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Original Article

The Effects of Phytobiotic-Enriched Diet on Immunity Index and Hematological-Biochemical Changes in Common Carp Fish (*Cyprinus carpio*)

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ABSTRACT

The present study compared the effects of four medicinal herbs on the growth, digestive enzyme activity, innate immunity, and oxidative status of common carp juveniles (*Cyprinus carpio*). The juvenile fish (average weight: 80.01 ± 0.34 g) were fed with four diets, including 1% *Zataria multiflora* oil (T2), Zingiber powder (T3), garlic essential oil extract (T4), garlic peel extract (T5), and control diet (T1) without any additives, for seven weeks (20 individuals per replicate). The results showed that weight gain and specific growth rate were highest in the treatment fed with garlic and ginger extracts (T3 and T4). The protease and lipase activities in the medicinal herb treatments (especially the garlic extract treatment; T4) were significantly higher than in the control. Fish fed with medicinal herb-supplemented diets (except for *Zataria multiflora* oil; T2) also displayed higher levels of serum lysozyme, alternative complement, total immunoglobulin, superoxide dismutase, and total protein levels compared to the control diet. The highest levels were observed at the 1% level of Zingiber powder (T3) and garlic extract (T4). Medicinal herb supplementation at a level of 1% is a natural immunostimulant and growth promoter supplement recommended for common carp.

Keyword: *Cyprinus carpio*, Medicinal plants, Nutrition, Health.

INTRODUCTION

Common carp (*Cyprinus carpio*) is an important species for aquaculture in many Asian and some European countries. It is commonly found in freshwater environments such as rivers, ponds, and lakes, and is seldom found in brackish water environments [1]. The use of antimicrobials and sanitizers has shown partial success in preventing or treating marine ailments. Currently, herbal and natural remedies have a particular position in the treatment of ailments due to their commercial value, low production cost, ecological friendliness (organic drugs), minimal side effects compared to chemical medicines, absence of pathogen resistance, the uniqueness of disease treatment with plants, and various medicinal skills regarding pharmaceutical plants [2]. Therefore, exploring different food additives is a very important goal for aquaculture researchers [3]. Garlic (*Allium sativum* L.) is a medicinal plant that has been used in Indian Ayurvedic medicine for a thousand years. Garlic has shown antimicrobial, antihypertensive, hepatoprotective, antioxidant, and immune
enhancing properties, including the promotion of lymphocyte proliferation, cytokine release, phagocytosis, and natural killer cell activity [4]. Ginger (*Zingiber officinale*) is another potential medicinal plant that contains natural antioxidants such as gingerols, shogaols, and zingerones that can improve disease resistance against pathogens [5]. *Zataria multiflora*, from the family Labiatae, is traditionally used as an antiseptic, anesthetic, and antispasmodic [6]. In Iran, it is more widely used as an herbal tea, flavoring agent (condiment and spice), and medicinal plant. It is also used as a tonic, carminative, digestive, antispasmodic, anti-inflammatory, antitussive, expectorant, and for the treatment of colds in Iranian traditional medicine [7]. Improving the immune system of fish is considered the most effective method of preventing fish diseases in aquaculture. This improvement can be achieved through the use of vaccines, which enhance the specific immune response of fish and are considered the most effective agents. As the concern about antibiotic resistance in aquaculture grows, there has been a rising trend towards the use of ancient herbal medications. The tendency towards ancient herbal medications is on the

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rise in aquaculture due to concerns about antibiotic resistance. According to several applied research studies in this area, herbal medicine has shown a potential capacity to boost the immune function of aquatic animals contributing to disease resistance enhancement [8]. Several applied research studies have shown that herbal medicine has the potential to boost the immune function of aquatic animals, which can contribute to enhancing disease resistance [9, 10, 11, 12, 13, 14 and 15]. Little information is available on the effects of garlic peel in aquaculture [13]. Therefore, this study aims to compare the effects of various herbal medicines on the growth performance, hematological factors, and intestinal enzymes of *C. carpio.*

MATERIAL AND METHODS

Animals

A total of 300 common carp specimens (*Cyprinus carpio*) were obtained from a carp farm located in Mazandaran province, Iran. The fish were transported to the Aquatic Animal Health Laboratory at Gonbad Kavous University, Iran. The fish were then acclimated to laboratory conditions for 10 days in fiberglass-reinforced plastic tanks and fed a commercial diet. The laboratory conditions were regularly monitored and maintained at a consistent level during the acclimation period.

