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Effect of dietary *Achillea wilhelmsii* extract on growth performance, and immune status of common carp (*Cyprinus carpio*).

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ABSTRACT

The use of natural products like plants and their extract as immunomodulator and growth motivator in fish has became a safer and cheaper choice than other synthetic material for fish farming industry. The effect of dietary administration of *Achillea wilhelmsii* extract on Common carp (*Cyprinus carpio*) immune status, growth performance and hematological parameters was studied. Fish were divided randomly into 4 groups before being fed with commercial diet supplemented with 0% (control), 1%, 2% and 3% of *Achillea wilhelmsii* for 8 weeks. The effects of the supplemented diets on fish were analysed for the immune parameters (lysozyme, bactericidal, respiratory burst activities and IgM content), haematological parameter and growth parameters (Final weight, Weight gain, Feed conversion ratio (FCR), Specific growth rate (SGR), and survival rate). The results recorded enhancement in all immune parameters in fish fed A. *wilhelmsii* extract. Particularly, 2% and 3% doses showed the highest significant values compared to control fish ($p < 0.05$). Also, increases in RBC, Hct and Hb values were observed in groups for all doses of *A. wilhelmsii* extract and highly significant increases were recorded in 2% and 3% doses ($p < 0.05$). Moreover, final weight, weight gain, and SGR improved in fish fed all doses of *A. wilhelmsii* extract. Surprisingly, the lowest dose (1%) showed the highest significant value in growth parmeters compared to control ($p < 0.05$). The present study suggests that the *A. wilhelmsii* extract, especially the highest dosage could be considered a good food supplement to enhance the immune status and general common carp health.

Keywords: *Achillea wilhelmsii*, immune response, growth performance, Common carp (*Cyprinus carpio*), immunostimulant.

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INTRODUCTION

The aquaculture industry faces many challenges including provision of an appropriate environment (eg, good nutrition, water quality and temperature), with good husbandry, hopefully, leading to fish better able to resist infectious disease. On the other hand, intensive and expanding aquaculture practices have led, too frequently, to disease outbreaks resulting in partial or total loss of fish production [1]. Furthermore, using antibiotics and other chemotherapeutics to control diseases may lead to development of drug resistant strains, environmental pollution and accumulation of residues in fish tissues [2]. Moreover, in many disease outbreaks pathogens take advantage of immunocompromised, stressed or malnourished fish. Thus the availability of diets that could enhance immunity and fitness at the same time would be a genuine boon for fish farmers. Recently, there has been a big trend toward using natural products , such as plants and their extracts as food supplements in fish diets due to their ability to biodegrade, cost effectiveness and safety for the environment [3]. In this context, many studies have reported enhancement in the fish immune system and growth rate after administration of diets supplemented with either plants or plant extracts. For example, respiratory burst activity, phagocytosis, weight gain and specific growth rate, significantly increased in grouper (*Epinephelus coioides*) after feeding with diets supplemented with 1.0 and 2.5 g/kg of *Sauropus androgynus* extract [4]. Also, gilthead seabream fed with fenugreek seed (*Trigonella foenum graecum*) showed improvement in growth and immune status, especially in the group administered with highest dose [5]. Conversely, some studies showed that the food which provided the best disease resistance may not have stimulated growth [6]. For example, 0.5% dose of garlic recorded an enhancement in the immune system of hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*), but did not improve growth [7]. Thus, the selection of the appropriate plant and dosage is the hard challenge. Moreover, duration of feeding and the method of administration, are also considered to be important factors which vary with different compounds and different fish species [8].

Achillea species are traditionally used as herbal teas for abdominal pain and flatulence in Turkey [9]. Moreover, *Achillea* species was used in Chinese medicine to treat the following conditions: to clear exterior wind (diaphoretic), as a tonic and to clear heart phlegm (anti-hypertension) [10]. *Achillea wilhelmsii* C. Koch, common name yarrow, a perennial medicinal herb belonging to family Asteraceae family, is widely distributed in different parts of Iran [11] and has been reported to occur in North America, Australia and New Zealand [12]. Several studies reported that the biological activities of *A. wilhelmsii* included antispasmodic [13], antacid [14], antioxidant [15-17], antihyperlipidemia [18], antihypertensive [19] and antitumoral effects [20]. Generally, *A. wilhelmsii* is rich in sesquiterpenes, flavonoids and monoterpenoids which have antioxidant activities and play an important role in elevation of the immune system to resist infection disease. [21-23].

