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Effects of *Arnebia benthamii* Extract on Growth Performance, Immunological Parameters and Disease Resistance against *Flexibacter columnare* in Spotted Snakehead *Channa punctata* (Bloch)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Herbal immunostimulants are known to improve disease resistance in fish by enhancing specific and non-specific defensive mechanisms. The present study was conducted to evaluate the effects of *Arnebia benthamii* extract (AE) on growth rate, immune status, and disease resistance towards

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Flexibacter columnare in spotted snakehead *Channa punctata*. Each experimental group of healthy *C. punctata* was fed on one of the four experimental diets viz. basal diet (without any AE supplementation), AE_{10} (supplemented with 10 mg AE/kg basal diet), AE_{20} (supplemented with 20 mg AE/kg basal diet) and AE_{40} (supplemented with 40 mg AE/kg basal diet) for 42 days. When the concentration of *Arnebia benthamii* was raised from 0 to 40 mg/kg, the weight gain (WG) and specific growth rate (SGR) were considerably enhanced. The lysozyme activity, C3, C4, and serum globulin levels were also significantly increased in all *Arnebia benthamii* supplemented groups. IgM concentration in fish fed on AE_{40} diet was significantly higher than in fish fed on basal diet. All *A. benthamii* treatment groups exhibited significantly higher survival rates after being challenged with *F. columnare*. These findings showed that supplementing diet of *C. punctata* with *A. benthamii* improved growth, immunological response, and disease resistance against *F. columnare*, with the greatest benefits in fish fed on AE_{40} diet for 42 days.

Keywords: Arnebia benthamii; specific growth rate; Flexibacter columnare; Channa punctata; immune response.

1. INTRODUCTION

The spotted snakehead, *Channa punctata* (Bloch) is a freshwater fish distributed to South-eastern countries of Asia. It is an air breathing and hardy fish [1]. These fish can live for up to four days out of the water if they are moist and have been observed wiggling up to 400 meters over wet ground to reach other bodies of water. They have two air-chambers (supra-branchial cavities) that develop from the roof of the bucco-pharynx and are lined with vascular epithelium. They take in air and operate in the same way as lungs. Snakeheads eat planktons, aquatic insects, and mollusks in their early stages of life, but become predatory and cannibalistic as they mature [2]. Snakehead meat has a pleasant flavor and is considered to be abundant in nutrients. It has excellent therapeutic value and contains all the necessary amino acids for wound healing, including glycine, which is required for the synthesis of human skin collagen [3]. *Channa punctata* is generally raised inlands and along with other inland fishes serves as the primary source of proteins, essential fats, and minerals for hundreds of thousands of people [4].

Fish diseases have long posed a major threat to productivity of long-term aquaculture thereby impacting the socioeconomic condition of fishermen in countries such as India. Infections in fishes can aggravate due to various conditions including stress, low physicochemical and microbiological quality of culture water, poor nutritional status, and excessive stocking density. Acute amounts of pollutants and suspended particles in seed fish and adults can also cause deformities and death [5]. Different opportunistic bacterial infections and parasites can take a steep toll on the fish business owing to high morbidity and mortality, reduced growth, and increased chemical expenditure as preventative and control measures [6]. Antibiotics are commonly employed in intensive aquaculture to reduce the spread of these bacteria. However, long-term antibiotic usage can have several negative consequences like increased antibiotic resistance in bacteria and presence of antibiotic residues in the environment as well as fish products [7]. Vaccines, although promising are costly and only effective against select infections [8]. Consequently, there is a need to search for alternative practices that have the potential to reduce the aforementioned adverse effects on fish farming.

