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Effects of *Viscum album* Linnaeus and *Nigella sativa* Linnaeus Extracts on Some Immune Responses of Common Carp *Cyprinus carpio* Linnaeus

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Abstract

The effects of oral administration of *Viscum album* (Family Loranthaceae) and *Nigella sativa* (Family Ranunculaceae) extracts on some immune responses of *Cyprinus carpio* were studied. Four hundred and fifty fish weighing 108±11.4 g were divided into two groups for immune and non-immune treatments. For immune treatment the fish were injected with *Aeromonas hydrophila* bacterin and the non-immune treatment received sterile phosphate buffer saline. Each treated group was divided into three treatments: Fish in treatments 1 and 2 were fed a diet supplemented with 0.5% (w/w) *V. album or N.sativa* extracts respectively while in treatment 3 fish were fed a free extract diet. Fish in all treatments were fed for 30 days. Blood samples were taken every 10 days and hematoimmunological indices were compared among treatments. Results showed that oral administration of *V. album* and *N. sativa* extracts were able to enhance lysozyme activity, serum bactericidal activity, serum protein and serum globulin in immunised and non-immunised groups ($P<0.05$) but complement activity showed no significant change among groups ($P>0.05$). Resistance to *A. hydrophila* infection was significantly higher in immunised fish treated with *V. album*. However, *N. sativa* did not reduce mortality following the challenge with bacteria. This study indicates that oral administration of *V. album* extract is a potential candidate for herbal based immunostimulants in common carp.

Introduction

Immunostimulants are substances that have the ability to increase resistance to disease by enhancing nonspecific and specific defense mechanisms (Chakrabarti and Rao, 2006) and offers a promising alternative to antibiotics and vaccines (Bricknell and Dalmo, 2005). Some immunostimulants, which have been tested for application in aquaculture, include glucan (Selvaraj et al. 2005), extracts from bacteria (Rairakhwada et al. 2007), levamisole (Findlay and Munday, 2000), chitosan (Gopalakannan and Aru, 2006), vitamins (Sahoo and Mukherjee, 2003), oligonucleotides from yeast RNA (Li et al. 2004), plant derived immunostimulants (Chakrabarti and Rao, 2006; Yin et al. 2009; Bilen et al. 2011). These immunostimulants may directly initiate activation of the innate defense mechanisms acting on receptors and triggering intracellular gene activation that may result in production of antimicrobial molecules (Bricknell and Dalmo, 2005). Herbal based immunostimulants are biocompatible, biodegradable, cost effective and safe for the environment (Ortuno et al. 2002; Bricknell and Dalmo, 2005). (*Viscum album* Linnaeus, commonly known as mistletoe, is a semi-parasitic woody plant perennial commonly found growing on oak and other deciduous trees. It has been shown that *V. album* extracts possess a variety of biological

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activities (Yoon et al. 2001; Yoon et al. 2003), such as induction of various cytokines (Mannel et al. 1991) and enhancement of natural killer cell activity in mammals (Kim et al. 1999). Moreover, it has been demonstrated that the extract of *V. album* augmented an anti-tumor effect in mammals by enhancing the cytotoxic activity of NK cells, lymphokine-activated killer cells and macrophages (Rostock et al. 2005).

Nigella sativa Linnaeus, commonly known as black seed, a member of botanical family Ranunculaceae, has been used in several Middle Eastern countries as a natural remedy against various gastrointestinal disorders and bronchitis for over 2000 years (Swamy and Tan, 2000). Black seed components display a remarkable array of biochemical, immunological and pharmacological actions including immunopotentiating (Swamy and Tan, 2000; Salem, 2005), anti-inflammatory (El-Dakhakhny et al. 2002), antibacterial, antiviral and anti-oxidant activities (Turkdogan et al. 2001) effects. Also, *N. sativa* extract showed immunostimulatory effects in mammals both *in vitro* and *in vivo* conditions (Haq et al. 1995;). However, no reports of possible imunostimulating effects of these herbs are available in coldblooded animals such as fish. The objective of this study was to assess the effect of *V. album* and *N. sativa* extracts on some specific and nonspecific immune responses as well as disease resistance in common carp *Cyprinus carpio* Linnaeus.