In addition, several studies have been carried out on the effect of *Achillea* species on the immune system and growth performance in animals. For example, extract of *A. wilhelmsii* showed a stimulatory effect on both humoral and cellular immune function in mice [24]. Furthermore, a 3% dose of *A. millefolium* induced immune response and reduced pathogenic bacteria in the digestive tract in broiler chickens, and thus it's use to improve intestinal health has been suggested [25]. Conversely, addition of yarrow powder to broiler chicks recorded a positive influence on growth performance but failed to elevate immune response [26]. In fish, 1% extract of *A. millefolium* revealed promotion in growth and immune parameters in rainbow trout [27]. It is worth mentioning that the immune system in fish is different from that of mammals and the response even differs from species to species in fish and to immunostimulant type.

Although, *Achillea* sp. are well established to contain antioxidant compounds, relatively few studies have been carried out on the protnetial effects on fish health. Therefore, the current study aims to examine the effect of dietary administration of *Achillea wilhelmsii* extract on the immune status and heamatological parameters as well as the growth performance of common carp (*Cyprinus carpio*), to determine the effective dose which could be used in fish farms, to improve the health status and production of the fish.

MATERIALS AND METHODS

Plant extraction and diet preparation:

Achillea wilhelmsii were collected from natural habitats in Chaharmahal and Bakhtyari province (southwest of Iran) and identification was done according to standard methods by Shahrekord University

Botany section [28]. One kg of aerial plant parts was dried in a well aired dark room before being ground into fine powder using a grinder. The resulting powder was mixed in 2 L volumetric flask with 90% ethanol for 48 h on a shaker and filtered off. This process was repeated 3 times as previous tell complete extraction. The collected filtrates were evaporated using rotator evaporator at low temperature to obtain a residue of 165 g.

Fish diets was prepared from the alcohol extract of *A. wilhemsii* by adding four concentrations as following; commercial diet non-supplemented (0%, control), commercial diet supplemented with 1 g (1%), 2 g (2 %) and 3 g (3%)/ 100 g of *A. wilhelmsii* extract.

Fish, experimental design and sampling:

Around 240 Common carp (*Cyprinus carpio*) of average weight 13.5 ± 0.72 g were obtained from a commercial fish farm (Mazandaran province, Iran) and transferred to the Caspian Sea Ecology Research Center. Fish were acclimatized for 2 weeks in fiber glass tanks (300 L) provided with Spongy filters (100 liters h^{-1} of flow). The water was maintained at 23.8±1.4°C, dissolved oxygen at 7.1 ± 0.5 mg l^{-1}, pH 8.1 ± 0.7 and electrical conductivity of 5225.7 ± 367.8 MM cm^{-1}. The photoperiod was 14 h light and 10 h dark cycle. During acclimatization fish were fed with commercial diet (Abzian diet, Mazandaran, Iran) three times a day at 3% of body weight/day. Fish were distributed randomly into 4 groups, each containing 60 fish before being fed with the four prepared diets, for 8 weeks. At the end of the experimental trial, fish were anesthetized with clove oil (80 mg l^{-1}). Blood samples were obtained from the caudal vein by insulin syringe before divided into two parts. One part was transferred to a tube containing anti-coagulant (heparin) for studying the respiratory burst assay and hematological parameter, while the other part was transferred to non-heparinized tubes for immunological studies. Serum was obtained from blood by centrifugation (600 g for 25 min at 4 °C) and then stored -20 °C until used.

Heamatological parameters:

Total red blood cells (RBC: 10^6 mm^{-3}) and white blood cells (WBC: 10^3 mm^{-3}) were enumerated in an improved neubaeur hemocytometer using Hayem and Turck diluting fluids [29]. Haematocrit (Ht %) was determined by the standard microhematocrit method and expressed as percentage. The haemoglobin (Hb, g dl^{-1}) level was determined according to Cyanomethemoglobin procedure. Differentiation of leukocytes was carried by preparing Giemsa stained smears and counted under light microcopy.

Immune parameters:

Lysozyme activity:

Serum lysozyme activity was measured according to Vendemiale, Grattagliano [30]. Briefly, 50 µL of serum was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1} in a 0.05 M sodium phosphate buffer (pH 6.2) and absorbance was measured at 530 nm after 0.5 and 4.5 min on a spectrophotometer. Lysozyme activity was evaluated by unit which defined as the sample amount caused decreasing in absorbance of 0.001 min.

Immunoglobulin content (IgM):

Serum immunoglobulin (IgM) content was measured according to the method described by Shaarawy, Tohamy [31]. Briefly, 100 µl of serum (100-fold dilutions in PBS) was mixed with an equal volume of 12% (v/v) solution of polyethylene glycol (10,000 218 MW, PEG; Sigma-Aldrich) before incubated for 2 h at room temperature to deposit the Ig molecules. The mixtures were centrifuged at 5,000 × for 10 min and the protein content was determined before and after centrifuged by the Bradford method [32]. The difference in the protein contents is considered as the IgM content (mg ml^{-1}).

