The dietary wood betony, *Stachys lavandulifolia* Vahl extract as a growth promoter and immune enhancer in common carp (*Cyprinus carpio*)

Bahrami Babaheydari, S.[1]; Paykan Heyrati, F.[2]; Akhlaghi, M.[3] and Dorafshan, S.[2*]

[1]MSc Student in Culture and Breeding of Aquatics, Division of Fisheries, Department of Natural Resources, Isfahan University of Technology, Isfahan, 84156-83111, Iran; [2]Division of Fisheries, Department of Natural Resources, Isfahan University of Technology, Isfahan, 84156-83111, Iran; [3]Aquatic Animal Health Unit, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

[*]***Correspondence:*** *S. Dorafshan, Division of Fisheries, Department of Natural Resources, Isfahan University of Technology, Isfahan, 84156-83111, Iran. E-mail: sdorafshan@cc.iut.ac.ir*

(Received 5 Oct 2013; revised version 7 Apr 2014; accepted 4 May 2014)

Summary

The present study was conducted to evaluate the efficacy of wood betony (WB), *Stachys lavandulifolia* extract on growth performance and some immune responses in common carp, *Cyprinus carpio*. Different concentrations of the WB extract 0, 2, 4 and 8% (g per 100 g of diet) were added to commercial diet. Each treatment was randomly assigned to triplicate groups of fish having average initial weight of 44 ± 0.62 g for 10 weeks. The results showed that final weight, food conversion ratio, specific growth rate and condition factor were significantly improved by WB in a dose dependent manner, where the best growth parameters were achieved in the group of fish receiving the highest concentration of WB ($P<0.05$). Feeding fish at 2 and 4% W/W by WB in the diet improved lysozyme activity, ACH_{50} and IgM levels significantly in comparison to the control ($P<0.05$). Group of fish fed on 4% WB in the diet had the best levels of the immune characteristics ($P<0.05$). Based on the results of this study, it is recommended to feed common carp with WB to improve growth and non-specific immunity.

Key words: Wood betony, *Stachys lavandulifolia*, Common carp, Growth, Immunity

Introduction

Aquaculture industry is one of the most important sectors of agriculture in the world. Different fish and shellfish species are under cultivation as human food. Among all cultivated species, the fresh water fish, common carp, *Cyprinus carpio* has the highest interest in cultivation areas including the Far East as well as Iran. Elevating the growth rate and immune response of cultivable organisms can significantly affect the benefits of fish culturists. There are many extensive researches on using different synthetic or natural compounds to improve growth and enhance fish health to overcome stressful culture condition (Anderson, 1992). Among different compounds, herbal components received much attention in aquaculture industry during the last decade for different purposes such as growth promotion, immunostimualtion, antiviral, antifungal, antibacterial, aphrodisiac as well as appetite stimulators (Citarasu, 2010; Chakraborty and Hancz, 2011). Wood betony (WB), *Stachys lavandulifolia* Vahl (Laminaceae) is a native plant growing in many parts of the middle east and eastern parts of the Europe in different countries including Iran, Iraq, Syria, Armenia, Turkey and Georgia (Javidnia *et al*., 2004). Several WB components such as leaves, flowers and roots (fresh or dried) have been used as traditional drugs to improve bruises, gum inflammation, wounds, arthritis and respiratory inflammatory disorders (Ody, 1993). Alkaloids (stachydrine and trigonelline), tannins, saponin, nicotinic acid, polyphenols and steroids are the major components of WB (Vundac *et al*., 2007; Bahrami Babaheydari *et al*., 2013) that can cause physiological and biological effects. Despite the importance of WB as a traditional drug in different areas of the world, based on our best knowledge, there wasno scientific literature on the effects of dietary administration of WB in fish as growth promoter and/or non-specific immune enhancer. Therefore, the present study was performed to determine the effects of dietary inclusion of WB extract on some growth and immune parameters in common carp as one of the most important cultivable fish species.