Treatments

For this study, five treatments (T1, T2, T3, T4, and T5) with three replicates were established. The control group was fed a basic diet (T1). *Zataria multiflora* oil (T2) and *Zingiber officinale* powder (T3) were added to the other experimental diets. The remaining groups were fed garlic extract (T4) and garlic peel extract (T5). The ingredients were added at a concentration of 1% [16, 17] to each experimental diet using 5% gelatin in sufficient water as a binder [18]. Each replicate consisted of 20 fish (80.01 ± 0.34 g) in a 200-liter tank with 120 liters of water at a salinity level of 0.56 ± 0.03 ppt [19]. Aeration was provided by a single air stone to maintain the dissolved oxygen level at 7.72 ± 0.18 mg/l. The water temperature was maintained at 25.19 ± 0.08°C and pH 7.08 ± 0.05 throughout the experimental period. The control diet was provided by enhancing the water with no crust power. The fish were fed 2.5% of their average body weight per day for seven weeks, divided into three feedings at 08:00, 11:00, and 18:00. Feces, molts, and dead fish were removed daily, and 30% of the water was changed with fresh water in each tank [5].

Plants Management

Garlic was purchased from a local marketplace. The garlic cloves and peels were dried at 37°C for three days and ground into a fine powder. The dried powders were soaked in a mixture of water and ethanol (1:1 ratio) for 48 hours. The extraction was filtered (Whatman No.1) and concentrated at 40°C using a rotary evaporator (HS-200S, Korea). Finally, the medicinal plant extracts were stored at 4°C until use [20].

Ginger (*Zingiber officinale*) was also purchased from a local marketplace and powdered for use in the diet. *Zataria multiflora* essential oil was prepared by Barij-Essence Company, Iran. The diets were then dried, sealed in plastic bags, and stored at 4°C until feeding [3, 21]. The proximate composition of the experimental diets (crude protein, crude lipid, crude ash, and crude fiber) was determined using AOAC [22] (Table 1).

Growth and Nutrition Parameters

At the end of the study, the final body weight (FBW), final body length (FBL), condition factor (CF), protein efficiency ratio (PER), lipid efficiency ratio (LER), feed conversion efficiency (FCE), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate were calculated for both the experimental and control groups. The following formulas were used to calculate the growth factors [4]:

Final body weight (FBW) = Mean weight of fish at the end of the study
Final body length (FBL) = Mean length of fish at the end of the study
Condition factor (CF) = (Fish weight (g) / Fish length (cm)3) × 100
Protein efficiency ratio (PER) = (Weight gain (g) / Protein intake (g))
Lipid efficiency ratio (LER) = (Weight gain (g) / Lipid intake (g))
Feed conversion efficiency (FCE) = (Weight gain (g) / Feed intake (g)) × 100

Specific growth rate (SGR) = [(ln(final weight) - ln(initial weight)) / Time (days)] × 100
Feed conversion ratio (FCR) = Feed intake (g) / Weight gain (g)
Survival rate = (Number of surviving fish / Initial number of fish) × 100

Table 1 Ingredients and Composition of the Experimental Diets

Ingredient	($g\ kg^{-1}$)	Composition	%
Fishmeal[1]	150	Dry matter	85.2
Poultry meal[2]	200	Crude protein	37.2
Soybean meal	200	Crude lipid	7.8
Wheat flour	378	Ash	6.2
Fish oil	15		
Soybean oil	15		
Corn flour	30		
L-Lysine[3]	4		
L-Methionine	6		
Vitamin premix a	1		
Mineral premix b	1		

