

Original Article

Impact of dietary leaf extracts of Black pepper *Piper nigrum* L. on the growth, hematological and immunological parameters of *Labeo rohita* (Hamilton, 1822) cultured in glass aquaria

Impacto de extratos de folhas da dieta de pimenta-do-reino *Piper nigrum* L. sobre o crescimento, parâmetros hematológicos e imunológicos de *Labeo rohita* (Hamilton, 1822) cultivado em aquários de vidro

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Abstract

A study was conducted to evaluate the effect of *Piper nigrum* (black pepper) leaf extract on the growth performance, proximate composition, hematological parameters, and immune response of *Labeo rohita* fingerlings with an average weight of 22.14 ± 0.98g. After acclimation for two weeks, fish (n=25) were randomly selected and placed in four glass aquaria (T₀, T₁, T₂ and T₃) at constant water temperature (30.0 ± 1.0 °C), pH (7.50 ± 0.5) and total hardness (200 ± 2.0 mgL⁻¹) for a period of 12 weeks, with three replicates each. Fish were fed with *P. nigrum* leaf extract supplemented feed @ 0.0%, 1.0%, 2.0% and 3.0% in T₀, T₁, T₂ and T₃, respectively. At the end of experiment, five fish were randomly selected from each aquaria for proximate composition, gut and skin microbial load, hematological parameters. Total proteins, albumins, and globulins were also recorded to evaluate immunological memory. The result revealed that fish in T₂ showed better growth performance with an average weight gain of 56.11 ± 0.51 g. Thus, it had been concluded that *Piper nigrum*, a medicinal plant, can also be used to enhance the growth performance and immune response of *Labeo rohita* as attractive alternatives against antibiotics and vaccines and has shown no negative side effects on fish health as well as on its environment.

Keywords: growth, disease resistance, immunity, *Labeo rohita*, *Piper nigrum*.

Resumo

Um estudo foi conduzido para avaliar o efeito do extrato da folha de *Piper nigrum* (pimenta-do-reino) sobre o desempenho de crescimento, composição centesimal, parâmetros hematológicos e resposta imune de alevinos de *Labeo rohita* com peso médio de 22,14 ± 0,98g. Após aclimação por duas semanas, os peixes (n = 25) foram selecionados aleatoriamente e colocados em quatro aquários de vidro (T₀, T₁, T₂ e T₃) em temperatura constante da água (30,0 ± 1,0 °C), pH (7,50 ± 0,5) e dureza total (200 ± 2,0 mgL⁻¹) por um período de 12 semanas, com três repetições cada. Os peixes foram alimentados com ração suplementada com extrato de folha de *P. nigrum* @ 0,0%, 1,0%, 2,0% e 3,0% em T₀, T₁, T₂ e T₃, respectivamente. Ao final do experimento, cinco peixes foram selecionados aleatoriamente de cada aquário para composição centesimal, carga microbiana intestinal e cutânea e parâmetros hematológicos. Proteínas totais, albuminas e globulinas também foram registradas para avaliar a memória imunológica. O resultado revelou que os peixes em T₂ apresentaram melhor desempenho de crescimento com ganho de peso médio de 56,11 ± 0,51 g. Assim, concluiu-se que *Piper nigrum*, uma planta medicinal, também pode ser usado para melhorar o desempenho de crescimento e resposta imunológica de *Labeo rohita* como alternativas atraentes contra antibióticos e vacinas e não mostrou efeitos colaterais negativos na saúde dos peixes, bem como sobre seu ambiente.

Palavras-chave: crescimento, resistência a doenças, imunidade, *Labeo rohita*, *Piper nigrum*.

