{ab}\pm0.44$

Data were presented as mean±SE (n=3). Values within the same column having different superscripts are significantly different (P<0.05).

Total Serum Protein, Albumin and Globulin

Serum total protein and albumin were determined using a kit (MERCK, Mumbai, India). Globulin content was calculated by subtracting the content of albumin from the total protein content.

Lysozyme Activity

For assay of serum lysozyme activity, serum samples were diluted with phosphate buffer (pH 7.4) to final concentration of 0.33 mg per ml. In a suitable cuvette, 3 ml of *Micrococcus luteus* suspension in phosphate buffer (A_{450} = 0.5-0.7) was taken, to which 50 microlitre of diluted serum sample was added. The content of the cuvette was mixed well for 15 seconds and reading was taken in a spectrophotometer at 450 nm exactly after 60 seconds of addition of serum sample. This absorbance was compared with standard lysozyme of known activity following the same procedure as above. The activity was expressed as U/min/mg protein.

Plasma Superoxide Dismutase (SOD)

SOD activity was assayed according to the earlier method (Mishra and Fridovich 1972), which is based on the oxidation of epinephrine to adrenochrome by the enzyme. 0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml of ethanol and 0.15 ml of chloroform (chilled in ice) and centrifuged. 0.5 ml of EDTA solution and 1 ml of buffer were added to 0.5ml of supernatant. The reaction was initiated by the addition of 0.5ml of epinephrine and the increase in absorbance (480nm) was monitored at 30 sec for 3min. Enzyme activity was expressed as 50% inhibition of epinephrine auto-oxidation/min/mg protein.

Catalase (CAT)

Catalase activity was assayed according to the method described earlier (Aebi 1984). To a reaction mixture of 2.45ml phosphate buffer (50 mM, pH 7.0), enzyme source was added and the reaction was started by the addition of 1.0 ml of H_2O_2 solution. The decrease in absorbance was measured at 240nm at 15sec intervals for 3min. The enzyme blank was run simultaneously with 1.0ml distilled water instead of H_2O_2 solution. Enzyme activity was expressed as nano moles H_2O_2 decomposed / min / mg protein.

Statistical Analysis

The data were expressed as average mean ± standard error (SE). Statistical analysis of data was done by one-way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). The levels of significance were expressed at 5% (P < 0.05).

RESULTS AND DISCUSSION

Growth Performance

Highest weight gain, SGR and PER was recorded in Cl-0.5 group, which was significantly different (P<0.001) than the control group. The HSI and GSI values were significantly higher than the control (P<0.005). The highest value of HSI and GSI was observed in Cl-0.5 group (Table 2).

Antioxidant Activity

The highest and lowest lysozyme values were recorded in Cl-0.5 group, respectively (Table 3). SOD activity of liver was higher than the gill. The highest activity was observed in the control and the lowest in Cl-0.5 group both in liver and gill (Table 3). Similarly, highest catalase activity was observed in control group and the lowest activity in Cl-0.5 group.

Hematological Parameters

Highest blood glucose level was observed in Cl-0.5 as compared to the control. RBC, WBC counts and Hb content were significantly (P<0.05) higher in Cl-0.5 group compared to the control. Platelet counts were significantly (P<0.05) higher in Cl-0.5, while lowest value was recorded in the control group.
There was significant difference (P< 0.001) in serum protein level among the different treatment groups. Cl-0.5

Table 3: Lysozyme activity, superoxide dismutase and catalase activity in serum, liver and gill of different experimental groups after challenge study.