Materials and Methods

Animals, feed preparation, feeding trail and growth measurements

The juvenile common carp, *C. carpio* (44 ± 0.62 g) were obtained from a fish propagation and breeding centre in Isfahan, Iran. Fish were kept under the natural environmental conditions, placed in 10 m^3 rectangular concrete tank for 2 weeks for acclimatisation during summer 2012. They fed on a commercial carp food (Isfahan Mokkamel, Iran) during acclimatisation period. The proximate composition of the commercial diet (wet basis %) was 9.2% humidity, 32% protein, 10.2% lipid and 11.1% ash (based on our analysis, data not shown). The aerial parts of WB including flowers and leaves

were collected from natural habitat, Isfahan province, Iran (Spring 2012). Hydro-alcoholic plant extraction was done based on Ghasemi Pirbalouti *et al*. (2010) with some modification. Briefly, aerial parts of the plants were washed thoroughly with distilled water and dried at room temperature under shading; finally, the plants were ground into powder. 100 g of powdered plant material was soaked in 500 ml of ethanol (75%) for 48 h, and shakene vigorously to allow for proper extraction. After filtering of the extract through Whatman paper no. 1, filtrate was concentrated using a rotary evaporator at around 50°C. Finally, 20 ml of concentrated liquid extract was obtained from l00 g of the plant powder; each ml of the concentrated extract was almost equal to 5 g of the plant powder.

In order to prepare the diets, the commercial pellet (Isfahan Mokkamel, Isfahan, Iran) was crushed, mixed with the appropriate WB liquid extract concentration (the extract volumes were adjusted by adding distilled water to final volume of 100 ml for each kg of diet), remade into pellets, and were allowed to dry for 72 h at room temperature and stored in refrigerator until used. The control diet was prepared by adding only 75% ethanol without any WB extract. The dietary WB was supplemented in four different groups of 0 (control), 2, 4 and 8% WB extract (defined as 2WB, 4WB and 8WB), each in three replicates. Each replicate contained 15 individual fish in a fibreglass tank (110 L water volume, 50% renewed each day). Water quality parameters were maintained as temperature 25 ± 1°C, pH 7.21 ± 0.5 and dissolved oxygen concentration 7.5 ± 0.06 mg/L during the experiment. Fish were fed at the rate of 2% of their body weight per day in the period of the experiment for 10 weeks, at 9:00, 14:00 and 19:00. Final weights, feed conversion ratio (FCR), specific growth rate (SGR) and condition factor (CF) were calculated for each group (Soosean *et al*., 2010).

FCR = Food given (g)/Weight gain (g)

SGR (%/day) = 100 × [(ln final weight - ln initial weight)/duration (days)]

CF (g/cm^3) = body weight (g)/[standard body length (cm)]^3

Immunological assays

Sampling

At the end of the experiment, a total of 10 fish (at least) from each treatment were taken for blood sampling after 24 h of starvation. About 1.5 ml of blood was collected from each fish via caudal vein using non-heparinised syringe (G.18 needle). The serum samples were separated and stored at -80°C till analysis.

Haemolytic assays

Three different non-specific immunological parameters including lysozyme activity (µg/ml), and immunoglobulin M (IgM, mg/ml) and alternative complement activity (ACH_{50}, units/ml) were determined. Briefly, lysozyme activity in serum was determined according to the method of Demers and Bayne (1997) based on the lysis of the lysozyme sensitive Gram-positive bacterium, *Micrococcus lysodeikticus* (Sigma). The dilutions of hen egg white lysozyme (Sigma) ranging from 0 to 20 ml/ml (in 0.1 M phosphate citrate buffer, pH = 5.8) were taken as the standard, and was placed along with the undiluted serum sample (25 ml) into wells of a 96-well plate in triplicate. One hundred and seventy five µL of *M. lysodeikticus* suspension (75 mg/ml) prepared in the same buffer was then added to each well. After rapid mixing, the change in turbidity was measured every 30 s for 5 min at 450 nm at approximately 20°C using a microplate reader.