1. Introduction

Aquaculture is the widely growing sector of human food production in the world (Tidwell and Allan, 2001). To sustain the aquaculture production rates all over the

world, it is quite necessary to increase the production of good quality fish feeds from various byproducts obtained from both plant and animal in origin (Francis et al., 2005);

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therefore, significant emphasis had been observed on the traditional uses of plant origin resources like soybean, peanuts, cotton and rice (Jackson and Capper, 1982; Bhosale et al., 2010). In most aquaculture practices, the production of valuable fish feed is not only a first major step, but it is quite necessary for sustainable fish production. Traditionally, fish feeds were prepared from byproducts (i.e., fish oil and fish meal) obtained by various marine fish species because of their high protein constituents in fish diets; however, their production cost has now become very high in the fish industry. So, in order to reduce all these kinds of fish feed production costs and sustain their quality in fish farming, recently some vegetable ingredients have now also been used in replacements of these marine fish origin ingredients during the preparation of these fish feed. But these plant origin ingredients sometimes make fish more susceptible to the development of certain pathogenic microorganisms (Lunestad et al., 2011). Nowadays, the fish feed also contains supplements/additives, which are particularly added in a very small quantity along with its major ingredients in fish feed preparation, because of their nutritive or non-nutritive ingredients for achieving some specific purposes, e.g., for improving fish flesh quality, feed quality in diet, and also for maintaining the quality of aquatic ecosystem (Bai et al., 2015). Besides, fish farmers nowadays are mostly interested in raising their fish culture in an environment free from antibiotics or the use of vaccines against certain pathogenic diseases. With prolonged use of all these antibiotics, many releases their residues in an aquatic environment that may later enter in fish body and usually produce a negative impact like decrease its nutritional quality, drug resistance, and suppress its immunity against certain diseases (FAO, 2002). Therefore, this issue has now raised the interest of most aquaculturists in the search for more safe dietary immunostimulants that can be used as an alternative to these antibiotics. But still, the significance of using all these additives or supplements in the form of immunostimulants in combination with major ingredients of fish feed has received little or no attention by several researchers throughout the world, particularly in developing countries. According to Baba et al. (2016), immunostimulants can enhance the growth performance, resistance against certain diseases, and also produced innate immune mechanisms in addition to the acquired immunity response of fishes. In aquaculture, due to use of artificial fish feed, fertilizers, and high stocking density in fish pond culture practices may sometimes cause diseases spread by several microbes. Therefore, the use of several antibiotics, chemicals, and vaccination against these microbes may sometimes lead to partial success in their prevention and control in fish culture, and also responsible for major economic losses. Alternative applications of various compounds have now been approaches to boost the immune response in fishes against several microorganisms. All these compounds are now commonly known as "immunostimulants" that play a beneficial role in disease management during aquaculture practices. These immunostimulants are grouped into different kinds based on their sources of origin, i.e.,

polysaccharides, bacterial preparations, animal byproducts, medicinal plant extracts, and cytokines. Mastan (2015) had classified various types of immunostimulants that are commonly used in aquaculture, such as, (1) **Muramyl dipeptide** is a glycoprotein, which is purified form of mycobacteria; (2) **Chitin** is a polysaccharides found as the major constituent in the exoskeletons of most crustaceans, insects and fungal cells; (3) **Chitosan** can be considered as a deacetylation product of chitin; (4) **Lentinan** is also a polysaccharide isolated from the Basidiomycete mushrooms that have anticancer properties and produced immunostimulant response by activation of B and T-lymphocytes and macrophages, which are involved in defense mechanisms of host; (5) **Schizophyllan** is another kind of polysaccharides isolated from *Schizophyllum commune* its composition is similar to lentinan; hence can produce immunostimulation and inhibits the growth of tumor cells (Yanaki et al., 1986; Mizuno, 1999; Lemieszek and Rzeski, 2012); and (6) **Glucans** is derived from outer cell wall of yeast i.e., *Saccharomyces cerevisiae* based byproduct and its dietary inclusion has been found to reduced the environmental stress and act as immunostimulator against certain bacterial and viral infections in many fish species as reported by Manners et al. (1973), Rychlik et al. 2013, Vetvicka et al. (2013) and Van den Abbeele et al. (2020). According to Shiau and Hsu (2002), ascorbic acid (Vitamin-C) and α -tocopherol (Vitamin-E) acts as antioxidants that are very essential for most cultured fish species i.e., Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*), because they have great impact on their immune system, growth and survival rates of these species. Besides, Yilmaz et al. (2018) had also reported that these plants origin supplements/additives in the fish diet may not the only used as an immunostimulant for fish culture practices but had also been found to produce a valuable impact on the growth performance, feed conversion ratio, liver function and specific growth rates in some tilapia and carp fish species. Black pepper belongs to the genus *Piper*, is a flavor plant of family Piperaceae, which is commonly called "**Ashanti pepper**" and also considered as a native plant of Central and Western African tropical regions (Atanda et al., 2016). Black pepper contains vitamin A, vitamin B1 and B2, vitamin C, and vitamin E which also act as antioxidants and also useful for better health because of the increase in the immune response in fish as well as its growth response (Chibuzor and Assumpta, 2014).