Bacterial culture and bactericidal activity:

Cultures of *Aeromonas hydrophila* were routinely grown in nutrient broth (Oxoid) for 24 h at 37 °C. The culture was centrifuged for 10 min at 3000 ×g at 4 °C. Then supernatant was discarded, and the pellet

resuspended in 0.9% of saline. The bacterial suspensions were counted using a hemocytometer slide at a magnification of 400× on a light microscope and adjusted to 10^7

Serum bactericidal activity was done according to [33] by using *A. hydrophila* as a model to examine the effectiveness of fish serum to kill the bacterial infection. Briefly, 100 µl of serum was added to 100 µl of bacterial suspension (10^7), mixed, and incubated for 1 h at 25 °C. A blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile 0.05 M sodium phosphate buffer, PBS (pH 6.2) at a ratio of 1:10. Around 100 µl of the mixture was plated onto the nutrient agar plates and incubated for 24 h at 25 °C before the number of colonies was counted.

Respiratory burst activity:

Respiratory burst activity of blood leukocyte was measured by Chemiluminescent assay, (measuring of light emission, CL) according to Jaffat, Hassan [34] with some modification using CL analysis (LUMI skan Ascent T392, Finland). A serial dilution of blood sample (prepared in Hanks balanced salt solution, HBSS) was used to determine the optimal dilution which gives a maximum CL peak. Fresh luminal solution (containing 0.014 g luminol (Sigma), 0.78 g KOH, 0.618 g boric acid and 10 ml distilled water) was prepared prior to the experiment. Then, 100 µl of luminol solution was added to 200 µl of diluted blood sample and immediately placed in the luminometer. Measurements were made for 60 min and the results of light emission are expressed in the form of relative light units per second (RLU s^{-1}) recorded by the luminometer.

Growth performance:

At the end of feeding trial, fish were deprived of food for 24 h before sampling, and the following parameters were measured:

Weight gain (g) = final body weight – Initial body weight

Specific growth rate (SGR) = [final mean body weight - initial mean body weight (g) x 100] / time interval (days).

Feed intake (g $fish^{-1}$) = dry feed intake (g)/ number of fish

Feed conversion ratio (FCR) = feed intake /weight gain (g)

Survival rate (SR) (%) = (initial fish number-dead fish number) / initial fish number×100

Statistical analysis

The data were subjected to statistical analysis using the SPSS software version no. 18 (SPSS Inc., Chicago, IL, USA). The statistical analysis was done by using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Heamatological parameters:

Fish fed for 8 weeks with *A. wilhelmsii* extract showed increased RBCs, Hct and Hb values notably at levels of 2% and 3% for which the highest significant values were recorded ($p < 0.05$) compared to control (Table 1). Also, 2% dose recorded the highest WBCs number compared to the other groups, although the observed differences were not statistically significant. Similarly, fish fed with 3% doses of *A. wilhelmsii* extract revealed increased monocytes and neutrophils percentages compared to the control but without significant difference (Table 1).

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Table 1. Heamatological parameters of Common carp in the 8-week feeding trial (Mean ±SE).

Parameters	Control	%1	%2	%3
RBC (10^6 ml^{-1})	1.32 ± 0.2[a]	1.38 ± 0.18[a]	1.52± 0.24[b]	1.63 ± 0.36[b]
WBC (10^3 ml^{-1})	7.25 ± 1.2[a]	7.16 ± 1.7[a]	7.34 ± 1.2[a]	7.21 ± 1.9[a]
Hct (%)	31.7 ± 1.18[a]	32.4 ± 1.23[a]	35.1 ± 1.42[b]	34.8 ± 1.5[b]
Hb (g dl^{-1})	7.52 ± 1.6[a]	7.82 ± 0.72[a]	8.39 ± 2.32[b]	7.84 ± 1.62[a]
Lymphocytes (%)	87.2 ± 1.62[a]	86.4 ± 0.86[a]	86.7 ± 2.08[a]	84.8 ± 1.38[a]
Neutrophils (%)	1.3 ± 0.08[a]	0.9 ± 0.12[a]	1.2 ± 0.16[a]	1.9 ± 0.18[a]
Monocyte (%)	11.0 ± 1.3[a]	12.8 ± 1. 8[a]	11.5 ± 1.9[a]	12.6 ± 2.1[a]

Significant differences between treatment and control groups were represented by letters (P<0.05)

Immune parameters:

The fish fed with all of the doses of *A. wilhelmsii* extract for 8 weeks showed enhancement in immune parameters including lysozyme activity, IgM content, bactericidal activity, and respiratory burst activity (Fig 1-4). Particularly, 2% and 3% doses of *A. wilhelmsii* extract showed statistically significant difference in respiratory burst activity and IgM content, as well as 2% dose in lysozyme activity, respective to the values found in control fish (fed non-supplemented diet) ($p < 0.05$). Bactericidal activity showed only a significant increase using 1% of *A. wilhelmsii* extract supplemented diet with no significant differences in the other groups compared to control.