Materials and Methods

Four hundred and fifty juvenile common carp, weighing an average of 108±11.4 g were obtained from a fish farm in Ahvaz, Khuzestan Province, south Iran. Fish were transferred to the aquaculture laboratory of Shahid Chamran University, Ahvaz, Iran and acclimated to the laboratory conditions for 1 week before the experiments. During the experiment, fish were kept in tanks supplied with aerated fresh water (temperature: 25±1°C; pH: 7.9±0.3; alkalinity (CaCO3): 20.15±1.2 mg L^{-1}; dissolved oxygen: 8-10 mg L^{-1}; NH_3 and NO_2<0.1 mg·L^{-1}). Temperature, pH and dissolved oxygen were measured with Multimeter WTW 340i (Germany), while alkalinity, NH_3 and NO_2 were measured with Aqualitic Photometer AL250 (Germany) every other day during the study. Also water exchange rate was 10% of total water volume per day.

For immunisation, *A. hydrophila* was grown in tryptone soy broth at 30 °C for 24 hr, centrifuged, washed and suspended in sterile phosphate buffer saline (PBS), and were then inactivated using 1% formalin for 1 hr before washing three times with PBS.

Hydro alcoholic extracts of *V. album* and *N. sativa* were prepared by the percolation method. The plant samples were cleaned, thoroughly dried under the shade, grounded finely and sieved. The individual sieved powder was then soaked in 80% aqueous ethanol (1:5 w/v) for 72 hr.

The supernatant was then filtered. The crude extracts were condensed with a rotary evaporator (Buchi, Switzerland) at 80 ºC. The clarified extracts were condensed at 40 ºC until the solvent residue had evaporated.

Experimental design

The fish were divided into six groups of 75 fish each and placed into 300 L tanks equipped with thermostat controlled heaters, aeration, external biofilters and automatic feeders. Three tanks were immunised by intraperitoneal injections with 100 µL of *A. hydrophila* at concentration 10^9 cell·mL^{-1}. Immunised fish received a booster dose of bacterial antigen after 2 weeks post immunisation. Non-immunised groups were intraperitnoneally injected with 100 µL sterile PBS. Two groups of both immunised and non-immunised treatments were fed with *V. album* and *N. sativa* extract treated diets and the rest of the groups were fed with extract free diets as control. The experimental diets were prepared by mixing the commercial carp food (Chineh Company, Iran), with 0.5% (w/w) *V. album* and *N. sativa* extracts. All treatments were fed twice daily at 5% body weight for 30 days.

Blood and serum collection

Blood samples were collected on 0, 10, 20 and 30 days post-experiment. Ten fish were sampled randomly from each experimental group, anaesthetised using MS_{222} at 0.1 mg·L^{-1} before blood sampling from the caudal vein. Sera samples were separated via centrifugation at 2000 g for 10 min and stored in separated microtubes (AHN Co, Germany) at -20 °C until used.

Lysozyme activity assay

Serum lysozyme activity was measured as described by Ellis (1990) with a slight modification. Briefly, 10 µL of individual serum was mixed with 200 µL of a *Micrococcus lisodeichticus* (Sigma) suspension at 0.2 mg·mL^{-1} in 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 27 °C, and its optical density was measured after 1 and 6 min at 530 nm using an ELISA plate reader (Dynatech, Netherland). One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1}·mL^{-1} serum.

Serum bactericidal activity

Bactericidal activity was studied following the procedure by Kajita et al. (1990) with slight modification. Sera samples were diluted three times with 0.1% gelatin-veronal buffer (GVBC2). *Aeromonas hydrophila* (live, washed cells) were suspended in the same buffer at a concentration of 10^5 CFU·mL^{-1}. The diluted sera and bacteria were mixed at 1:1 v/v and incubated for 90 min at 25 °C with shaker. The number of viable bacteria was then calculated by counting the colonies from the resultant incubated mixture on TSA plates in duplicate. The bactericidal activity of test serum was expressed as percentage of CFU in test group to that in control group.