2. Materials and Methods

2.1. Study area

The present experimental trial was conducted for 12 weeks in glass aquaria placed in the research laboratory of Fisheries Research Farms, Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences (UVAS), Ravi Campus, Lahore, Pakistan. A total of 300 samples of *Labeo rohita* fingerlings were obtained

from the fish ponds located in Fisheries Research Farm and kept in cemented tanks for acclimation for two weeks.

2.2. Physico-chemical conditions of glass aquaria

The physicochemical characteristics of water samples i.e., temperature ($^{\circ}\text{C}$), pH, electrical conductivity (mScm^{-1}), total hardness (mgL^{-1}), total ammonia (mgL^{-1}), dissolved oxygen (mgL^{-1}), and carbon-dioxide (mgL^{-1}) concentrations were determined in 12-hour intervals in each glass aquarium by following the methods of American Public Health Association (APHA, 1998) and Rongoei and Outa (2016). Each glass aquaria was supplied with continuous aeration under controlled laboratory conditions at constant controlled water temperature (30°C), pH (7.5), and total hardness (200 mgL^{-1}), respectively. The fish was subjected to 12-hr photoperiod during the whole experimental study.

2.3. Preparation of black pepper leaf extract

Fresh leaves of *Piper nigrum* were collected during the period from October and December 2019 from the Pattoki nursery farms near Sajohwal link road of Tehsil Pattoki, District Kasur in Pakistan. Black pepper leaves were washed with cold water thoroughly and dried in shady places at room temperature 25°C for three to four days until got their constant dry-weight. Thereafter, the dried leaves were crushed by an electrical grinder into fine powder form, and sieved to an average 105 mm of particle size, and stored in a sealed glass jar at 4°C in a refrigerator before their extraction process. About 50 (fifty) grams of shade-dried leaves powder of *Piper nigrum* was soaked sequentially in 500 mL of 80% methanol in a conical flask at room temperature 25°C for 72 hours and filtered from Whatman No. 1 filter paper. Methanol from the filtrate was then completely evaporated in a rotary evaporator (ZZKD RE-5002 rotary evaporator) under reduced pressure at 40°C to get dark-greenish residues as a leaf extract. The remaining residues were weighed and stored in sterile sealed glass bottles refrigerated at 4°C , which later used as feed additives in fish diets to observe its impact on the immune system and growth condition of fish in three glass

aquaria by following the methods of Das et al. (2013) with some modification.

2.4. Fish feeding

Fish were fed with the experimental diets twice per day at the rate of 3% of its total body weight at 9:00 am and 5.00 pm. Isonitrogenous (34% crude protein) and isocaloric (3.00 Kcal g^{-1}) diets were prepared from locally available feed ingredients. The feed formulation with ingredient percentages recorded in Table 1 as given by Khan et al. (2012) and Das et al. (2013) mention below.

2.5. Experimental protocol

After acclimation, healthy fish ($n=25$) size ranged from 21.16 to 23.12g were randomly selected and divided into four equal groups and placed in separate glass aquaria with 70 liters water capacity, with three replicates each. The aquaria were designated as a controlled group (T_0) were growing in the diet as mentioned in Table 1 but devoid of black pepper leaf extract, and three experimental groups (T_1 , T_2 and T_3) that were growing with black pepper leaf extract supplements with above mentioned given diet formulation in Table 1 with 1.0% (T_1), 2.0% (T_2) and 3.0% (T_3), respectively.