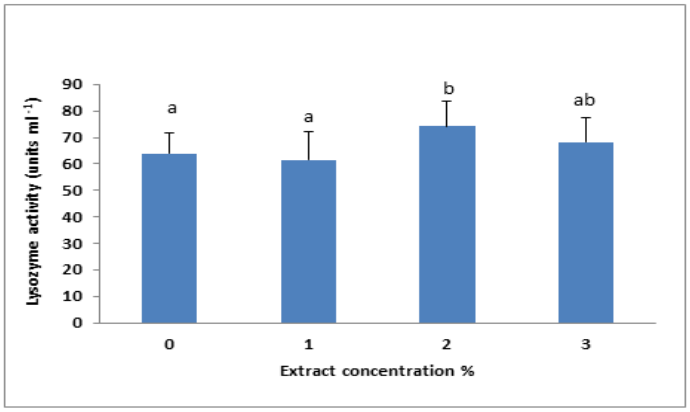

Fig. 1. Lysozyme activity in common carp fed diets supplemented with different doses of *A. wilhelmsii* extracts. Significant differences between treatment and control groups were represented by letters (P<0.05). Bars= mean ± S.E.

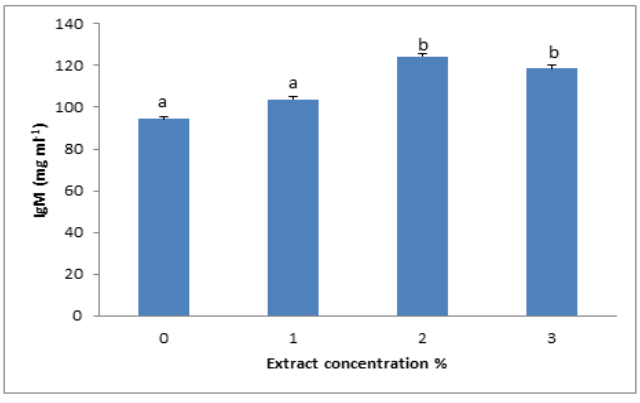

Fig. 2. IgM content in common carp fed diets supplemented with different doses of *A. wilhelmsii* extracts. Significant differences between treatment and control groups were represented by letters (P<0.05). Bars= mean ± S.E.

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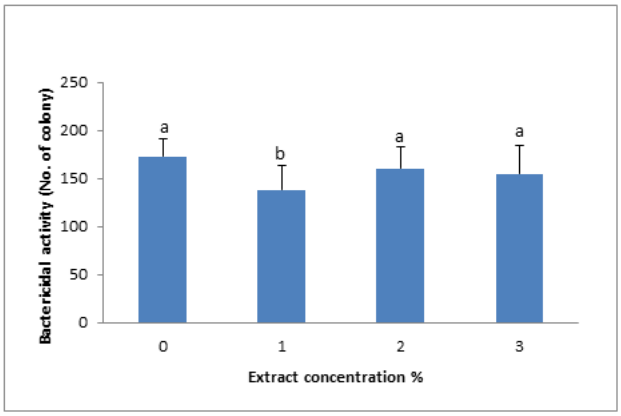

Fig. 3. Bactericial activity in common carp fed diets supplemented with different doses of *A. wilhelmsii* extracts. Significant differences between treatment and control groups were represented by letters (P<0.05). Bars= mean ± S.E.

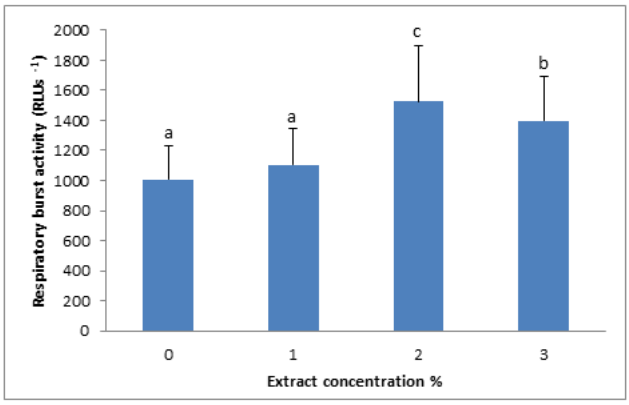

Fig. 4. Respiratory burst activity in common carp fed diets supplemented with different doses of *A. wilhelmsii* extracts. Significant differences between treatment and control groups were represented by letters (P<0.05). Bars= mean ± S.E.