Serum total protein, globulin and albumin

The total serum protein level was determined following the protocol of Lowry et al. (1951) using the standard protein estimation kit (Zistchem Diagnostics, Iran). Total albumin content of serum was measured using the bromocresol green method (Durgawale et al. 2005). For globulin estimation, 50 µL of saturated ammonium sulphate solution was added drop wise to 50 µL of the serum samples followed by vortexing. Samples were then centrifuged at 8,000 g for 5 min, and 20 µL of each sample was then dissolved with 80 µL of carbonate-bicarbonate buffer (pH 9.3). The protein content was then estimated using the standard protein estimation kit (Zistchem Diagnostics, Iran).

Specific antibody titer

Anti *A. hydrophila* antibody titer was conducted in 'U' shaped microtiter plates. Two-fold serial dilutions of 25 µL serum in equal volume of PBS were prepared in each well. Aliquot of 25 µL of formalin-killed *A. hydrophila* (10^7 cells·mL^{-1}) suspension was then added. The plates were incubated overnight at room temperature and the titer was calculated as the reciprocal of the highest dilution (based on $\log_2$) of serum showing complete agglutination of the bacterial cells. Antibody titer was tested in triplicate for each sample.

Alternative complement activity

Alternative complement activity was assayed following the procedure of Yano (1992), using rabbit red blood cells (RaRBC).

Relative Percentage survival (RPS)

Virulent strain of *A. hydrophila* was used for disease resistance assay. Thirty fish from each treatment group were intraperitoneally injected with the bacterial suspension ($2.1 \cdot 10^7$ CFU per fish = $2LD_{50}$) and mortality of challenged fish was recorded daily for 10 days. The cause of death was ascertained by re-isolating the infecting organism from kidney and liver of dead fish. Cumulative daily mortality curve was drawn according to Misra et al. (2006).

Statistical analysis

Statistical analyses were performed using SPSS 16 software. Data were tested for normal distribution with Shapiroe Wilk's test and for homogeneous variance with Levene's test. Differences among the extract supplemented fed and control groups were tested with One-Way ANOVA followed by Duncan's multiple range test (1955) using a probability level of 0.05.

Results

Lysozyme activity

The serum lysozyme activity increased significantly in the fish fed for 20 and 30 days with feed supplemented with *V. album* and *N. sativa* in immunised fish (Fig. 1). Besides, lysozyme activity increased in days 10, 20 and 30 in non-immunised groups fed with two extracts (Fig. 2) ($P < 0.05$). The highest lysozyme activity was observed in the immunised fish fed with *V. album* supplemented feed for 20 days.