Immunostimulants hold great significance in fish disease management because they provide a cost-effective alternative to the medicines and antibiotics [9]. These are dietary additives that have shown the ability to develop a non-specific immune response and increase resistance to pathogenic infection. Fish, like other vertebrates, respond to pathogens in both specific as well as nonspecific ways [10,11]. Herbal immunostimulants can improve disease resistance in fish by enhancing these specific and non-specific defensive mechanisms as they improve the fish's immunological health by increasing the number of phagocytes, increasing lysozyme secretion and complement activity, and enhancing immunoglobulin levels [12]. Herbal remedies are important in disease prevention because of their antioxidant and antibacterial properties [13]. Earlier research has found that a combination of herbs improved the nonspecific immunity in fish and shrimp [14]. Herbal extracts also improve fish survival and reproduction by boosting immunity. They are biodegradable and easy to prepare with a simple process [15]. In the

commercial aquaculture industry, herbs are used as nutrient-supplements and growth promoters [16]. Immunostimulants can be given as an injection, submerged in, or taken orally. However, incorporating immunostimulants into the diet orally appears to be the most logical and successful method because it causes the least amount of stress [16].

Arnebia benthamii has been reported to possess antibacterial, antifungal, antioxidant, cytotoxic, anti-inflammatory, and wound-healing activities [17]. In traditional medicine, this plant is used to treat fever and a variety of tongue, throat and heart issues while its roots also show anthelmintic, antipyretic, and antiseptic properties [17]. Phytochemical analysis shows that *A. benthamii* contains various phytochemical constituents such as deoxyshikonin, acetylshikonin, naphthoquinones, stigmasterol, arnebinone, and arnebin-7 [18].

There seems to be no reported study on physiological impact of *Arnebia benthamii* extract on fish to the best of our knowledge. As a result, the current study was conducted to explore the effects of *Arnebia benthamii* extract (AE) on growth parameters, immunological parameters, antioxidant status, and disease resistance against *Flexibacter columnare* in *C. punctata*.

The aquaculture industry faces significant challenges in maintaining optimal fish health and growth rates. Dietary supplements have emerged as a crucial strategy to address these issues [19]. Herbal immunostimulants, in particular, have shown promise in enhancing fish immunity and disease resistance [20]. However, the search for effective and sustainable supplements continues. Recent studies have highlighted the immunomodulatory properties of *Arnebia benthamii*, a plant-based compound [21]. Yet, its effects on fish growth and immunity remain unexplored.

While existing research has investigated various herbal immunostimulants, the potential applications of *Arnebia benthamii* in aquaculture remain understudied. This knowledge gap necessitates further investigation into its efficacy as a dietary supplement. Considering the significant gaps in research vis-à-vis effects of *Arnebia benthamii* extract on commercially important fish species, the current study was conducted to evaluate the effects of *Arnebia benthamii* extract on growth parameters, immunomodulatory response and disease resistance against *Flexibacter columnare* in *C. punctata*.

2. MATERIALS AND METHODS

2.1 Experimental Fish Specimens, Collection, Transport, and Acclimatization

This study employed a completely randomized design (CRD) with four treatments and three replicates each. Healthy specimens of spotted snakehead, *C. punctata* with about 50 g of average weight were collected from Amravati, Maharashtra, India (20° 56′ 14.73″ N 77° 46′ 46.38″ E). The fish were cleaned in 1% $KMnO_4$ for nearly 3 minutes and acclimatized in 400 L tanks filled with dechlorinated water for 4 days before trials. During the period of acclimatization, the tank was fully aerated using electric pump with air stone diffusers. They were fed with only basal diet two times a day at the rate of 3-4% of their body weight during this period. Basic physicochemical parameters of water were also monitored.

2.2 Preparation of Plant Extracts

A. benthamii was collected at higher elevations in Gulmarg, Jammu and Kashmir Union Territory (34.05° 06′ 3.73″ N 74.38° 22′ 60.18″ E), India. The roots and the remainder of the plant were dried separately in the shade and then crushed into a fine powder with a grinder. The powder was soaked in 100% ethanol overnight with powder to ethanol ratio of 1:5. The entire mixture was then placed in a Soxhlet extractor for 24 h at the boiling temperature of the solvent (78.3°C), and the aqueous mixture extracted was filtered through Whatman's filter paper (No.1). Using a Rotary evaporator, the filtrate collected was concentrated under vacuum at 40°C. The deposit attained was stockpiled in deep freezer at -70°C till further use.