Growth performance:

After 8 weeks of treatment, all fish fed *A. wilhelmsii* extract supplemented diets showed a higher increase in final weight, weight gain, and SGR compared to fish from the control group (non-supplemented diet) (Table 2). Interestingly, the lowest dose (1%) recorded the highest significant values ($p < 0.05$) in final weight, weight gain, and SGR followed by 2% and 3% doses, respectively. Moreover, all treatment groups showed a highly significant difference in SR ($p < 0.05$) compared to control (95% ± 2.5) (Table 2).

Table 2: Growth performance of Common carp in the 8-week feeding trial (Mean ±SE).

Parameters	Control	%1	%2	%3
Initial weight (g)	13.6±0.50^a	13.54±0.60^a	13.52±0.53^a	13.56±0.67^a
Final weight (g)	22.5±2.2^a	29.8±2.7^b	26.2±2.3^a	23.1±1.85^a
Weight gain (g)	8.9±0.8 ^{a}	16.2±1.4^c	12.6±2.3^b	9.6±1.1^b
FCR	2.96±0.7^a	2.82±0.5^a	2.44±0.4^a	2.12±0.4^b
SGR (%)	14.84±0.09^a	27.1±0.14^c	21.13±0.11^b	15.9±0.08^a
SR (%)	95± 2.5 ^{a}	100±0.00 ^{b}	100±0.00 ^{b}	100±0.00 ^{b}

Significant differences between treatment and control groups were represented by letters (P<0.05)

DISCUSSION

Many medical plants have compounds that possess several biological activities, and thus used in traditional folk medicine. The present results revealed that *A. wilhelmsii* could be considered as a promising plant to be used in aquaculture for obtaining healthy fish that could resist a disease outbreak.

Haematological parameters, including erythrocyte count, leukocyte count, haemoglobin concentration and haematocrit are important factors for fish biologists to consider in the evaluation of fish health as well as in monitoring stress responses [35, 36]. Previous studies reported increases in hematological parameters of fish species after administration of either plants or their extracts as immunostimulants [37, 38]. Our study supports previous studies, where all doses of *A. wilhelmsii* extract resulted in increases RBCs count, Hct and Hb values. Similarly, common carp showed an increase in RBCs, Hb and WBCs after feeding with a herbal mixture prepared with the extract of 5 plants (elecampane, *Inula helenium*; coltsfoot, *Tussilago farfara*; black mustard, *Brassica nigra*; purple coneflower, *Echinacea purpurea* and greater celandine, *Chelidoniume mjus*) for 20 and 40 days [38]. Moreover, using herbal mixture (medicated leaven, *medicata fermentata*; Japanese hawthorn fruit, *Crataegi fructus*; wormwood flower, *Artemisia capillaries*, and senkyu, *Cnidium officinale*) for 8 weeks resulted in increasing the Hb and Hct values of juvenile flounder [39]. Monocytes and granulocytes are considered as mobile phagocytic cells found in the blood and head kidney. They play a vital role in inflammation as a direct response to microbial invasion [40]. The number of monocyte was increased in groups fed with *A. wilhelmsii* extract compared to control. The enhancement in most haematological parameters in common carp could be attributed to the antioxidant compounds which improve fish health to assist in resistance to an invading pathogen [23].

Lysozyme is considered an important parameter in the immune defence of both invertebrates and vertebrates. In fish, lysozyme has been identified to be an opsonin, and activates the complement system and phagocytes [41]. Thus, lysozyme plays a vital role in the host defense mechanisms against infectious diseases [42]. The results of this study revealed a significant increase in lysozyme activity in groups fed with 2% and 3% doses of *A. wilhelmsii* extract for 8 weeks. Indeed several authors recorded enhancement in lysozyme activity after administration of different doses of plant extract for various durations. For example, Feeding Nile tilapia (*Oreochromis niloticus*) with 0.1 and 0.5% doses of *Astragalus radix* extract significantly enhanced the lysozyme activity especially after 3 and 4 weeks [43]. Similarly, lysozyme activity was raised in common carp after feeding with 0.5% of chinese herbs, *A. radix* and *Ganoderma lucidum* extracts for 5 weeks [44]. Furthermore, different doses of aqueous extract of *Eclipta alba* leaf showed significant increase in the lysozyme activity of common tilapia, *Oreochromis mossambicus* after 2 and 3 weeks [45].