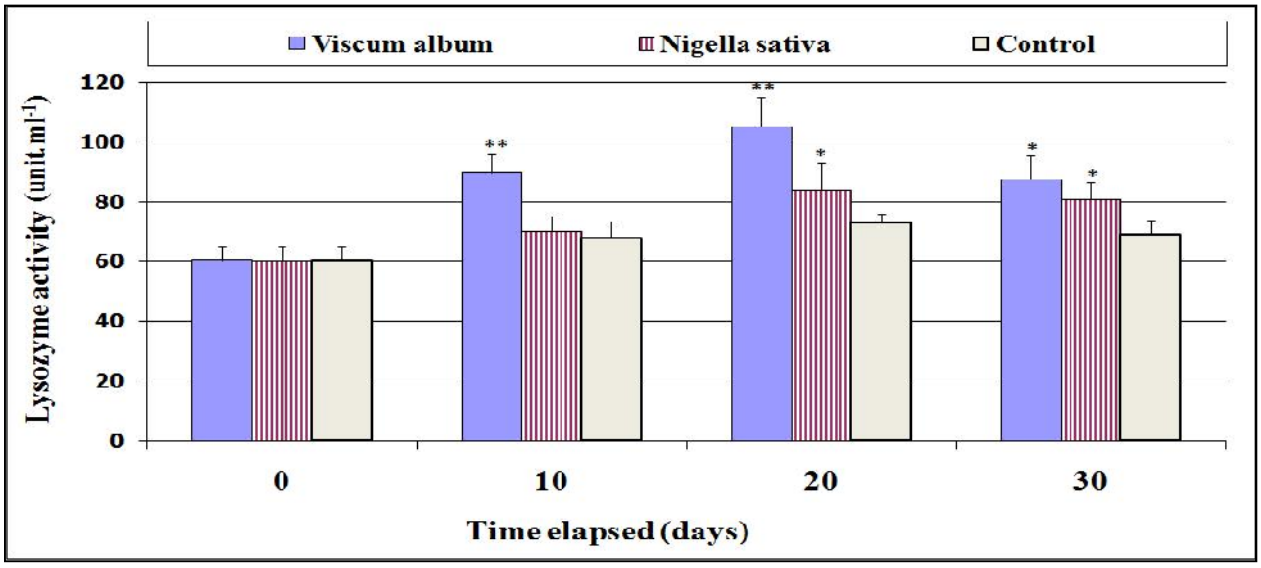

Fig.1.The effects of *V. album* and *N. sativa* on serum lysozyme activity (units·mL^{-1}) of immunised common carp in each sampling period (Values are Mean ± SD, n= 10). Mean values bearing** are significantly different from control ($P<0.01$) and mean values bearing* are significantly different from control ($P<0.05$).

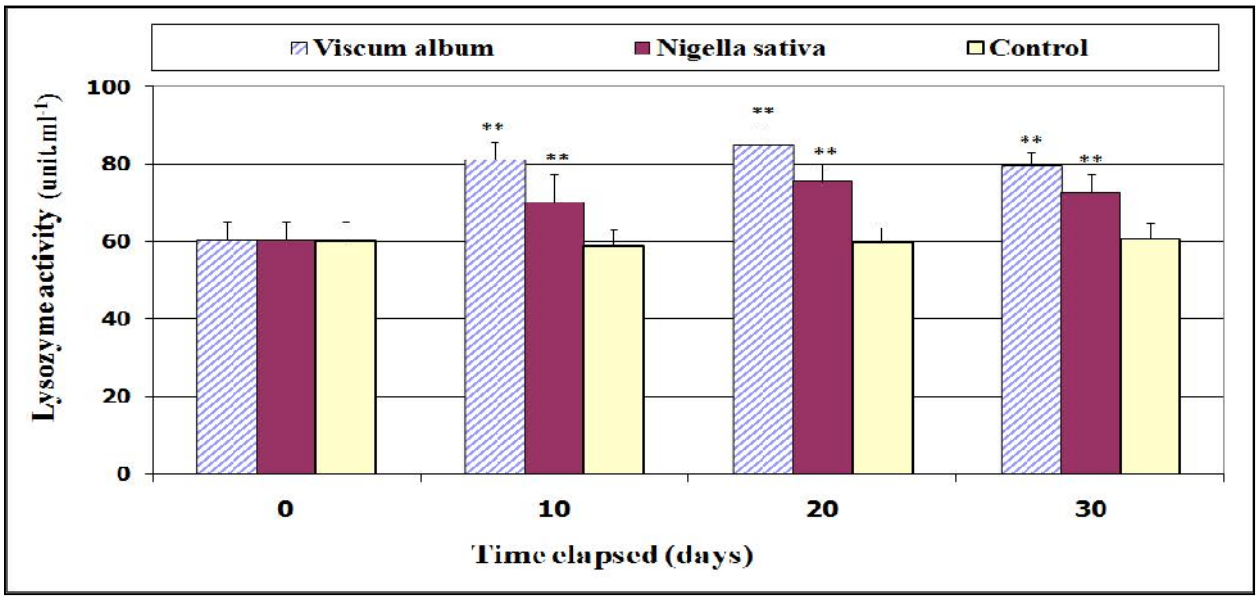

Fig. 2. The effects of *V. album* and *N. sativa* on serum lysozyme activity (units·mL^{-1}) of non immunised common carp in each sampling period (Mean±SD, n= 10). Mean values bearing** are significantly different from control with 99% confidence (P<0.01) and mean values bearing* are significantly different from control with 95% confidance (P<0.05).