IgM group level and alternative complement activity (ACH_{50}, units/ml) were determined based on the methodology described by Tahmasebi-Kohyani *et al*. (2011). For IgM determination, 96-well plates were coated with 100 ml serially diluted (1:200) serum samples, incubated overnight at 4°C and washed three times with buffered Tween-20 PBS (50 mM sodium phosphate, pH = 7.4, containing 150 mM NaCl and 0.1% Tween-20). The wells were blocked for 2 h at room temperature with 5% skim milk and underwent three washes with Tween-20 PBS. Anti fish serum (100 µL at a 1:2000 dilution) was added to each well and incubated for 1.5 h at 37°C prior to rinsing with Tween-20 PBS. Identical conditions were used for incubation with the secondary anti-mouse antibody. The plates were revealed by incubation (30 min, room temperature) in the dark with 100 ml of a 0.42 mM solution of O-phenylenediamine dihydrochloride (OPD) in 100 mM citrate/sodium acetate buffer, pH = 5.4, containing 0.03% hydrogen peroxide as a substrate. The reaction was stopped by adding 25 ml of 2 M H_2SO_4 per well. Absorbance of the wells was read at 490 nm. Negative controls consisted of samples without primary antibody. The mean absorbance of the negative controls for each plate was subtracted from the optical density at 490 nm (Yeh *et al*., 2008).

To determine alternative complement activity level (ACH_{50}, units/ml), sheep red blood cells (SRBCs) were used as target cells in the presence of gelatin veronal buffer (GVB, Sigma, St. Louis, MO, USA). Individual 20-µL aliquots (2-fold dilutions) of a serially diluted serum with EGTA-$Mg2^+$-GVB buffer (10 mM ethyleneglycoltetraacetic acid and 10 mM $MgCl_2$ in GVB) as a complement source were mixed with 6 µL of SRBC suspension (4×10^8 cells /ml), and the mixture was incubated at 21°C at pH = 7.2 for 2 h. The haemolytic reaction was stopped by adding 200 µL of GVB containing 10 mM EDTA. The mixtures were centrifuged at 1600 g for 10 min at 4°C. The optical density (OD) of the supernatants was measured at 414 nm using an enzyme-linked immunosorbent assay (ELISA) reader (A). The reactions were supplemented with 6 µL EDTA-GVB, 20 µL EDTA-GVB, and 220 µL distilled water to replace the SRBC suspension, the diluted serum, and the diluted serum + EDTA-GVB buffer, respectively, as the SRBC blank (B), serum blank (C), and 100% haemolysis sample (D). The degree of haemolysis (Y) was defined as:

$Y = [A - (B-C)] (D-C)^{-1}$

and calculated, and the lysis curve for each specimen was obtained by plotting Y $(1\text{-}Y)^{-1}$ against the volume of complements added on a log/log scaled graph. The volume of serum complement producing 50% haemolysis (ACH_{50}) was determined, and the number of ACH_{50} units/ml was calculated for each experimental group.

Statistical analysis

Statistical analysis was performed by one way ANOVA at 5% significance level. A multiple comparison test (Duncan multiple range test, DMRT) was conducted to compare the significant differences among the groups using SPSS V.19. Values are presented as mean ± SD for growth parameters and mean ± SE for haemolytic assays.

Results

Survival and growth parameters

There was no mortality or signs of disease attributed to the treatments over the 10 weeks of the experiment. Fish fed diets with 4% and 8% WB had the highest final weight, followed by fish that had the diets with 2% WB. The lowest final weight was in fish fed the WB-free control diet (Table 1). Dietary WB resulted in better FCR and the best one was observed in fish fed diet with 8% WB. There was a significant ($P<0.05$) decrease in condition factor (CF) between the control and the fish fed diet with 8% WB (Table 1). Therefore, Dietary inclusion of WB could cause significant improvement in all the tested growth parameters in common carp including final weight, FCR, SGR and CF at the end of experiments (Table 1).

Lysozyme activity

The serum lysozyme activity ranged from 5.85 to 12 µg/ml, where the highest level of lysozyme activity (12 ± 0.78 µg/ml) was measured for the group that received 4%, followed by (8 ± 0.31 µg/ml) 2% WB group ($P<0.05$, Fig. 1A). There were no significant differences in the lysozyme activity between control and fish which received the highest dose of WB ($P>0.05$, Fig. 1A).