2.6. Growth performance

Total body weight (g), fork length (mm), and total lengths (mm) were noted for each fish sample before stocking in experimental trials. Now growth parameters, i.e., the average increase in average total body length and fork length (mm), the average increase in weight gain (WG) in grams, feed conversion ratio (FCR), average daily weight gain (ADG), survival rate (SR), and condition factor (K) recorded at every fortnight of this experimental period were calculated by formula followed by Khan et al. (2012) as follows:

1. Weight gain (WG) (g) = Final weight - Initial weight (g/fish)
2. Feed conversion ratio (FCR) = Total feed / Total weight gain

Table 1. shows the composition of control & experimental diets with different doses of dried leaf extract of black pepper for *Labeo rohita* fingerlings in three experimental trials.

Ingredient used/100 g feed	Control group diet %		Experimental group diets %		
	T_0 (%)	T_1 (%)	T_2 (%)	T_3 (%)	
Fish meal (g)	39.01	39.01	39.01	39.01	
Corn gluten (30% CP*)	40.54	40.54	40.54	40.54	
Cotton seed meal (g)	1.72	1.72	1.72	1.72	
Rice polish (g)	7.17	7.17	7.17	7.17	
Wheat flour (g)	5.0	4.0	3.0	2.0	
sun flower oil (mL)	3.56	3.56	3.56	3.56	
Vitamins-mineral premix (quantity in g/2.5 kg)	3.0	3.0	3.0	3.0	
Dried leaf extract of black pepper (g)	0.0	1.0	2.0	3.0	

*CP = Crude Protein.

3. Condition factor (K) = $W \cdot 100 / TL^3$ Fulton's equation (Fulton, 1902)

Whereas, 'W' weight of fish in grams and 'TL' is the total length of fish in mm.

4. Average daily weight gain (ADG) = $(\text{Final weight} - \text{Initial weight}) / \text{Experimental period (g/fish)}$

5. Survival rate = $(\text{Number of fishes at the end} / \text{Number of fishes initial stocked}) \times 100 (\%)$.

2.7. Proximate analysis of fish

Five fish samples were taken from each experimental trial (T_0 , T_1 , T_2 and T_3) and analyzed for crude proteins, crude lipids, moisture, ash, and mineral contents to determine their nutritional value according to the procedures described in Association of Official Analytical Chemists (AOAC, 2003) and Matiullah et al. (2016), at the end of trial.

2.8. Hematological parameters and Liver Function Tests (LFT)

After completion of feeding trial, fish blood plasma, and serum tests were performed with methods followed by Baba et al. (2016) with some modifications. About five fish samples were randomly selected from each experimental tank and pooled these samples for analysis of TLC (total leukocyte count), neutrophil, lymphocytes, monocytes, eosinophil, platelets, total RBC, hemoglobin, while for liver function test (LFT), total proteins, albumins, and globulins were calculated by methods followed by Shah and Altindag (2004), Rao and Chakrabarti (2005), Hrubec and Smith (2010), and Das et al. (2013).

2.9. Challenge experimental trials (dropsy)

At the end of experiment, each aquarium was introduced with a group of 10 diseased fish (dropsy) out of 25 fish samples per experimental trial for three weeks. During this phase, the feeding strategy with leaf extracts in three experimental trials and one control group has remained the same. Then the immune response of these fishes already fed with and without in terms of diseased fish percentage was determined by methods of Das et al. (2013).

2.10. Microbial load

To calculate the growth of microbial fauna inside (gut) and outside (skin) of fish, two diseased fish samples were collected and sacrificed by killing and sterile with 70% ethanol. Gut and skin samples were taken properly from these fish samples of each group in sanitized containers

for taking their bacteriological samples after disinfection. All bacterial isolates were growing in the form of colonies by using nutrients agar media and 20 g/1L distilled water by using the methods of Aly and Ismail (2016).

2.11. Statistical analysis

The effects of the plant extracts as supplemented feed (0.0%, 1.0%, 2.0% and 3.0%) on growth, hematological parameters, and the physicochemical parameters correlate with growth performance were analyzed by using one-way analysis of variance (ANOVA) and significant differences among treatment means were compared using Duncan's multiple range test (DMRT) using SAS version 9.1. Significance was tested at 5% level ($p \leq 0.05$).