Serum bactericidal activity

Serum bactericidal activity significantly (P<0.01) increased in fish fed with *V. album* supplemented feed in immunised and non-immunised fish on days 10, 20 and 30 post-experiment (Table 1), while serum bactericidal activity in *N. sativa* administrated groups increased in non-immunised fish on days 20 and 30 of treatment(P<0.05).

Anti A. hydrophila antibody titer

Results of anti *A. hydrophila* antibody titer in experimental treatments are shown in Table 1. The titer was significantly higher in immunised fish fed with *V. album* supplemented feed on days 20 and 30 of treatment (P<0.01). However no significant difference in the agglutination titer was recorded in other groups (P>0.05).

Serum proteins

The levels of serum total protein and globulin in treatments showed significant increase in fish fed with *V. album* and *N. sativa* supplemented feed in both immunised and non-immunised treatments on 10, 20 and 30 days of study(P<0.05). However, no difference was seen in albumin concentration among groups (P<0.05) (Table 1).

Table 1. The effect of *V. album* and *N. sativa* on alternative complement activity and serum proteins of *C. carpio* (Mean±SD, n=10). [a]Mean values for a parameter bearing the same superscripts are not significantly different (P>0.05) from each other in each row. [*] Means in the same column which are significantly different (P<0.05) from control.

Parameters	Groups	Treatments	Day Zero	Day 10	Day 20	Day 30
Haemolytic complement activity ($u \cdot L^{-1}$)	Immune	*Viscum album*	442±19[a]	441±24[a]	439±22[a]	446±14[a]
		Nigella sativa	443±19[a]	444±19[a]	446±18[a]	439±22[a]
		Control	442±19[a]	439±22[a]	445±21[a]	439±22[a]
	Non-immune	*Viscum album*	442±19[a]	434±21[a]	445±16[a]	436±21[a]
		Nigella sativa	442±19[a]	438±23[a]	442±21[a]	447±15[a]
		Control	442±19[a]	447±15[a]	436±21[a]	442±20[a]
Anti *A.hydrophila* Ab titer (Log2)	Immune	*Viscum album*	0.94±0.93[c]	4.8±1.6[b]	7.60±1.14[*b]	7.40±1.94[*b]
		Nigella sativa	0.94±0.93[c]	4.2±1.31[a]	5±1.58[a]	4.8±1.79[a]