1- Pars kilka Co., Mazandaran, Iran (Kilka powder analysis; Protein: 70–72%, Fat: 8–11%, Ash: 11.6%, Moisture: 7–9%).
2- Makianmehr Co., Golestan, Iran. 3- Morghenojan.Co., Tehran, Iran.
a Vitamin premix (per kg of diet): vitamin A, 2000 IU; vitamin B1 (thiamin), 5 mg; vitamin B2 (riboflavin), 5 mg; vitamin B6, 5 mg; vitamin B12, 0.025 mg; vitamin D3, 1200 IU; vitamin E, 63 mg; vitamin K3, 2.5 mg; folic acid, 1.3 mg; biotin, 0.05 mg; pantothenic acid calcium, 20 mg; inositol, 60 mg; ascorbic acid (35%), 110 mg; niacinamide, 25 mg.
b Mineral premix (per kg of diet): $MnSO_4$, 10 mg; $MgSO_4$, 10 mg; KCl, 95 mg; NaCl, 165 mg; $ZnSO_4$, 20 mg; KI, 1 mg; $CuSO_4$, 12.5 mg; $FeSO_4$, 105 mg; Co, 1.5 mg.

Sampling and Processing

After seven weeks of feeding, the fish were fasted for 24 hours before blood and serum collection. Nine fish from each treatment group were randomly selected for blood sampling from the caudal veins [4]. The sampled blood was then centrifuged at 2000 g (at 4°C) for 10 minutes. Additionally, 12 fish were randomly selected, euthanized, and dissected to collect the entire digestive tract [23]. The serum and intestine were extracted and frozen at -80°C for serum and digestive enzyme analyses [24].

Digestive Enzyme Parameters

The digestive tract samples were homogenized, centrifuged at 25000 g for 20 minutes, and the supernatants were collected [25]. Amylase activity was measured using the method described by Langlois et al. [26] with 0.3% soluble starch as the substrate dissolved in NaH_2PO_4 buffer (pH 7.4). Protease activity was determined following the method described by Walter [27], using casein (Sigma) at a concentration of 1% w/v as the substrate in 0.2 M phosphate buffer at pH 7.0. To measure the lipase activity, p-nitrophenol myristate was used as the substrate dissolved in 0.25 M Tris-HCl (pH 9.0) [28]. These parameters were used to assess the effects of the different herbal additives on the digestive enzyme activity of *C. carpio*.

Immune and Biochemical Parameters

The lysozyme levels were determined using the method described by Ellis [29]. The total immunoglobulin was evaluated according to the Siwicki and Anderson method [30]. Alternative hemolytic complement activity (ACH50) was defined based on the hemolysis of rabbit red blood cells (RaRBC) using the Sunyer and Tort method [31]. The content of alternative complement activity (ACH50) was measured and used to calculate the complement activity of the samples (the value of ACH50 is in units per ml). Serum total protein (TP) and glucose were measured using Parsazmon's kits (Parsazmon Company, Iran) based on the company's protocol.

Antioxidant Responses

Superoxide dismutase (SOD) activity was determined using the method described by Fridovich [32] which is based on the inhibition of nitroblue tetrazolium reduction by the xanthine / xanthine oxidase system as a superoxide generator. Catalase (CAT) activity was measured according to the method described by Beutler [33]. Malondialdehyde (MDA) levels, which are an indicator of free radical generation, were estimated according to Lowry et al. [34].

Data Analyses

The normality of the data was determined using the Shapiro-Wilk Test. Significant differences between treatments were evaluated using One-way analysis of variance (ANOVA). Duncan's test was used at a significant level of ($P< 0.05$) to compare means (Mean ± SEM). Statistical analyses were performed using SPSS 21 and Excel 2013 software.