IgM level

The serum IgM level was found to be significantly ($P<0.05$) improved by adding WB in the diet as low dose of 2% W/W in comparison to the control (Fig. 1B). However the highest IgM levels were measured in fish treated with 4% and 2% WB, respectively ($P<0.05$, Fig. 1B). Feeding fish with the highest (8%) concentration of WB did not affect IgM level in comparison to the control group ($P>0.05$, Fig. 1B).

Complement activity (ACH_{50})

The alternative complement activity (ACH_{50}) in the serum was found to be significantly ($P<0.05$) greater in fish fed diets with 2% and 4% WB compared with those of other experimental groups ($P<0.05$, Fig. 1C).

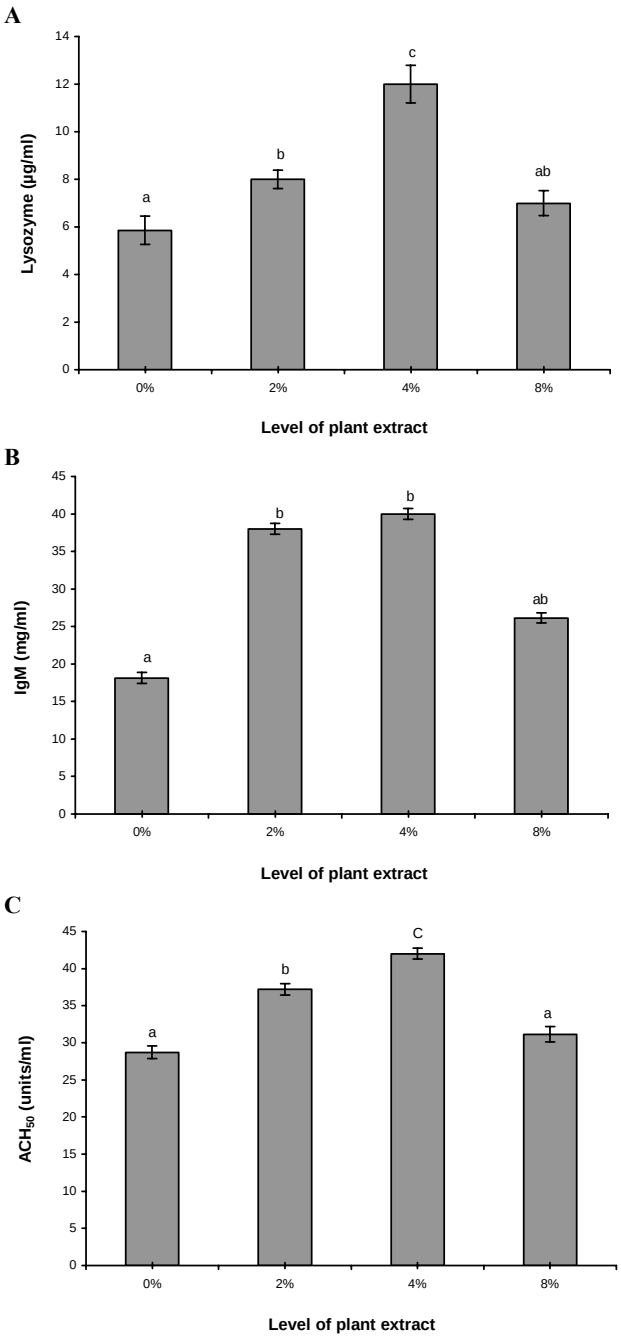

Fig. 1: The effects of dietary wood betony extract on some humoral immunity of carp. **A**: Lysozyme activity, **B**: IgM level, and **C**: Alternative complement activity (ACH_{50}). Data are presented as mean with standard error as error bars; Significant differences ($P<0.05$) between groups is indicated by diferent letters on the bars for each non-specific immunity parameter

Discussion

Increasing demands for producing environmentally friendly aquatic products caused significant elevation in using traditional herbs in aquaculture. Various medicinal herbs have been used in aquaculture industry to achieve different goals such as enhancing growth rates, increasing immune response to pathogens and reducing stress reactions (Galina *et al.*, 2009; Citarasu, 2010). Herbal compounds have been used in different ways