		Control	0.94±0.93[c]	4±1.11[a]	4.60±0.90[a]	4.80±1.31[a]
	Non-immune	*Viscum album*	0.94±0.93[a]	1±1.22[a]	0.8±0.84[a]	0.8±1.3[a]
		Nigella sativa	0.94±0.93[a]	1±1.1[a]	0.8±0.83[a]	1±0.71[a]
		Control	0.94±0.93[a]	0.8±0.45[a]	0.7±0.55[a]	0.8±0.84[a]
Bactericidal activity (cfu. $Control^{-1}$)	Immune	*Viscum album*	72.9±10.1[a]	39.2±5.8[*b]	43.6±6.6[*b]	45.2±5.2[*b]
		Nigella sativa	72.9±10.1[a]	58.4±7.6[b]	63.2±7.6[b]	63.6±7[b]
		Control	72.9±10.1[a]	62.0±5.1[a]	60.8±6.3[a]	58.4±6.6[a]
	Non-immune	*Viscum album*	72.9±10.1[a]	52.4±6.6[*b]	50.4±6.6[*b]	54.4±7[*b]
		Nigella sativa	72.9±10.1[a]	67.2±4.4[a]	65.2±6.4[b]	64.4±7.8[b]
		Control	72.9±10.1[a]	71.2±5.8[a]	71.2±5.2[a]	72.4±6.1[a]
Serum protein level ($g \cdot dL^{-1}$ of serum)	Immune	*Viscum album*	3.2±0.5[a]	4.48±0.64[*b]	4.22±0.74[*b]	4.14±0.74[*b]
		Nigella sativa	2.96±0.63[a]	3.82±0.75[*b]	3.94±0.81[*b]	3.82±0.56[b]
		Control	3.18±0.24[a]	3.58±0.42[a]	3.36±0.45[a]	3.5±0.6[a]
	Non-immune	*Viscum album*	3.08±0.41[a]	3.92±0.44[*b]	3.9±0.63[*b]	3.88±0.6[*b]
		Nigella sativa	3.09±0.65[a]	3.8±0.63[*b]	3.62±0.65[*b]	3.18±0.28[ab]
		Control	2.92±0.4[a]	3.02±0.58[a]	3.12±0.52[a]	2.82±0.24[a]
Globulin ($g \cdot dL^{-1}$ of serum)	Immune	*Viscum album*	2.3±0.46[a]	3.6±0.66[*b]	3.34±0.65[b]	3.2±0.1[*b]
		Nigella sativa	2.02±0.65[a]	2.88±0.85[b]	3±0.58[b]	2.9±0.59[b]
		Control	2.22±0.13[a]	2.98±0.31[a]	2.88±0.36[a]	2.76±0.49[a]
	Non-immune	*Viscum album*	2.24±0.71[a]	3 ±0.54[*b]	3.02±0.8[*b]	2.96±0.58[*b]
		Nigella sativa	2.14±0.68[a]	2.88±0.76[*b]	2.77±0.7[b]	2.26±0.24[ab]
		Control	2.06±0.14[a]	2.12±0.69[a]	2.24±0.55[a]	1.9±0.28[a]
Albumin ($g \cdot dL^{-1}$ of serum)	Immune	*Viscum album*	0.9±0.12[a]	0.88±0.13[a]	0.88±0.16[a]	0.94±0.33[a]
		Nigella sativa	0.82±0.13[a]	0.94±0.27[a]	0.94±0.34[a]	0.92±0.13[a]
		Control	0.9±0.16[a]	0.9±0.24[a]	0.88±0.16[a]	0.92±0.13[a]
	Non-immune	*Viscum album*	0.84±0.11[a]	0.92±0.31[a]	0.88±0.19[a]	0.92±0.13[a]
		Nigella sativa	0.94±0.15[a]	0.92±0.22[a]	0.92±0.13[a]	0.92±0.13[a]
		Control	0.86±0.15[a]	0.9±0.12[a]	0.88±0.19[a]	0.92±0.13[a]