Treatment	Lysozyme*	SOD (Liver)	SOD (Gill)	Catalase (Gill)	Catalase Liver
Control	26.23^{a} ±0.01	21.35^{e} ±0.52	19.66^{e}±0.97	0.48^{c}±0.06	1.10^{e} ±0.18
CI (0.5%)	86.83^{e} ±2.78	61.11^{a} ±0.42	41.10^{a}±1.32	2.36^{a}±0.23	3.91^{a} ±0.07
CI (1.0%)	30.60^{b} ±4.37	46.00^{bcd}±1.73	37.28^{d}±1.57	1.49^{b}±0.02	2.49^{b} ±0.38
Cd (0.5%)	52.45^{bcd}±2.27	42.89^{bc} ±1.11	26.15^{b}±0.28	1.69^{bc}±0.12	3.38^{de}±0.08
Cd (1.0%)	43.71^{c}±8.74b	39.07^{b} ±1.17	25.53^{b}±0.43	1.39^{b}±0.17	3.20^{cd}±0.10
CC (0.5%)	52.45^{cd} ±2.00	49.60^{cd} ±0.57	23.10^{c}±1.96	1.84^{bc}±0.48	3.09^{bcd}±0.20
CC (1.0%)	78.68^{cd} ±7.57	53.68^{de} ±1.64	30.43^{c}±0.62	1.73^{bc}±0.21	2.61^{bc}±0.08

Data were presented as mean±SE (n=3). Values within the same column having different superscripts are significantly different (P<0.05). * One unit of lysozyme producing a decrease in absorbance of 0.001/min.

Table 4: Respiratory burst activity, serum total protein, albumin, globulin and A:G ratio of different experimental groups after challenge study.

Treatment	NBT (OD/540nm)	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	A:G ratio
Control	0.13^{a} ±.006	4.84^{a} ±0.01	1.88^{e} ±0.01	3.41^{b} ±0.01	0.95^{e} ±0.02
CI (0.5%)	0.41^{e} ±.004	7.56^{e} ±0.01	3.51^{f} ±0.06	6.64^{e} ±0.04	0.81^{d} ±0.02
CI (1.0%)	0.31^{d} ±.004	6.51^{c} ±0.32	1.36^{b} ±0.02	5.15^{c} ±0.35	0.26^{a} ±0.02
Cd (0.5%)	0.21^{c} ±.009	5.39^{b} ±0.07	0.44^{a} ±0.03	1.22^{a} ±0.01	0.38^{b} ±0.01
Cd (1.0%)	0.16^{b} ±.002	5.45^{b} ±0.04	1.75^{d} ±0.02	3.69^{b} ±0.01	0.47^{c} ±0.005
CC (0.5%)	0.20^{c} ±.012	5.21^{ab} ±0.07	1.38^{b} ±0.01	4.87^{c} ±0.03	0.28^{a} ±0.00
CC (1.0%)	0.16^{b} ±.001	7.05^{d} ±0.02	1.58^{c} ±0.01	5.98^{d} ±0.00	0.26^{a} ±0.005

Data were presented as mean±SE (n=3). Values within the same column having different superscripts are significantly different (P<0.05).

Table 5: Haematological parameters of different experimental groups after challenge study.

Treatment	Blood glucose (g/dL)	RBC (10^6/µL)	WBC (10^3/µL)	HGB (g/dL)	PLT (10^3/µL)
Control	110.85^{e} ±0.90	7.04^{a} ±0.03	7.80^{e} ±0.10	20.02^{a} ±0.02	7.04^{a} ±0.03
CI (0.5%)	65.73^{a} ±0.85	11.25^{e} ±0.02	12.25^{a} ±0.15	51.26^{g} ±0.03	11.25^{e} ±0.02
CI (1.0%)	77.41^{b} ±0.36	9.24^{c} ±0.03	10.20^{d} ±0.10	41.35^{d} ±0.01	9.24^{c} ±0.03
Cd (0.5%)	90.50^{c} ±2.41	8.26^{b} ±0.03	8.35^{b} ±0.15	37.16^{c} ±0.03	8.26^{b} ±0.03
Cd (1.0%)	93.04^{cd} ±1.08	8.16^{b} ±0.03	9.45^{c} ±0.15	31.22^{b} ±0.02	8.16^{b} ±0.03
CC (0.5%)	92.36^{cd} ±1.56	10.23^{d} ±0.02	9.35^{c} ±0.25	48.36^{f} ±0.02	10.23^{d} ±0.02
CC (1.0%)	96.21^{d} ±0.54	9.35^{c} ±0.02	8.35^{b} ±0.15	42.85^{e} ±0.04	9.35^{c} ±0.02

Data were presented as mean±SE (n=3). Values within the same column having different superscripts are significantly different (P<0.05).

group showed significantly (P<0.05) higher value as compared to the control. Significantly higher value (P<0.001) was observed in serum albumin and globulin level in CI-0.5 as compared to control. Lowest A:G ratio value (P<0.005) was observed in Cd-1.0 as compared to control. Significant difference was recorded in NBT among the different treatment groups (P<0.001). The highest and lowest value was observed in CI-0.5 and control group, respectively (Table 4 & 5).