RESULTS

Growth Performance

The effect of the experimental diets on the growth performance of common carps (*C. carpio*) is shown in Table 2. The final weight of the fish showed significant differences among the groups ($P < 0.05$). The highest significant final weights were recorded in the ginger and garlic groups compared to the control and other experimental groups. The specific growth rate (SGR) was significantly increased ($P < 0.05$) in all groups compared to the control group. Fish fed with ginger and garlic showed the highest SGR compared to other experimental groups. All the fish fed with the experimental diets had lower feed conversion ratio (FCR) compared to the control group. The lowest FCR was recorded in the fish fed with the ginger and garlic diet compared to other experimental groups. Additionally, the fish receiving diets supplemented with ginger and garlic had significantly higher growth parameters, including condition factor (CF) as well as protein efficiency ratio (PER) ($P < 0.05$). The survival rate was not affected ($P > 0.05$) by the herbal-supplemented diets. These results suggest that the addition of ginger and garlic to the diets of *C. carpio* can improve their growth performance. (Table 2).

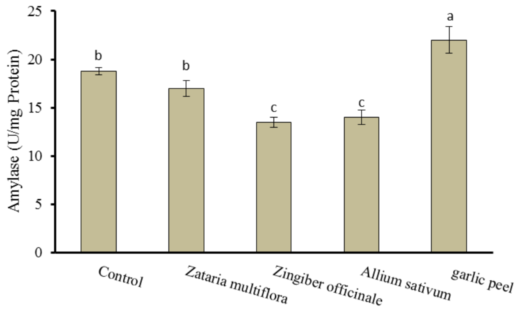
Table 2 Growth and Feed Performance of Common Carp Fed with Medicinal Plants after 7 Weeks (Mean ± SEM)

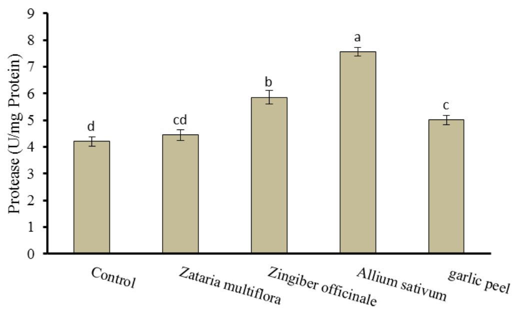
	Treatments				
Growth parameters	T1 (Control)	T2 (garlic extract)	T3 (garlic peel)	T4 (*Z. multifora* oil)	T5 (*Z. officinale* powder)
FBW (gr)	115.09±0.47 c	129.18±0.67 a	121.95±0.97 b	120.29±0.38 b	131.81±0.57 a
CF (gr/cm^3)	0.91±0.04 ab	1.24±0.07 a	1.13±0.05 ab	1.18±0.06 a	1.37±0.05 b
SGR (%/day)	0.78±0.02 c	0.94±0.017 a	0.85±0.026 b	0.86±0.019 b	0.94±0.017 a
FCE (gr/cm^3)%	23.65±9.50 d	65.39±9.22 b	43.96±13.25 c	39.03±5.24 c	73.19±7.78 a
FCR	2.75±0.06 a	2.11±0.03 c	2.53±0.08 b	2.48±0.06 b	2.09±0.03 c
PER (gr/gr)	1.05±0.024 c	1.35±0.023 b	1.15±0.03 4c	1.16±0.025 c	1.47±0.02 6a
LER (gr/gr)	1.33±0.13 c	3.68±0.14 a	2.49±0.20 b	2.15±0.13 b	4.13±0.13 a
Survival (%)	100	100	100	100	100

* Final body weight (FBW), Condition factor (CF), Specific growth rate (SGR), Feed conversion efficiency (FCE), Feed conversion ratio (FCR), Protein efficiency ratio (PER), Lipid efficiency ratio (LER)

Digestive Enzymes

The activities of digestive enzymes in common carp fed diets supplemented with 1% of medicinal plants for 7 weeks are shown in figure 1. The activity of amylase was significantly affected by the ginger and garlic groups. The protease and lipase activities were significantly higher in fish fed *Allium sativum* (garlic extract) and *Zingiber officinale* (ginger), respectively (figure 1).