Alternative complement activity

Fish fed with *V. album* or *N. sativa* supplemented feed (immunised and non-immunised) did not show any change of alternative complement pathway compared to control groups (P>0.05).

Relative percentage survival (RPS)

The mortality pattern in *V. album* and *N. sativa* treated groups during 10 days post-challenge period is shown in Figs. 3 and 4. No significant changes in mortality were seen in immunised fish fed with extract supplemented feed (immunised and non-immunised) and control feed (P>0.05). The percentage mortality was significantly reduced in non-immunised fish fed with *V. album* supplemented diet (P<0.05). *N. sativa* didn't induce any significant change not only in immunised but also in non-immunised groups (P>0.05).

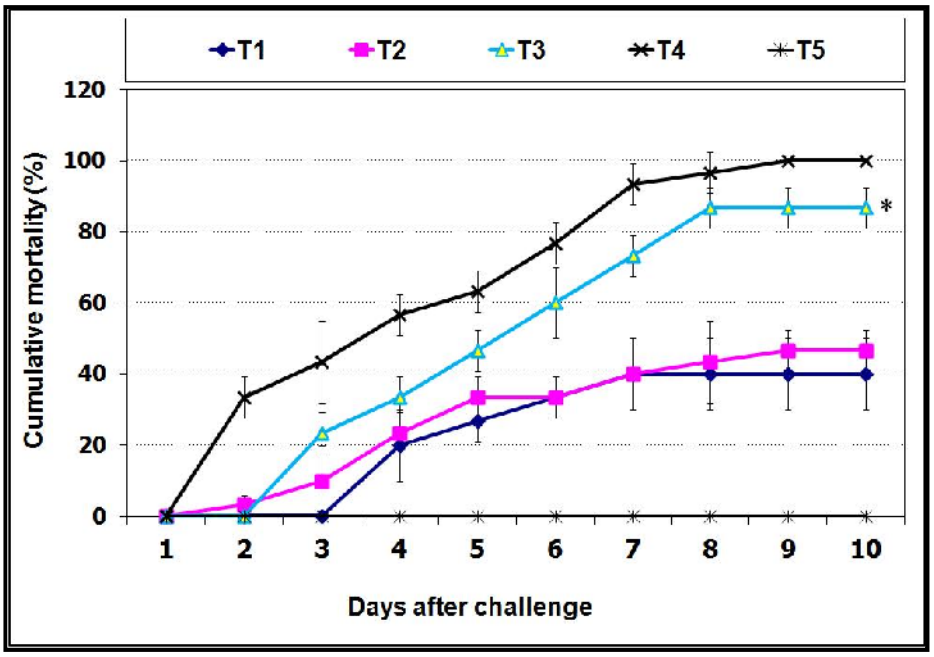

Fig. 3. Cumulative mortality of common carp in bacterial challenge after oral administration of *V. album* supplemented food, T1: Immunised carp fed with *V. album* supplemented feed, T2: Immunised carp fed with free extract feed, T3: Non-immunised carp fed with *V. album* supplemented feed, T4: Non-immunised carp fed with basal feed, T5:'Shieh'' stands for a control challenge without bacteria. The results represent Mean ± SD (n = 20) in each treatment. Statistical significance between treatments: *P < 0.05.

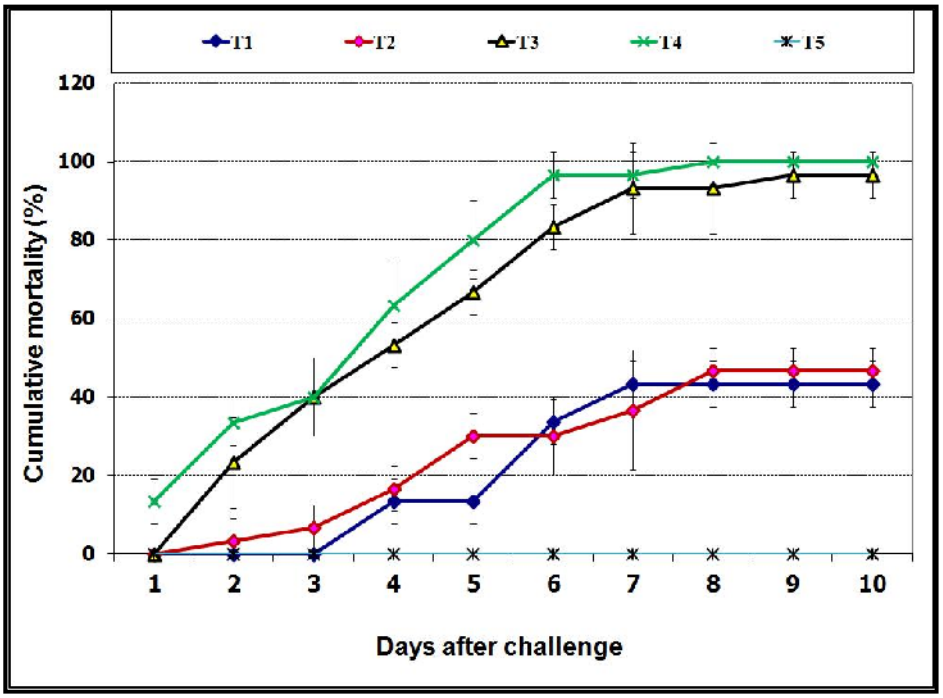

Fig. 4. Cumulative mortality of common carp in bacterial challenge after oral administration of *N. sativa* supplemented feed, T1: Immunised carp fed with *N. sativa* supplemented feed, T2: Immunised carp fed with basal feed, T3: Non-immunised carp fed with *N. sativa* supplemented feed, T4: Non-immunised carp fed with basal feed, T5:‘Shieh’’ stands for a control challenge without bacteria. The results represent Mean ± SD (n = 20) in each treatment.