3. Results

The obtained results of experimental trials can be classified as; physicochemical properties of water in each experimental tank, growth performances and its correlation with these water quality parameters, and the impact of different concentrations (0.0%, 1.0%, 2.0% and 3.0%) of dietary supplement black pepper leaf extracts on immunology, hematology and proximate composition of fish fillets raised in each experimental trial as mentioned below.

3.1. Physicochemical characters of water quality parameters

During the experimental trial, the mean value of water temperature, pH, total hardness, total ammonia, dissolved oxygen, electrical conductivity, calcium and magnesium in all experimental groups (T_0 , T_1 , T_2 and T_3) were recorded as 29.99 ± 0.08 °C, 7.07 ± 0.02 , 199.99 ± 0.47 mgL⁻¹, 0.54 ± 0.15 mgL⁻¹, 6.57 ± 0.22 mgL⁻¹, 2.68 ± 0.16 mScm⁻¹, 15.13 ± 0.22 mgL⁻¹ and 40.25 ± 0.05 mgL⁻¹, respectively, as shown in Table 2.

3.2. Growth performance

The data regarding the growth performance recorded every fortnight and presented in Table 3. It showed the significant differences for all parameters among four experimental groups (T_0 , T_1 , T_2 and T_3). The maximum weight gain, fork, and total length of fish were observed in T_2 as compared to the control group (T_0). The maximum feed intake was observed in T_1 and the Feed conversion ratio

Table 2. Physico-chemical parameters recorded during experimental period.

Treatment	Temperature (°C)	pH	Total Hardness (mgL ⁻¹)	Total Ammonia (mgL ⁻¹)	Dissolved Oxygen (DO) (mgL ⁻¹)	Electrical Conductivity (EC) (mScm ⁻¹)	Calcium (Ca) (mgL ⁻¹)	Magnesium (Mg) (mgL ⁻¹)
T_0 (0%)	$29.90 \pm 0.23a$	$7.03 \pm 0.11a$	$199.61 \pm 0.97a$	$0.46 \pm 0.13a$	$6.28 \pm 0.14a$	$2.54 \pm 0.12a$	$15.10 \pm 0.37a$	$40.26 \pm 0.36a$
T_1 (1%)	$30.10 \pm 0.26a$	$7.03 \pm 0.11a$	$200.65 \pm 1.30a$	$0.43 \pm 0.30a$	$6.79 \pm 0.29a$	$2.92 \pm 0.18a$	$15.33 \pm 0.36a$	$40.18 \pm 0.33a$
T_2 (2%)	$30.01 \pm 0.33a$	$7.07 \pm 0.10a$	$199.67 \pm 1.33a$	$0.51 \pm 0.18a$	$6.51 \pm 0.24a$	$2.66 \pm 0.08a$	$15.26 \pm 0.41a$	$40.26 \pm 0.40a$
T_3 (3%)	$29.98 \pm 0.22a$	$7.01 \pm 0.12a$	$199.95 \pm 1.29a$	$0.76 \pm 0.25a$	$6.70 \pm 0.38a$	$2.60 \pm 0.10a$	$14.83 \pm 0.57a$	$40.31 \pm 0.52a$
Means \pm SD	29.99 ± 0.08	7.07 ± 0.02	199.99 ± 0.47	0.54 ± 0.15	6.57 ± 0.22	2.68 ± 0.16	15.13 ± 0.22	40.25 ± 0.05

Table 3. Growth Responses of *Labeo rohita* Fed with and without Black Pepper Leaf Extract.

Treatments	Growth Parameters					
	Average Increase in wet weight (g)	Average Increase in Fork Length (mm)	Average Increase in Total Length (mm)	Average Feed intake (g)	Feed Conversion Ratio	Condition Factor (K)
Treatment # 1	47.70 ± 0.06b	38.96 ± 0.11 c	45.50 ± 0.63 b	140.74 ± 1.23 a	2.95 ± 0.01 a	2.87 ± 0.01 a
Treatment # 2	56.11 ± 0.51a	65.06 ± 0.25 a	53.01 ± 0.02 a	93.26 ± 0.98 b	1.66 ± 0.01 d	2.05 ± 0.14 b
Treatment # 3	41.28 ± 0.35c	25.92 ± 0.61 d	25.67 ± 0.40 c	91.63 ± 0.85 b	2.21 ± 0.01 b	2.55 ± 0.03 a
Control fish	38.24 ± 0.33c	56.68 ± 0.42 b	47.16 ± 0.55 b	67.79 ± 0.65 c	1.77 ± 0.01c	2.22 ± 0.04 b

Means with same letters in a single column are statistically similar at $p < 0.05$. All values are presented in Means ± SD.