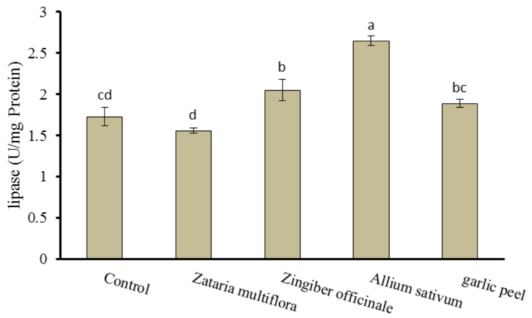


Fig. 1 Digestive Enzyme Activities of Juvenile Common Carp Fed Medicinal Plants for 7 Weeks (Mean ± SEM). Different Lowercase Letters within a Column Show Significant Effects of the Treatments ($P < 0.05$).

Immune and Biochemical Response

After 7 weeks, the serum lysozyme activity and Total immunoglobulin of common carps in the ginger, garlic, and garlic peel treatments were significantly higher ($p < 0.05$) than those in the control group (Fig 2). The treatments inoculated with garlic and ginger exhibited the highest total immunoglobulin level ($P < 0.05$). However, the Total immunoglobulin was not statistically significant ($P > 0.05$) between the ginger and garlic groups ($P < 0.05$). The carps' alternative hemolytic complement activity (ACH50) was influenced ($P < 0.05$) by ginger, garlic, and garlic peels as food additives compared to the control group. However, a numerically higher ACH50 level was found in the fish receiving ginger. The glucose level was significantly increased in those treated with garlic peel ($P < 0.05$). The glucose level of fish after 4 weeks of experimental diet was significantly increased in fish treated with garlic peel ($P < 0.05$) (Fig 2).