Table 1: Growth performance and feed utilization of juvenile common carp fed diets contacting various percentage of wood betony extract for 10 weeks

Variable (units)	Control (0)	2%	4%	8%
Initial weight (g)	43.88 ± 0.34^{a}	43.42 ± 0.68^{a}	44.53 ± 0.42^{a}	44.02 ± 1.52^{a}
Final weight (g)	72.56 ± 2.18^{b}	81.01 ± 4.27^{ab}	84.51 ± 7.17^{a}	89.74 ± 4.08^{a}
FCR	2.94 ± 0.01^{a}	2.68 ± 0.27^{ab}	2.64 ± 0.31^{ab}	2.42 ± 0.11^{b}
SGR (%/day)	0.72 ± 0.08^{b}	0.89 ± 0.05^{ab}	0.91 ± 0.14^{a}	1.02 ± 0.07^{a}
CF (g/cm)	1.73 ± 0.38^{a}	1.66 ± 0.18^{ab}	1.63 ± 0.18^{ab}	1.52 ± 0.26^{b}

Values are expressed as mean ± SD. At the same row, means with at least one same letter are not significantly different ($P<0.05$)

including injection, immersion and/or as a edible component in diet for aquatic species (Chakraborty and Hancz, 2011). Despite the importance of WB as a traditional medicine (Ody, 1993), to the best of our knowledge, the present study is the first experiment conducted to evaluate the effectiveness of WB in aquaculture. The results of this study showed positive effects of dietary WB on growth performance and some immunstimulation activities in common carp. The positive effects of different plant components as a growth promoter in aquaculture has been reported previously, e.g. in common carp by using *Rheum officinale* extract (Xie *et al*., 2008) or in African catfish, *Clarias gariepinus* by adding *Garcinia mangostana* as a food additive (Soosean *et al*., 2010). For millennia, phytochemicals have been considered as food additive or drugs without any specific knowledge of their cellular actions or mechanisms and it is very difficult to evaluate the physiological effects of a specific component in medicinal plant extract, since a large number of individual compounds may occur in a single extract and their fate *In vivo* cannot be measured. For example, different chemical ingredients such as saponin, polyphenols and carotenoids are determined in WB extract (Vundac *et al*., 2007; Bahrami Babaheydari *et al*., 2013). It has been well documented that polyphenols and caretenoids can act as natural antioxidants and may affect cell to cell signalling, receptor sensitivity, enzyme acting and gene expression regulations (Virgili *et al*., 2008), which may responsible for growth improvements in the common carp. Some other compounds such as saponin may affect the growth rate in opposite ways. Saponin is usually categorised as an antidigestive component (Mahadkar *et al*., 2012). But saponin derived from *Quillaja saponaria* showed positive effects on fish growth by increasing RNA transcription, amino acids and proteins production rates (Francis *et al*., 2005). Such differences may be because of the alternation in saponin sources, concentrations, processing techniques as well as species specific characteristics of animals. Some other components of biomedical plant such as vitamins and trace elements may act as digestive enzyme stimulators which lead to evaluation in feed digestion rates and weight gain (Galina *et al*., 2009).