Table 4. Effect of black pepper leaf extract on hematological parameters of *Labeo rohita* during the experimental study.

Parameters	T ₁	T ₂	T ₃	T ₀
Hemoglobin/μL	7.21 ± 0.98	9.85 ± 0.99	11.30 ± 1.20	6.40 ± 0.75
TLC/μL	4.7×10 ⁴ ± 0.42	4.9×10 ⁴ ± 0.44	5.1×10 ⁵ ± 0.59	4.6×10 ⁴ ± 0.42
Total RBC counts/μL	2.33×10 ⁶ ± 0.13	3.00×10 ⁶ ± 0.25	4.10×10 ± 0.31	2.52×10 ⁶ ± 0.14
Platelets/μL	1.53×10 ⁵ ± 0.11	1.68×10 ⁵ ± 0.13	1.69×10 ⁵ ± 0.14	1.52×10 ⁵ ± 0.10
Neutrophil (%)	84.10 ± 5.19	85.0 ± 5.24	86.0 ± 6.41	80.0 ± 4.02
Lymphocytes (%)	5.90 ± 0.78	8.0 ± 0.89	8.0 ± 0.88	6.0 ± 0.82
Monocytes (%)	2.90 ± 0.12	3.70 ± 0.23	3.60 ± 0.20	3.0 ± 0.19
Eosinophil's (%)	2.10 ± 0.02	2.30 ± 0.13	2.40 ± 0.16	3.0 ± 0.20
Total protein (g/L)	6.90 ± 0.86	8.50 ± 0.98	7.30 ± 0.87	6.8 ± 0.82
Albumin (g/L)	4.10 ± 0.62	5.90 ± 0.78	5.10 ± 0.72	4.90 ± 0.69
Globulin (g/L)	2.80 ± 0.01	2.60 ± 0.02	2.20 ± 0.01	1.90 ± 0.02

All values are presented in Means ± SD. T₁ = Treatment # 1, T₂ = Treatment # 2, T₃ = Treatment # 3, T₀ = Control.

Table 5. Effect of black pepper leaf extract on proximate composition of *Labeo rohita* during experimental study. Data expressed as Mean ± SE.

Treatments	Moisture (%)	Dry matter (%)	Fat (%)	Ash (%)	Fiber (%)	Protein (%)	Carbohydrates (%)
T ₀	0.02 ± 0.01d	99.98 ± 2.65a	6.50 ± 0.05c	17.00 ± 1.11a	0.24 ± 0.06b	57.00 ± 3.02b	0.55 ± 0.15c
T ₁	0.35 ± 0.05b	99.65 ± 1.23a	9.17 ± 0.65a	15.90 ± 1.12a	0.37 ± 0.05a	70.18 ± 2.35a	8.83 ± 0.23a
T ₂	0.64 ± 0.06a	99.36 ± 2.25a	8.53 ± 0.25ab	16.21 ± 1.31a	0.20 ± 0.09b	61.25 ± 2.16ab	9.35 ± 0.21a
T ₃	0.17 ± 0.05c	99.82 ± 1.98a	8.59 ± 0.36ab	9.53 ± 0.09b	0.15 ± 0.02c	65.84 ± 1.65ab	4.63 ± 0.32b

All values are presented in Means ± SD.

was recorded better for T₂. Nonsignificant differences were also recorded in condition factor (K) values throughout the experiment. The growth rate was significantly higher in fish fed with black pepper leaf extract in all three treatment trials as compared to the control group.