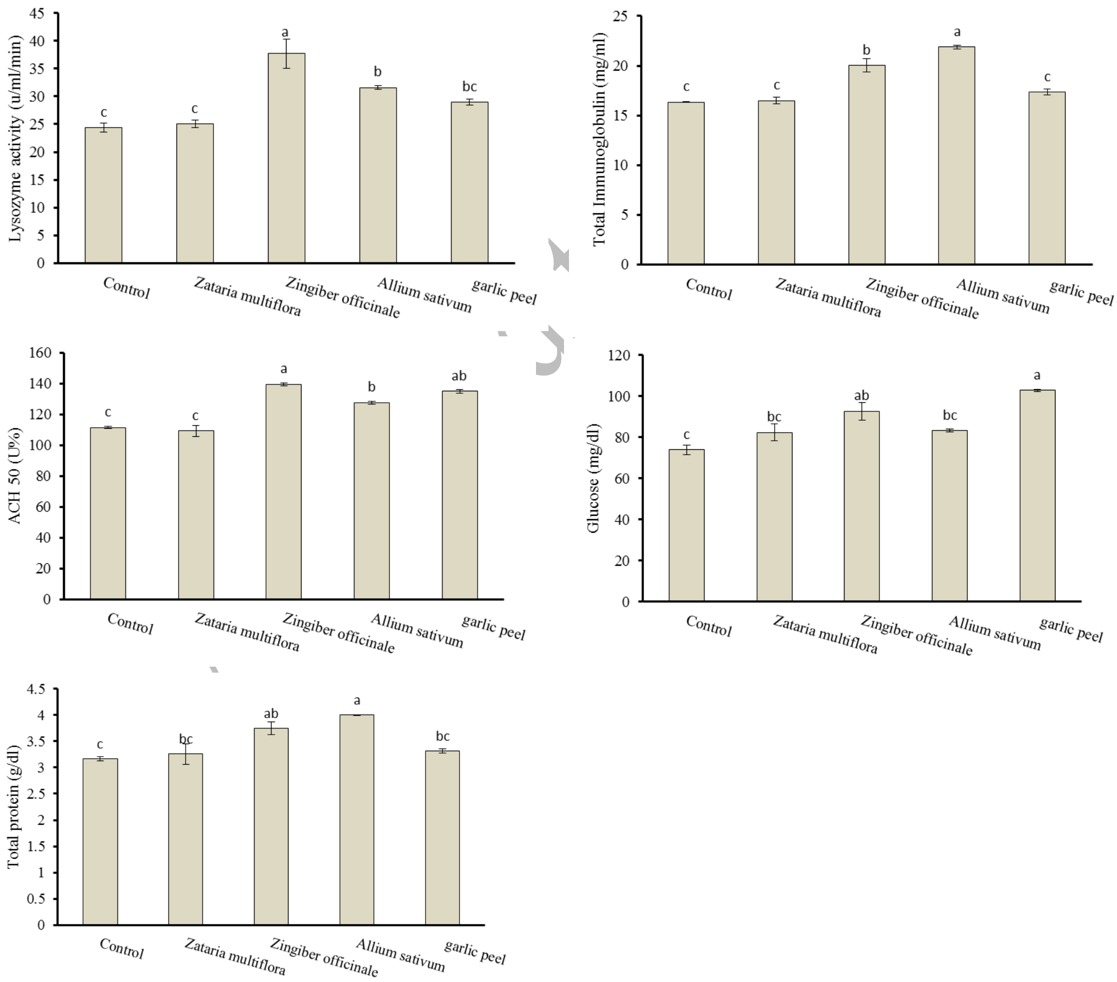


Fig. 2 Innate Immune Responses of Common Carp Fed Medicinal Plants for 7 Weeks (Mean ± SEM). Different Lowercase Letters within a Column Show Significant Effects of the Treatments ($P < 0.05$).

Antioxidant Enzymes Activities

After 7 weeks of oral administration of four different herbal diets, the serum SOD activity of common carps in the ginger and garlic treatments was significantly higher ($p < 0.05$) than in the *Zataria multiflora* and control groups (Fig 3). Catalase activity of fish fed with ginger and garlic treatment diets was significantly ($P < 0.05$) higher than the other treatments. The MDA level was decreased significantly in fish fed medicinal plants compared to the control group, with the lowest being in fish fed with ginger (*Z. officinale*) diet (Fig 3).

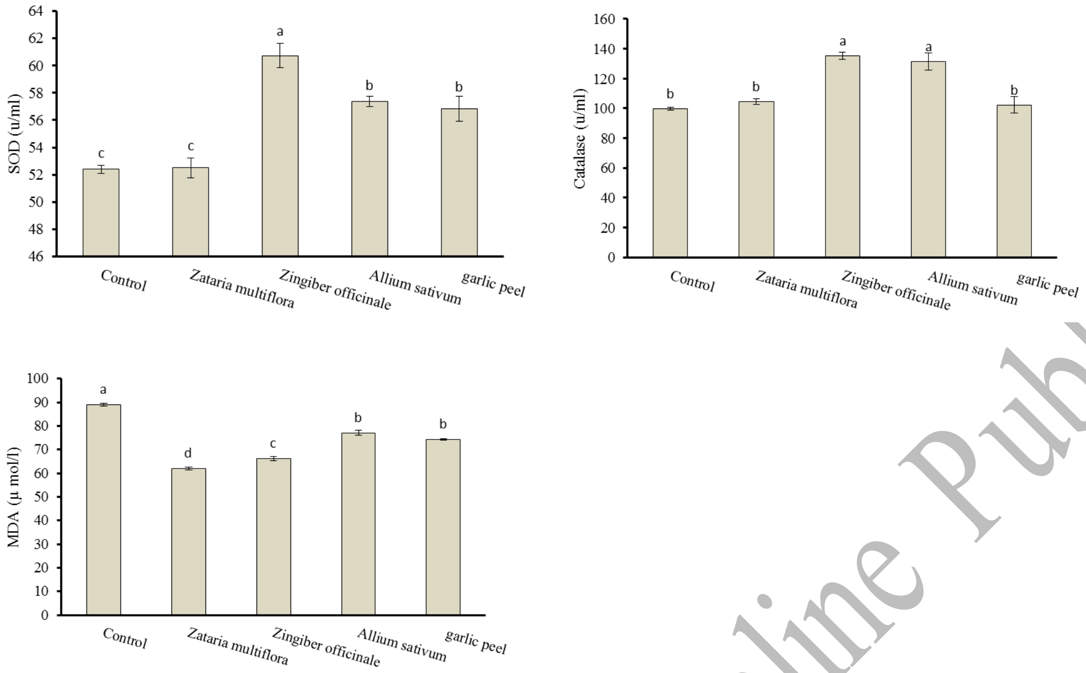


Fig. 3 Antioxidant Enzyme Activities of Common Carp Fed Medicinal Plants for 7 Weeks (Mean ± SEM). Different Lowercase Letters within a Column Show Significant Effects of the Treatments ($P < 0.05$).