The results of the present study showed that dietary administration of WB could improve some non-specific defence mechanisms in common carp, However, the highest dose of WB (8%) reduced the level of complement and lysozyme activities as well as IgM in comparison to the control ($P>0.05$). An immuno-stimulant is a chemical, drug, stressor or action that enhances the defence mechanisms or immune response (Anderson, 1992). The immunostimulatory effects of herbal biomedicines were usually measured as lysozyme activity (Yin *et al*., 2006; Sahu *et al*., 2007; Sharma *et al*., 2010), leucocytic phagocytosis (Yin *et al*., 2006; Sahu *et al*., 2007), serum bacterial activity (Sahu *et al*., 2007), total immune globulin level (Sharma *et al*., 2010) as well as antigen-specific serum antibody (Rao and Chakrabarti, 2004). Lysozyme, IgM and ACH_{50} are some of the major components of immune system in the fish (Tahmasebi-Kohyani *et al*., 2011) which can act as anti-bacterial enzymes, especially for Gram-positive species (Chipman and Sharon, 1969), main immunoglobulin, as a major component of humoral immune system in fish (Watts *et al*., 2001) and protecting the fish from a wide range of potentially invasive organisms respectively (Muller-Eberhard, 1988). The immunostimulatory effects of herbal biomedicine in aquaculture have been extensively reviewed (Galina *et al*., 2009; Citarasu, 2010). It has been reported that several components such as alkaloids, tannin and polysaccharides which are present in WB extract have immunostimulatory effects in difference fish species such as common carp, Rohu carp, *Labeo rohita* and tilapia (Chakraborty and Hancz, 2011). Yuan *et al*. (2008) showed that polysaccharides from a *Astragalus* spp. (Fabaceae) can increase some immune-related gene expression in different tissues such as head kidney of common carp. While Divyagnaneswari *et al*. (2007) proposed that the immunostimulatory effect of the Indian herb *Solanum trilobatum* may result from either proliferative responses of the leukocytes or elevation of the phagocytosis, respiratory burst activity and expression of interleukins in tilapia. These differences come from the diversity of phytochemicals presented in different plant and their action pathways. The highest dose of WB (8%) could not affect antibody-mediated system in comparison to the control ($P>0.05$) which may show the overdosing of WB in the diet. The potential overdosing of the phytochemicals has been reported previously and must be considered in all studies related to using herbal biomedicine (Yin *et al*., 2006). Turker *et al*. (2009) demonstrated that overdoses of some phytochemicals could cause imbalance of bacterial fauna in the intestine of fish which could affect the growth or immunity performance. Bacteria in the gut perform many important functions for animals, including breaking down and aiding in the absorption of otherwise indigestible food, stimulating cell growth, repressing the growth of harmful bacteria, training the immune system

to respond only to pathogens and defending against some infectious diseases (Cahill, 1990). So, any changes in gut flora may affect the growth and immunity of the fish. Based on the results of this study it could be concluded that common carp feed added o WB extract could promote the growth rate as well as non-specific immunity of the fish. However, dosage optimization is strongly recommended.

Acknowledgements

The authors would like to thank Isfahan Aquatic Propagation and Breeding Center for supplying fish. This research was supported with funding from the Isfahan University of Technology under grant No. 502.90.53949 awarded by Dr. S. Dorafshan. The authors would like to thank to all persons who helped us in doing the experiment.

References

Anderson, DP (1992). Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Ann. Rev. Fish Dis., 2: 281-307.

Bahrami Babaheydari, S; Heyrati, FP and Dorafshan, S (2013). Chemical components of the hydroalcoholic extract of *Stachys lavandulifolia* Vahl from Isfahan province. The Second International Conferences on Agriculture and Natural Resources. Razi University, Kermanshah. PP: 446-449.

Cahill, MM (1990). Bacterial flora of fishes: a review. Microb. Ecol., 19: 21-41.

Chakraborty, SB and Hancz, C (2011). Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in finfish culture. Rev. Aquacult., 3: 103-119.

Chipman, DM and Sharon, N (1969). Mechanism of lysozyme action. Science. 165: 454-465.

Citarasu, T (2010). Herbal biomedicine: a new opportunity for aquaculture industry. Aquacult. Int., 18: 403-414.

Demers, NE and Bayne, CJ (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Dev. Comp. Immunol., 21: 363-373.

Divyagnaneswari, M; Christybapita, D and Michael, RD (2007). Enhancement of nonspecific immunity and disease resistance in *Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. Fish Shellfish Immunol., 23: 249-259.

Francis, G; Harinder, P; Makkar, S and Becker, K (2005). *Quillaja* saponins - a natural growth promoter for fish. Anim. Feed. Sci. Tech., 121: 147-157.

Galina, J; Yin, G; Ardo, L and Jeney, Z (2009). The use of immunostimulating herbs in fish. An overview of research. Fish Physiol. Biochem., 35: 669-676.

Ghasemi Pirbalouti, A; Jahanbazi, P; Enteshari, S; Malekpoor, F and Hamedi, B (2010). Antimicrobial activity of some of the Iranian medicinal plants. Arch. Biol. Sci., 62: 633-642.