3.3. Hematological parameters and liver function test

Hematological parameters of fish samples collected from each treatment were calculated for each group as shown in Table 4. TLC (Total leukocyte count), RBCs (Red blood cells) and lymphocytes were observed higher in all the experimental treatments (T₁, T₂ and T₃) than in the control group (T₀). Total proteins were observed at 8.50 g/L highest value in T₂. Albumin was observed higher in T₂, while globulin was recorded higher in T₁.

3.4. Proximate composition of *Labeo rohita*

For analysis of the proximate composition of *Labeo rohita*, the values of parameters viz. crude protein, crude fat, moisture, dry matter, ash, carbohydrates, and crude fiber were calculated and presented in Table 5. The maximum protein content was observed in T₂ and minimum protein content was observed in control (T₀).

3.5. Microbial analysis

The highest value was recorded of microbial flora both in gut and skin was higher in the control group with average values 1.08×10⁴ and 0.73×10⁴, respectively, while the same lowest values were recorded in T₂ as 0.12×10⁴ and 0.20×10⁴, shown in Table 6.

Table 6. Effect of black pepper leaf extracts on disease resistance of *Labeo rohita* in all treatments by counting the bacterial isolates from digestive tract and skin samples.

Treatments	Digestive tract/gut (cfu/mL)	Skin (cfu/mL)
T ₀	1.08 x 10 ⁴	0.73 x 10 ⁴
T ₁	0.55 x 10 ⁴	0.63 x 10 ⁴
T ₂	0.12 x 10 ⁴	0.20 x 10 ⁴
T ₃	0.08 x 10 ⁴	0.05 x 10 ⁴

All values are presented in Means \pm SD.

Table 7. Diseased Fish Percentage during Challenge Trials.

Weeks	Diseased fish Percentage (%)			
	T ₀	T ₁	T ₂	T ₃
Week-1	22.22 \pm 3.84	---	---	---
Week-2	59.99 \pm 6.66	---	---	---
Week-3	100.00 \pm 0.00	17.78 \pm 3.85	15.55 \pm 3.85	---
Week-4	100.00 \pm 0.00	28.88 \pm 3.85	15.55 \pm 3.85	13.33 \pm 0.00

All values are presented in Means \pm SD.

3.6. Challenge trials

After 12 weeks of the growth phase, each experimental trial was exposed to diseased fish to check immune response of fish. Spreading percentage of dropsy in experimental fish in T₁, T₂, T₃, and T₀ was recorded as follows, 28.88 \pm 3.85%, 15.85 \pm 3.85%, 13.33 \pm 0.00%, and 100.00 \pm 0.00% as shown in Table 7, which reveals that black pepper leaf extract can enhance the immune system of fish in three experimental groups (T₁, T₂, T₃) against specific diseases than in control group. All fish in control group were got infected however, less disease was spreaded in treatment groups.

4. Discussion

Plants extracts used as immune stimulant as well as for growth promoting effects in aquaculture has received great attention in the last many years. It has been shown that plants extracts are capable to enhance immune responses and reduce mortality due to different pathogenic agents also promoted the growth responses of the fish (Citarasu, 2010). Matiullah et al. (2016) reported the effect of black pepper seeds (BPS) on growth performance in *L. rohita*, with different levels (0.0, 0.5, 1.0, and 2.0% and concluded that the fish fed with 0.5% (BPS) exhibited significantly higher ($P < 0.05$), % weight gain. The results matched to the present findings in a way that inclusion of black pepper leaf extract in diets improve significant growth during the first phase of the trials experimental fish fed with plants extract and control group have significant differences in weight gain showed that black pepper leaf extract is beneficial for fish growth. Growth was enhanced significantly higher in T₂ fed with 2% black pepper leaf extract supplemented diet, wet weight (g) and total length (mm) accordingly, 56.11 \pm 0.51 and 65.06 \pm 0.25 during 12 weeks of experimental trials

as compared to Govind et al. (2012) used black pepper seed as additive feed with 0.5%, 1.00% and 2.00% while recommended 0.5% supplemented feed for the better growth of *L. rohita*. The present study revealed more better performance of growth parameters, while used black pepper leaf extract as a supplemented feed for the same species. Feed intake was recorded higher 140.74 \pm 1.23 T₁ fed with 1% leaf extract, but its growth was observed less than T₂ which was fed with 2% leaf extract showed that leaf extract enhance the growth of fish in T₂ more than the later. Zheng et al. (2009) had also reported that adding 50 mg/kg synthesized allucin to tilapia feed helped to increase more than 2–3% of its weight gain and survival rates after 45 days of culture. The feed conversion ratio also increased by 11% and the biological appraisal was 12% higher than in the control group which is much more closer to the results recorded in the present research trial.