Mortality

After challenged with *Aeromonas hydrophila*, mortality of fingerlings was recorded for 10 days. There was no mortality up to the first 12 h. After 8th day there was again no mortality. Lowest survival was recorded in the control compare to the treatment groups. The relative percentage survival (RPS) was significantly higher (P<0.05) in CI-0.5 group compare to control group (Table 6).

Many compounds from plants have been used to enhance the immunity of fish when given through different routes of administration. Intraperitoneal administration has been found to be the most rapid and effective route. However, incorporation in the diet is regarded as the most suitable method for fishes. Being non-stressful, dietary administration is the most ideal method, permitting a larger number of fish to be treated with minimum effort and cost as well (Anderson 1992). Many herbal extracts have been reported to be immunostimulants for fish which are generally given through diet (Citarasu and Sivaram 2006). There is growing evidence that spices can acts as free radical scavenger and have protective function against oxidative stress (Owen and Johns 2002). These anti-oxidative and

Table 6: Number of mortalities and relative percentage of survival of *Labeo rohita* fingerlings of different experimental groups after challenge with *Aeromonas hydrophila*.

Treatment	Total no. of fishes (3 replicates)	No. of mortalities	Survival (%)	RPS(%)
Control	15	12	20.00	0.00
Cl (0.5%)	15	2	86.67	86.67
Cl (1.0%)	15	7	53.34	53.34
Cd (0.5%)	15	8	46.67	46.67
Cd (1.0%)	15	7	53.34	53.34
CC (0.5%)	15	6	60.00	60.00
CC (1.0%)	15	7	53.34	53.34

immunomodulatory role of these spice extracts have not been reported in fish, especially in rohu, *Labeo rohita*,.a tropical popular fish, Hence, present study was aimed at to investigate the growth antioxidative and immuno-modulatory effects of clove and or cardamom extracts in the diet of *L.rohita*.

Results of the present study revealed that weight gain, specific growth rate, feed conversion efficiency and protein efficiency ratio were increased in the Cl-0.5 group as compared to the other groups. Clove as a digestive stimulant has been reported earlier, but these are largely empirical; however, the beneficial attribute has been authenticated in exhaustive animal studies only recently (Platel and Srinivasan 2004). Though combination of clove and cardamom extract also supported higher growth but feeding clove extract only (0.5%) exhibited higher growth indicating the enhanced digestive role of clove extract.

Neutrophils play an important role in producing O_2^- and OH^- radicals by respiratory burst which is substantial mechanism for eliminating the pathogenic microorganisms. Glass-adherent NBT (+) cell activation has been reported to increase after stimulation by an immunostimulant (Jeney and Anderson 1993). Although a steady increase was recorded in NBT (+) cell activation in the treated groups, but there was significant difference between Cl-0.5 and the control group ($P<0.05$). This suggests the immunostimulating role of clove extract was more effective in *L. rohita* compare to the cardamom alone or in combination. Enhanced respiratory burst activity of fish phagocytes due to feeding of herbal based immunostimulants has also been reported. Rao *et al*. (2006) reported that superoxide anion production by the blood leucocytes was enhanced after feeding with *Achyranthes aspera* seed in *Labeo rohita*.