Discussion

The results presented in this paper demonstrate that oral administration of extracts of *V. album* and *N. sativa*, popular medicinal herbs, are able to stimulate some specific and non-specific immune parameters in common carp. Resistance to *A. hydrophila* infection significantly increased in the non-immunised fish fed with *V. album* extract compared to control. However, *N. sativa* did not reduce mortality after challenging fish with *A. hydrophila*. Resistance against bacterial infection in fish have been shown after oral administration of *Aloe vera* (Kim et al. 1999; Alishahi et al. 2010), *Eclipta alba* (Christybapita et al. 2007), and *Solanum trilobatum* (Divyagnaneswari and Cristybapita, 2007). Similarly oral administration of *V. album* enhanced resistance against *A. hydrophila* in Japanese eel (*Anguilla japonica)* (Choi et al. 2008). Also these results showed that the serum lysozyme activity significantly increased in carp fed with *V. album* and *N. sativa* supplemented feed, not only in immunised fish but also in non-immunised groups. It was previously observed that immunostimulants, vaccines and probiotics can enhance the plasma lysozyme activity in fish (Alishahi and Buchmann, 2006). Elevated lysozyme level was also measured in large yellow croaker and common carp (Jian and Wu, 2004) after feeding the fish with various herbal extracts.

Choi et al. 2008 also reported elevated serum lysozyme activity in Japanese eel fed with *V.album* supplemented feed.

Controversial results have been reported concerning the effect of immunostimulators on the complement activity; some reports confirmed increased complement activity (Bagni et al. 2005; Cheng et al. 2007), but others reported lack of effect on complement activity after administration of immunostimulants (Robertsen, 1999). In this study *N. sativa* and *V. album* did not induce any change in alternative pathways of complement activity compared to control animals. One of the indicators of humoral defence mechanisms, plasma proteins levels and globulin were also found to increase in fish fed with food supplemented with these plant extracts. The increase in serum protein content might be correlated with an increase of proteins like serum lysozyme and bactericidal peptides. Probably, the increase in the leukocyte count and their functions might have resulted in the enhancement of the serum protein and globulin level. Misra et al. (2006) reported the correlation among serum bactericidal activity, total leukocytes, total serum protein, and globulin of Indian carp after injection of Tusfin. Besides Dügenci et al. (2003) showed that administration of feed supplemented with *V. album* increased blood leukocytes and total plasma protein and globulin level in rainbow trout.

Incorporation of *V. album* in the carp diet enhanced serum antibody level against *A. hydrophila* in immunized treatment ($P<0.05$). Therefore, it seems that *V. album* is able to enhance humoral immune responses of carp. However, *N. sativa* did not induce significant change in antibody titer in treated fish in all sampling periods. Although immunostimulants stimulate mostly non-specific immunity, various studies confirmed specific immunity enhancement followed by administration of immunostimulants. Selvaraj et al. (2005) reported higher antibody production in rainbow trout fed with glucan supplemented feed. Similar results have been reported in catfish and Atlantic salmon (Findlay and Munday, 2000) and common carp (Alishahi et al. 2010) using glucan, levamisole and *Aloe vera* respectively. Probably the immuno stimulating mechanisms of *V. album* and *N. sativa* in carp are basically different as these two herbal extracts increased some non-specific immune responses. However, only *V. album* enhanced specific immune response in carp. *Viscum. album* significantly decreased the cumulative mortality of non-immunized carp after challenge with *A. hydrophila.* However, *N. sativa* had no effect on survival rate of treated carps. This can be related to either the plant species, the extraction procedure, the percentage of extract in the diet or the effect ofgenetics of the experimental animal used in this assay.

Conclusion

The oral administration of *N. sativa* and *V. album* extracts were able to enhance some immunological parameters of common carp including lysozyme activity, complement activity, serum bactericidal activity and serum proteins up to one month post-administration. But *V. album* alone was able to improve the humoral immunity in immunised fish. Also, survival of fish fed these herbs was increased after challenging fish with virulent strain of *A. hydrophila* .Therefore, it may be useful to use *V. album* extract at 500 $mg{\cdot}kg^{-1}$ diet in this fish species, particularly under stress conditions. However, the actual immunostimulatory active compounds of these herbs used in fish is unknown and warrant further investigation.

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