The proximate composition analysis exposed significantly higher values of proteins, crude fat, fiber and percent carbohydrates due black pepper leaf extract supplementation, while the remaining values of proximate composition like moisture, dry matter and ash were recorded non-significant ($p > 0.05$).

The results regarding microbial flora both in gut contents and skin of the fish cultured under different feeding regimes including 1.00%, 2.00%, 3.00% black pepper leaf extract and control group were observed maximum in control group which was not fed with leaf extract reveal that leaf extract minimize the microbial fauna of the fish body inside and outside as compared to Pratheepa and Sukumaran (2014), who also reported the same response of *Euphorbia hirta* plant extract effect as immunosimulant and had concluded that plant extract helped to remove some pathogenic microbes from kidney and improved blood functions. Microbial fauna indicated the fish health and immunity, higher microbial load indicated less health and immunity as compared to those which have less microbial fauna in their body.

Hematology of fish was recorded promoted by the black pepper leaf extract as supplemented feed which is almost similar to Javed et al. (2016) who reported the effect of different schedules of administration of water based infusion of Aloe vera gel, *Allium sativum*, *Berberis lycium*, *Silybum marianum*, *Cara mcopticum* and *Trigonella foenumgreacum* on hematology of broiler chicks. It was concluded that mixed infusion of the mentioned plants at the rate of 20 mL/L of drinking water on daily basis could be effectively utilized to improve hemoglobin and packed cell volume in broiler chicks which shows similarity to the hematological concentration in *L. rohita* in which total leukocytes (TLC), total RBCs and lymphocytes were observed significantly higher in T₃ and T₂, while T₁ showed better results than control group with the comparison to Pratheepa and Sukumaran (2014) who reported positive response of *Euphorbia hirta* plant extract effect as immunosimulant and he also concluded that plant extract helped to remove some pathogenic microbes from kidney and improved blood functions.

Total protein, albumin and globulin were recorded highly significant in black pepper leaf extract given treatments respectively in T₂, T₃ and T₁ in contrast to Haghghi (2013)

concluded Aloe vera effect on the specific and non specific immunity of rainbow trout which showed higher significant values of total protein, albumins and globulins in those fish which were fed with aloevera than the control group he kept.

All the physicochemical parameters of water were described in results showed that all mean values were non-significant among all treatments which reveal that fish present in all treatments have same water condition due to which the comparison of growth and health of fish were recorded in best way.

Fish was challenged against dropsy disease by adding diseased fish to the aquarium, the results indicated the percentage of the spreading of dropsy was much lower in the fish fed with the black pepper leaf extract which reveals that black pepper leaf improve the immune system of which shows similarity to the report of Das et al. (2013) observed the efficiency of water extract of *Ocimum sanctum* leaf in immune response on fingerlings of rohu against the *Aeromonas hydrophila* infection by using as supplemented feed with different concentration and then fish was challenged against *A. hydrophila* after 42 days and 18 days post infectious mortalities were recorded. The contents were enhanced in treatment groups than control group. At the concentration of 2% higher relative percentage of survival against *A. hydrophila* infection than control which shows that leaf extract enhance the immunity and makes *labeo rohita* more resistant to infectious bacteria *A. hydrophila*.

5. Conclusion

It is concluded that black pepper leaf extract supplemented diet improves the growth and immune responses in fish. The total protein content is also increased which shows increase protection against bacterial disease in challenge trial after three months dietary inclusion. It seems beneficial to use black pepper leaf extract supplemented diets to improve growth rate and immunity of fish to improve aquaculture production however more extensive field trials and economic studies are recommended before large scale and commercial use.

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