Javidnia, K; Mojab, F and Mojahedi, A (2004). Chemical constituents of the essential oil of *Stachys lavandulifolia* Vahl from Iran. Iran. J. Pharm. Res., 3: 61-73.

Mahadkar, S; Valvi, S and Rathod, V (2012). Screening of anti-nutritional factors from some wild edible plants. J. Nat. Prod. Plant Resour., 2: 251-255.

Muller-Eberhard, HJ (1988). Molecular organization and function of the complement system. Ann. Rev. Biochem., 57: 321-347.

Ody, P (1993). *The complete medicinal herbal, a practical guide to medicinal herbs, with remedies for common ailments.* 1st Edn., New York, USA, DK Adult Publication. P: 192.

Rao, YV and Chakrabarti, R (2004). Enhanced anti-proteases in *Labeo rohita* fed with diet containing herbal ingredients. Indian J. Clin. Biochem., 19: 132-134.

Sahu, S; Das, BK; Mishra, BK; Pradhan, J and Sarangi, N (2007). Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. J. Appl. Ichtyol., 23: 80-86.

Sharma, A; Deo, AD; Riteshkumar, ST; Chanu, TI and Das, A (2010). Effect of *Withania somnifera* (L Dunal) root as a feed additive on immunological parameters and disease resistance to *Aeromonas hydrophila* in *Labeo rohita* (Hamilton) fingerlings. Fish Shellfish Immunol., 29: 508-512.

Soosean, C; Marimuthu, K; Sudhakaran, S and Xavier, R (2010). Effects of mangosteen (*Garcinia mangostana* L.) extracts as a feed additive on growth and haematological parameters of African catfish (*Clarias gariepinus*) fingerlings. Eur. Rev. Med. Pharma. Sci., 14: 605-611.

Tahmasebi-Kohyani, A; Keyvanshokooh, S; Nematollahi, A; Mahmoudi, N and Pasha-Zanoosi, H (2011). Dietary administration of nucleotides to enhance growth, humoral immune responses, and disease resistance of the rainbow trout, *Oncorhynchus mykiss* fingerlings. Fish Shellfish Immunol., 30: 189-193.

Turker, H; Birinci Yıldırım, A and Pehlivan Karakaş, F (2009). Sensitivity of bacteria isolated from fish to some medicinal plants. Turk. J. Fish. Aquatic Sci., 9: 181-186.

Virgili, F and Marino, M (2008). Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. Free Radical Biol. Med., 45: 1205-1216.

Vundac, VB; Brantner, AH and Plazibat, M (2007). Content of polyphenolic constituents and antioxidant activity of some *Stachys* taxa. Food Chemis., 104: 1277-1281.

Watts, M; Munday, BL and Burke, CM (2001). cDNA sequences and organization of IgM heavy chain genes in two holostean fish. Dev. Comp. Immunol., 19: 153-164.

Xie, J; Liu, B; Zhou, Q; Su, Y; He, Y; Pan, L; Ge, X and Xu, P (2008). Effects of anthraquione extract from rhubarb *Rheum officinale* Bail on crowding stress response and growth of common carp *Cyprinus carpio* var. *Jian*. Aquaculture. 281: 5-11.

Yeh, SP; Chang, CA; Chang, CY; Liu, CH and Cheng, W (2008). Dietary sodium alginate administration affects fingerling growth and resistance to *Streptococcus* sp. and iridovirus, and juvenile non-specific immune responses of the orange spotted grouper, *Epinephelus coioides*. Fish Shellfish Immunol., 25: 19-27.

Yin, G; Jeney, G; Racz, T; Pao, X and Jeney, Z (2006). Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non-specific immune response of tilapia, *Oreochromis niloticus*. Aquaculture. 253: 39-47.

Yuan, C; Pan, X; Gong, Y; Xia, A; Wu, G; Tang, J and Han, X (2008). Effect of *Astragalus* polysaccharides (APS) on the expression of immune response genes in head kidney, gill and spleen of the common carp, *Cyprinus carpio* L. Int. Immunopharmacol., 8: 51-58.