The serum protein level is an important indicator of humoral defense system of fish and increases especially in the fish fed with different immunostimulants. Dugenci *et al*. (2003) reported that serum plasma protein level was higher in fish fed with 1% ginger in the diet. The herbals immunostimulant incorporated diets induced to increase the humoral elements in the serum. The results of this study showed that feeding *L.rohita* with supplemented diets containing clove and cardamom enhanced the total plasma protein, albumin and globulin values in treatment groups. This is also in agreement with other worker Rao *et al*. (2006) who found higher serum protein level in rohu fingerlings (*L.rohita*) after feeding with *Achyranthes aspera* seed. Sahu *et al*. (2007) reported that serum protein, albumin and globulin levels in *L.rohita* fingerlings fed with *Mangifera indica* kernel were higher than the control. Since serum protein includes 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 clove extracts at lower dose significantly enhanced the total serum protein, albumin and globulin. The reason could be that clove essential oil displayed the greatest inhibition of a radical known as DPPH, or diphenylpicrylhydrazyl when compared to the other spice extracts in the present study. Cloves also have a strong inhibitory effect against microbes and are able to kill species of bacteria and fungi. This property is a result of the spice's main antimicrobial chemical ingredient: eugenol, the phenol compound. Eugenol makes up the majority of clove bud oil, at 60-90% (Jacobsen 2010).

Lysozyme causes lysis of bacterial cell, triggers the complement system and phagocytes by acting as opsonin (Magnadottir 2006). It is bactericidal in action by hydrolyzing β (1-4) linkages of bacterial cell wall peptidoglycans. In the present study highest lysozyme activity was found in the Cl-0.5 than the control, though higher activity was recorded in all the treatment groups after challenged with *A.hydrophila*, confirming immunostimulating role of clove extract during infection. Elevated lysozyme activity was also observed in Japanese ell (*Anguilla japonica*) after feeding with Korean mistletoe extract (KM-110, *Viscum album Coloratum*) (Choi *et al*. 2008).

The production of free radicals in the body is countered by antioxidant enzymes like SOD and catalase. The mechanism of their protective function is different: superoxide dismutase mainly catalyzes cell defense reaction against potentially harmful effects of superoxide (O_2^-) produced during metabolism. SOD activity was highest in the CL-0.5 group, suggesting minimum production of free radicals as much of these free radicals are being scavenged by clove extract at 0.5%, which also

showed highest anti-oxidant activities. The antioxidant activity of clove could have attributed to its phytochemical contents for the protective effect (Rock *et al*. 1996), or due to their trace element contents which are required for the antioxidant enzyme activity (Lampe 1999). 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). CI-0.5 group exhibited significantly lower blood glucose level than the control groups. This is in agreement with other workers (Sahu *et al.* 2007) and (Citarasu *et al*. 2006) that glucose level reduced in aquatic animals fed on herbal immunostimulant. Hemoglobin content, WBC and RBC counts in the present investigation were significantly higher in CI-0.5 group, which is supported by the findings of (Sahu *et al.* 2007) & (Gopalakannan and Arul 2006).

In the present study after challenge with *A.hydrophila,* all experimental groups exhibited higher survival rate compared to the control group. This might be due to enhancement of the non-specific immune system of the fish by feeding spice extracts. The CI-0.5 group exhibited significantly higher survival throughout the experimental period post challenge infection, when compared with the control group. Feeding of Polinaceas extract (*Echinacea angustifolia* root extract) demonstrated immunostimulatory activity by reducing the mortality in mice, *Candida albicans* both in treated or without treated mice (Morazzonia et al. 2005). Earlier studies revealed that intraperitonial injection of *Ocimum sanctum* leaf extract enhanced the non-specific immune parameters and reducing the mortality thereby protecting the fish against *V.carchariae* infection (Logambal *et al.* 2000). The present finding is also in agreement with the results of other worker (Abutbul *et al*. 2004), in tilapia fed with a diet containing ethyl acetate extract of *Rosmarinus officinalis*.

CONCLUSION

It may be concluded that, clove extract at lower concentration (0.5%) was found to have enhanced digestive ability, which was reflected as higher growth rate. Though clove as digestive stimulant has been recorded earlier, but its effect in *labeo rohita* was confirmed in this study. Beside its role for enhancing growth, the non-specific immune system was stimulated by feeding 0.5% clove extract, which was evident as higher NBT, lower SOD, catalase and lysozyme activity. The antioxidant activity of clove was found to be highest among the eleven commonly available spices. The immunoprotection of clove extract was confirmed by the higher survival of fish after challenged with *Aeromonas hydrophilla*. The immuno stimulatory role of clove extract was found at 0.5% in this study. The efficiency may be studied at still lower doses, which may be species specific. Further study in this aspect will be helpful for its use in aquafeed.

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