2.3 Preparation of Experimental Diet

Basal diet (100 g) was prepared by mixing wheat flour (60 g), fish meal (35 g), chelated mineral mix (Vedmantra Aqua Grow) (3 g) and Cod liver oil (2 mL) (Table 1). Four different experimental diets were prepared with differing concentrations of plant extract: basal diet (without any AE supplementation), AE_{10} (supplemented with 10 mg AE/kg basal diet), AE_{20} (supplemented with 20 mg AE/kg basal diet) and AE_{40} (supplemented with 40 mg AE/kg basal diet). The extracts were

carefully homogenized with a little quantity of water before being formed into pellets with a hand pelletizer [22]. The pellets were then dried for 24 h and kept in an airtight container at -4°C for future usage.

Table 1. Composition of basal diet

Sr. No.	Ingredient	Amount
1	Wheat flour	60 g
2	Fish meal	35 g
3	Chelated Mineral Mix	3 g
4	Cod liver oil	2 mL

2.4 Feeding Trial

After acclimatization, fish were randomly selected and separated into four experimental sets of 20 fish each, with three replicates of each group in fiberglass tanks containing 100 L of water for each experimental diet. These tanks were stocked with chlorine-free water and electric pumps were installed for aeration. During culture period animals were fed one of the four experimental diets viz. basal diet (without any AE supplementation), AE_{10} (basal diet supplemented with 10 mg AE/kg basal diet), AE_{20} (basal diet supplemented with 20 mg AE/kg basal diet) and AE_{40} (basal diet supplemented with 40 mg AE/kg basal diet) for 42 days. All physicochemical parameters were also maintained during the whole experimentation period.

2.5 Growth Performance

Before weighing and sampling, all fish were fasted for 24 hours, and the following parameters were assessed at the conclusion of the feeding trial (42 days):

Weight Gain (WG) = FW − IW
FW: represents the Final Weight in g
IW: represents the Initial Weight in g

Specific growth rate (SGR) was calculated as per the formula [23].

$$SGR = (Ln\ FW - Ln\ IW) \times 100/T$$

T is the experimental period in days.

2.6 Sample Collection

The samples were taken from the 24-hour-starved fish. Three fish were randomly taken from every replica tank and sedated with MS-222 (Tricaine methanesulfonate) at a dosage of 100-120 mg/L of tank water for sample collection. A hypodermic syringe was used to draw blood from the caudal vein. Non-EDTA tubes were used to conduct the tests.

2.7 Preparation of Bacterial Inoculum

In this investigation, a virulent strain of *F. columnare* was isolated from infected fish *C. punctata*. The pathogen was cultured for 24 h at 25°C in 100 mL of tryptone soya broth (HiMedia) and the culture fluid was centrifuged for 10 min at 4°C at 8000 rpm. The supernatant was then collected, and sedimentary bacteria were diluted in a 0.85% NaCl solution. The bacterial population was expressed as number of cfu/mL. 10^5 cfu/mL concentration was used for intraperitoneal injection during the present study.

2.8 Determination of Dose (LD_{50})

Four experimental tanks in duplicate were prepared prior to experimentation. Ten fish were placed in each tank and kept without food for 24 h.

Fish were given 200 µL of bacterial inoculum with the following concentrations: 1×10^8 cfu/mL, 1×10^6 cfu/mL, 1×10^4 cfu/mL, and 0 cfu/mL (control, using 200 µL of NaCl 0.85%) intraperitoneally. The percentage of mortality was calculated after 24 hours, 48 hours, 72 hours, and 96 hours by Abbott's formula [24]. Probit analysis was used to establish the LD_{50} at 96 h.