In fact, teleost IgM appears similar to mammalian IgM in structure, physiological haracteristics and soluble forms [46, 47]. Furthermore, it plays a vital role as an immune effector molecule in the blood [48]. Previous studies have reported a major individual variation in serum IgM levels among fishes related to size and/or age [49, 50], environmental conditions [51, 52], disease status [53, 54] or immunostimulant types [55, 56]. On the other hand, dosage and time of immunostimulant administration created other variation factors. It should be noted that all doses of *A. wilhelmsii* extract increased the IgM levels after 8 weeks compared to the control group, especially with 2% and 3% doses which provok the highest significant values. In agreement with our study, freshwater murrel (*Channa punctatus*) recorded enhancement in IgM and total protein after administered 5% dose of *Ficus benghalensis* root extract.

In fish, bactericidal activity is a mechanism has been noted for the killing and clearing of pathogenic organisms [57]. In this assay, *A. hydrophila* was used as a model to examine the effectiveness of fish serum which fed with *A. wilhelmsii* extract to kill the bacterial invader. The lowest number of bacterial colonies grown on TSA media indicated the efficiency of immune molecules in serum to kill this pathogen when infecting the fish. Our results revealed higher serum bactericidal activity in all treated groups compared to the control. Similarly, serum bactericidal activity was significantly increased in common carp following administration of a dose of 0.75% and 1% of herbal extracts (mixture of *Inula helenium, Tussilago farfara, Brassica nigra, Echinacea purpurea* and, *Chelidoniume majus*) [38]. Interestingly, the fish immune system responds differently with different plant extracts. For example, greasy grouper (*Epinephelus tauvina*) showed enhancement in serum bactericidal activity after administered different concentrations of *Ocimum sanctum* and *Withania somnifera* extract whereas the extract of *Myristica fragrans*, did not record any effect [58].

Many forms of reactive oxygen radicals are generated during the phagocytosis process, which are considered toxic to bacterial fish pathogens [59]. The first form released from the respiratory burst is superoxide anion, thus measurement of O_2^- has been accepted as an accurate method of measuring this activity [60]. Interestingly, our results demonstrated enhanced respiratory burst in all treatment groups compared to the control, especially at the highest doses (2% and 3% doses). In agreement with our study, the highest doses of aqueous, ethanol, and methanol solvent leaf extracts of *Punica granatum* showed increases in

respiratory activity of olive flounder *Paralichthys olivaceus* fed for 8 weeks [61]. Similarly, higher doses of mixture extracts of *Astragalus membranaceus* and *A. sinensis* recorded significant increase in respiratory burst activity of yellow croaker and Jian carp after 20 days [62, 63]. It should be emphasized that a high dose does not necessarily enhance or inhibit the immune response in fish. For example Ndong and Fall [7] recorded significant increased respiratory burst activity in hybrid tilapia with 0.5% garlic more than a 1% dose fed for 4 weeks.

The nutritional status of fish is very important for the ability to resist infectious disease. Thus, there is a major need for a proper diet to improve health and to prevent disease outbreaks at the same time. In this issue, several studies have been carried on aspects of immunity and nutrition in fish [64, 65]. Some of them demonstrate a positive correlation between increasing disease resistance and growth rate and survival rate [66]. Indeed, the present results supported this idea, where using diets supplemented with *A. wilhelmsii* extract lead to increased growth parameters (final weight, weight gain, FCR and SGR) and survival rate and these were synchronous with the enhancement of most immune activities of common carp. In agreement with this study, rainbow trout (*Oncorhynchus mykiss*) recorded significant increase in growth performance as well as immune parameters after feeding diets supplemented with 0.1%, 0.5% and 1% of yarrow extract (*Achillea millefolium* L.) for 30 days [27]. On the other hand, the optimal dosage which enhance the immune status did not enhance the growth performance. This fact was clearly observed in our study, where the lowest dose of *A. wilhelmsii* (1%) showed the highest growth rate and vise versa in immune parameters. Similarly, 2.5% dose of katuk (*Sauropus androgynus*) recorded the highest immune parameters in grouper (*Epinephelus coioides*), while the dose of 1% recorded the highest growth rate [4]. This could be attributed that higher dosage of plant extracts has caused activation of enzyme inhibitors as most plants contain inhibitors to protect their major components from unintended degradation [4]. Some authors observed that antinutrients substances contained in plants such as protease inhibitors, tannins, amylase and lipase, saponins and antivitamins [67] may cause disturbance in the gastrointestinal tract [68]. Such condition could affect the digestive processes and feeding efficiency, and thus nutrients cannot be utilized for growth maximization.