DISCUSSION

Hematological indicators are an important criterion for evaluating the effects of dietary treatments on animals [13]. For instance, Thanikachalam et al. [35] reported a significant increase in the WBC count of African catfish (*Clarias gariepinus*) following a 20-day post-feeding with garlic peel . Chitsaz et al. [11] also reported significant increases in the Hb level in *H. huso* fed with a diet incorporated with garlic peel compared to the control treatment. The use of immunostimulants in fish diets as potential protective food additives has been reviewed by Wang et al. [36]. In the present study, the introduction of herbal additives to the fish diet improved growth performance, including WG, SGR, FCR, PER, and LER, notably in the ginger and garlic treatments, followed by garlic peel and *Zataria multiflora*, compared to the control group. Consistent with our study, garlic powder supplementation administered dietary to common carps could enhance growth parameters. Norhan et al. [37] demonstrated that 1% garlic administration improved growth rate and feed utilization in sea bass. Conversely, no significant variations were detected in the body weight of tilapia fed with dietary supplements of 0.5% and 1% garlic for four weeks [38]. Digestion is an important process in animal metabolic rate because it regulates the availability of nutrients required for all biotic purposes and is the main instrument in considering the dietary situation and conformity of the creature to nutritional variation [11]. It has been revealed that medicinal plants stimulate the secretion of pancreatic enzymes, which are imperative parameters in nutrient breakdown and absorption [39].

According to this study, carps fed with garlic and garlic peel diets showed enhanced activity of digestive enzymes such as protease and lipase, which improved digestion and intake of essential nutrients for fish development. These results are similar to the findings of Chitsaz et al. [11], who reported increased digestive enzyme activity in *Huso huso* fed with garlic peel. Similarly, many researchers have reported improved amylase activity under various herbal additives [40]. Jang et al. [41] also demonstrated that herbal medicine administration could enhance the pancreatic digestive enzyme activities, such as α-amylase and trypsin, as well as intestinal enzymes, improving the capability to enhance fish growth efficiency [42]. Moreover, this improvement is potentially due to the development of intestinal microflora, contributing to a decrease in pathogenic bacteria growth and an increase in the colonization of beneficial bacteria [43, 44]. Therefore, the use of medicinal plants can improve the digestive process, leading to better nutrient absorption and utilization, ultimately improving the growth performance of aquatic animals.

Humoral and cellular immunity are the specific defense mechanisms in fish. Cellular immunity includes neutrophils, phagocytic cells, natural killer cells, and lymphocytes, while humoral immunity includes immunoglobulins, hemolysins, lysozyme, and complement molecules [42]. In the present study, garlic and ginger treatments positively affected the total protein concentration in common carps. Similarly, garlic and ginger oil increased the total protein concentration in sea bass [45].

Moreover, the supplementation of fish diets with ginger extracts has been shown to increase TP levels in fish plasma [46]. Similarly, powdered or oil forms of garlic have been found to improve TP in the blood of *Oreochromis niloticus* [47, 48]. An increase was observed in the alternative complement activity (ACH50) in carps fed with ginger, garlic, and garlic peel, potentially improving phagocytosis by enhancing the recognition and elimination of bacterial agents. Several studies have reported enhancement of complement activity following the administration of different immunostimulants such as herbal derivatives [49]. Serum immunoglobulin mainly neutralizes exogenous pathogens and is considered a component of the teleost humoral immune system. In this study, feeding fish with ginger and garlic-supplemented diets resulted in higher levels of immunoglobulin in carps compared to the control. Sarhadi et al. [50] also reported that artemisia (*Artemisia annua*) leaf extract improved the immune response, including lysozyme, immunoglobulin, and protease. The remarkable enhancement in serum immunoglobulin levels may be due to the active ingredients in garlic (known as allicin), which can actively stimulate the secretion of IgM. Lysozymeis the first line of defense in the innate immune system [51]. Its function is to act as a marker of innate immunity in leukocyte respiratory burst activity [52]. Blood lysozyme actively lyses peptidoglycans in the bacterial cell wall, and it is considered an important factor in the defense against bacterial infections. In the present study, garlic (1%) significantly enhanced lysozyme activity, suggesting that the use of medicinal plants can improve the innate immune response of aquatic animals. Additionally, lysozyme works as an opsonin and activates phagocytes and the complement system, further enhancing the immune response [3].