In conclusion, our results demonstrated that dietary administration of *A. wilhelmsii* extract to common carp promotes the growth, stimulated the immune response and increased hematological parameters after 8 weeks. Surprisingly, the highest doses (2% and 3%) enhanced the immune response but did not show as high a growth rate as recorded for lower dose (1%). Such results are encouraging to further investigations on antinutritional factors in *A. wilhelmsii* plant that retard growth promotion at higher dose.

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REFERENCES

1. Bondad-Reantaso MG, Subasinghe RP, Arthur JR, Ogawa K, Chinabut S, Adlard R. Veterinary parasitology. 2005; 132:249-72.
2. Alexander CP, John Wesly Kirubakaran C, Michael RD. Fish Shellfish Immunol. 2010; 29:765-72.
3. Wu C-C, Liu C-H, Chang Y-P, Hsieh S-L. Fish Shellfish Immunol. 2010; 29:258-63.
4. Samad APA, Santoso U, Lee M-C, Nan F-H. Fish Shellfish Immunol. 2014; 36:582-9.
5. Awad E, Cerezuela R, Esteban MÁ. Fish Shellfish Immunol. 2015; 45:454-64.
6. Galindo-Villegas J, Hosokawa H. Immunostimulants: Advances en Nutricion Acuicola VII Memorias del VII Simposium Internationale de Nutricion Acuícola. 2004;16-9.
7. Ndong D, Fall J. Journal of Clinical Immnunology and Immunopathology Research. 2011; 3:1-9.
8. Sakai M. Aquaculture. 1999; 172:63-92.
9. Honda G, Yeşilada E, Tabata M, Sezik E, Fujita T, Takeda Y. Journal of Ethnopharmacology. 1996; 53:75-87.
10. Ross J, Press G. Greenfields Press Seattle; 2003, pp. 54-65
11. Mozaffarian V. A dictionary of Iranian plant names: LatinEnglish–Persian. Farhang Mo'aser. 1966, pp. 50-55
12. Dokhani S, Cottrell T, Khajeddin J, Mazza G. Plant foods for human nutrition. 2005; 60:55-62.
13. Yaeesh S, Jamal Q, Khan Au, Gilani AH. Phytotherapy Research. 2006; 20:546-51.

14. Niazmand S, Khooshnood E, Derakhshan M. Pharmacognosy magazine. 2010; 6:282.
15. Candan F, Unlu M, Tepe B, Daferera D, Polissiou M, Sökmen A. Journal of Ethnopharmacology. 2003; 87:215-20.
16. Nemeth E, Bernath J. Current pharmaceutical design. 2008; 14:3151-67.
17. Fathi H, Lashtoo Aghaee B, Ebrahimzadeh M. Pharmacologyonline. 2011; 2:942-9.
18. Asgary S, Naderi G, Sarrafzadegan N, Mohammadifard N, Mostafavi S, Vakili R. Drugs under experimental and clinical research. 2000; 26:89-94.
19. Niazmand S, Esparham M, Rezaee S, Harandizadeh F. Aveicennal J Phytomed. 2011; 1:51-6.
20. Csupor-Löffler B, Hajdú Z, Zupkó I, Réthy B, Falkay G, Forgo P. Phytotherapy Research. 2009; 23:672-6.
21. Jaimand K, Rezaee M. Journal of Essential Oil Research. 2001; 13:354-6.
22. Saeidnia S, Gohari AR, Yassa N, Shafiee A. DARU Journal of Pharmaceutical Sciences. 2005; 13:34-6.
23. Alfatemi SMH, Rad JS, Rad MS, Mohsenzadeh S, da Silva JAT. 3 Biotech. 2015; 5:39-44.
24. Sharififar F, Pournourmohammadi S, Arabnejad M. 2009; 47:668.
25. Yakhkeshi S, Rahimi S, Hemati Matin H. Journal of Agricultural Science and Technology. 2012; 14:799-810.
26. Toghyani M, Tohidi M, Toghyani M, Gheisari A, Tabeidian SA. J Med Plants Res. 2011; 5:2748-54.
27. Bahabadi MN, Banaee M, Taghiyan M, Haghi BN. 2014; 2:275-85.
28. Pastor A, Collado PS, Almar M, González-Gallego J. Journal of Hepatology. 1997; 27:363-70.
29. Baumgardner JN, Shankar K, Hennings L, Albano E, Badger TM, Ronis MJ. The Journal of nutrition. 2008; 138:1872-9.
30. Vendemiale G, Grattagliano I, Caruso ML, Serviddio G, Valentini AM, Pirrelli M. 2001; 175:130-9.
31. Shaarawy SM, Tohamy AA, Elgendy SM, Elmageed Z, Bahnasy A, Mohamed MS. Int J Biol Sci. 2009; 5:549-57.
32. Bradford MM. Foreign Patent Documents. Biochemistry. 1976; 72:248-54.
33. Kajita Y, Sakai M, Atsuta S, Kobayashi M. Fish Pathology. 1990; 25:93-8.
34. Jaffat HS, Hassan AS, A.B. N. International Journal of Scientific & Engineering Research. 2014; 5:852-8.
35. Blaxhall PC. Journal of fish biology. 1972; 4:593-604.
36. Soivio A, Oikari A. Journal of fish biology. 1976; 8:397-411.
37. Awad ES. Heriot-Watt University; 2010, pp.159.
38. Mohamad S, Abasali H. Research Journal of Animal Sciences. 2010; 4:26-34.
39. Ji SC, Jeong GS, Im GS, Lee SW, Yoo J, Takii K. Fisheries Science. 2007; 73:70-6.
40. Sarder MRI, Thompson KD, Penman DJ, McAndrew BJ. Developmental & Comparative Immunology. 2001; 25:37-46.
41. Grinde B. J Fish Dis. 1989; 12:95-104.
42. Tuvikene A. Annales Zoologici Fennici: JSTOR; 1995, p. 295-309.
43. Yin G, Jeney G, Racz T, Xu P, Jun X, Jeney Z. Aquaculture. 2006; 253:39-47.
44. Yin G, Ardo L, Thompson K, Adams A, Jeney Z, Jeney G. Fish Shellfish Immunol. 2009; 26:140-5.
45. Christybapita D, Divyagnaneswari M, Dinakaran Michael R. Fish and shellfish immunology. 2007; 23:840-52.
46. Ellis A. Fish Shellfish Immunol. 1999; 9:291-308.
47. Wilson MR, Ravenstein E, Miller NW, Clem LW, Middleton DL, Warr GW. Developmental & Comparative Immunology. 1995 19:153-64.
48. Ross DA, Wilson MR, Miller NW, Clem LW, Warr GW. Immunological reviews. 1998; 166:143-51.
49. Picchitti S, Scapigliati G, Fanelli M, Barbato F, Canese S, Mastrolla L. Journal of fish biology. 2001; 59:1503-11.
50. Magnadóttir B, Jónsdóttir H, Helgason S, Björnsson B, Jørgensen TØ, Pilström L. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 1999; 122:181-8.
51. Klesius P. Vet Immunol Immunopathol. 1990; 24:187-95.
52. Magnadóttir B, Jónsdóttir H, Helgason S, Björnsson B, Jørgensen TØ, Pilström L. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 1999; 122:173-80.
53. Magnadóttir B, Gudmundsdóttir S, Gudmundsdottir B. Vet Immunol Immunopathol. 1995; 49:127-42.
54. Nielsen M. Journal of helminthology. 1999; 73:227-32.
55. Awad E, Austin D, Lyndon AR. Aquaculture. 2013; 388:193-7.
56. Cuesta A, Meseguer J, Esteban M. Vet Immunol Immunopathol. 2004; 101:203-10.
57. Ellis A. Developmental & Comparative Immunology. 2001; 25:827-39.
58. Sivaram V, Babu M, Immanuel G, Murugadass S, Citarasu T, Marian M. Aquaculture. 2004; 237:9-20.
59. Hardie L, Ellis A, Secombes C. Diseases of aquatic organisms. 1996; 25:175-83.

60. Secombes CJ. Techniques in fish immunology. 1990; 1:137-54.
61. Harikrishnan R, Heo J, Balasundaram C, Kim M-C, Kim J-S, Han Y-J. Fish Shellfish Immunol. 2010; 29:668-73.
62. Jian J, Wu Z. Fish Shellfish Immunol. 2004; 16:185-91.
63. Jian J, Wu Z. Aquaculture. 2003; 218:1-9.
64. Blazer VS. Annual Review of Fish Diseases. 1992; 2:309-23.
65. Lall S, Olivier G. Colloques de l'INRA (France). 1993.
66. Fjalestad KT, Gjedrem T, Gjerde B. Aquaculture. 1993; 111:65-74.
67. Francis G, Makkar HP, Becker K. Aquaculture. 2001; 199:197-227.
68. Jobling M. Aquaculture International. 2012; 20:601-2.