Free radicals are produced during the oxidative stress process to protect living organisms from invading pathogens. However, these free radicals can be harmful to living organisms themselves, and an antioxidant system is applied to protect them from free radicals. The most important indicators of antioxidant activities are the increase in the SOD and CAT enzyme levels [53, 54, 55]. SOD is the first line of defense against oxygen toxicity, using its inhibitory effects on oxyradical formation and contributing to the dismutation of the superoxide anion radical into water and hydrogen peroxide, which is then detoxified by CAT [56]. In the present study, fish treated with ginger and garlic diet had the highest SOD and catalase levels. In two similar studies on tilapia, garlic and ginger were found to increase antioxidant enzyme activity (SOD and CAT) [57, 58, 59]. In other research, the addition of purslane extract (*Portulaca oleracea*) to the diet of grass carp *(Ctenopharyngodon idella)* led to a statistically significant increase compared to the control [60]. In general, the results show that ginger reduced the oxidative stress caused by stress by inhibiting radicals, because the highest level of superoxide dismutase (SOD) and the lowest level of malondialdehyde (MDA) were observed in the treatment fed with ginger [16].

Malondialdehyde (MDA) is one of the end products of lipid peroxidation caused by oxygen free radicals, and it is an important oxidative stress indicator that shows the degree of lipid peroxidation. An increase in MDA levels is an important indicator of cellular damage [61]. In this study, low MDA values were obtained in all experimental groups, especially in ginger-treated fish, when compared to the control. Similarly, it has been reported by Sahan et al. [59] and Islam et al. [58] that MDA levels decreased in tilapia fed ginger. Our results were also in accordance with the findings of Giannenas et al. [62] in the ginger-fed group. It has been emphasized that this maybe associated with various bioactive substances (shagol, gingerols, zingeron, etc.) found in the composition of ginger, which provide antioxidant properties to ginger. Phenolic compounds of ginger (gingerols, shogaols, volatile oils, flavonoids, and phenolic ketone derivatives) have been reported to promote antioxidant activity against free radicals and inhibit lipid peroxidation [63].

CONCLUSION

Based on the results obtained in the present study for common carps fed with ginger and garlic-added diets at a 1% concentration, it can be concluded that these medicinal plants can enhance the digestive activity, growth function, and general health status of aquatic animals. The findings suggest that the use of phytobiotics, especially garlic administration in fish diets, can improve growth functions and reduce oxidative stress. These results are consistent with previous research indicating that the use of medicinal plants in aquaculture can enhance the immune response and antioxidant defense system of aquatic animals, ultimately leading to better disease resistance and improved overall health. Therefore, the use of medicinal plants such as ginger and garlic in aquaculture can be a promising and sustainable approach to improve the performance and health of aquatic animals, while also reducing the use of synthetic chemicals in aquaculture.

Ethical Statement

This work was conducted in accordance with the guidelines and regulations approved by the Ethics Committee of Gonbad Kavous University, Iran. All procedures involving animal handling and experimentation were carried out in strict accordance with the guidelines and regulations for animal welfare and protection. Special attention was given to minimize the stress and discomfort of the experimental animals throughout the study. No animals were harmed or sacrificed unnecessarily during the course of this research.

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Conflict of Interest

The authors declare that there is no potential conflict of interest related to this research.

Authors Contributions

All authors have contributed equally to this research project. They provided critical feedback, designed the study, analyzed the data, and helped shape the manuscript.

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