2.9 Intraperitoneal Injection

The $LD_{50\text{-}96h}$ *F. columnare* were harvested from the diluted stock culture. Appropriate volume (1 mL/100 g body weight) of the selected serial dilution (10^5 cfu/mL) was injected intraperitoneally, using a tuberculin syringe into the fish. Twenty fishes (60 ±3 g) from each concentration and control group were inoculated with of the chosen dose of the pathogen.

2.10 Disease Resistance

A challenge test was done after 42 days of feeding to see how *Arnebia benthamii* supplemented diets affected disease resistance against *F. columnare.* The fish was considered dead, when there was no respiratory gill movement and no response to gentle prodding [25]. *In this way,* regular mortality was monitored for 10 days [26]. The experimental settings during the bacterial challenge were not altered and no experimental meals were supplied during 10 days of mortality testing. All the fish were fed on a basal diet.

2.11 Cumulative Percentage Mortality and Survival Rates

The cumulative percentage mortality rate was calculated by monitoring the number of fish which perished throughout the investigation and presenting it as a percentage.

Mortality rate (%) = (No. of deaths during experimental period / Total number of fishes) x 100

Survival rate (%) = (No. of fishes surviving during experimental period / Total number of fishes) x100

2.12 Immunological Parameters

2.12.1 Lysozyme activity

By using the Lysozyme Activity Kit (LY0100 Sigma-Aldrich), lysozyme activity was assessed through turbidimetric technique [27]. 30 µL of test sample was mixed with 800 µL of *Micrococcus lysodeikticus* (*luteus*) suspension and initial absorbance at 450 nm was measured. After incubating this solution for 5 min at 25°C absorbance at 450 nm was measured which was then used for calculating lysozyme activity in sample. Lysozyme causes lysis of *Micrococcus lysodeikticus* cells leading to diminution in absorbance.The manufacturer of Lysozyme Activity Kit defines one unit (1 U) of lysozyme activity as the quantity of lysozyme that causes a decrease of 0.001/min. in absorbance value at 450 nm (A_{450}) in a suspension of *Micrococcus lysodeikticus* as substrate, at a pH of 6.24 in a 2.6 mL reaction mixture at 25°C (1 cm light path).

2.12.2 Total serum protein, albumin and globulin measurements

The concentration of total serum protein was measured using the Biuret technique and the Delta Total Protein kit (DL2301) while the concentration of serum albumin was measured using Bromocresol green end point assay (84LS100-60). Amount of albumin was subtracted from total protein to get the total globulin protein fraction.

2.12.3 Serum complement C3 level

The Complement C3 Assay Kit, GB670M (Weldon Biotech India Pvt. Ltd.) was used to assess the serum complement C3 level using the immunoturbidimetric approach. The antigen-antibody reaction was carried out by mixing the samples with the antibodies provided in the kit, which resulted in a change in the sample's absorbance, which was measured at 340 nm. C3 levels were measured in mg/L.

2.12.4 Serum complement C4 level

The test is based on reaction between antigen and antibody. The reaction generates an insoluble complex causing increase in turbidity which is then measured spectrophotometrically at 340 nm. The quantity of complex generated in the sample is proportional to the amount of C4. The Assay Kit for Complement C4, GB680M (Weldon Biotech India Pvt. Ltd.) was used for determination of serum complement C4 level.

2.12.5 Serum Immunoglobulin M (IgM)

This test is based on the interaction between an IgM antigen and an anti-IgM antibody. Turbidity is measured spectrophotometrically at 340 nm as an insoluble compound produced by this reaction. The amount of complex produced in the sample is proportional to the amount of IgM in the sample. Immunoglobulin M Assay Kit (GS661M) manufactured by Weldon Biotech India Pvt. Ltd was used for the measurement.

2.12.6 Estimation of total immunoglobulin in plasma

The overall immunoglobulin in plasma was estimated using Siwicki and Anderson modification of the Lowry method [28]. This method uses polyethylene glycol for precipitating the immunoglobulin out of the plasma. Blood samples were first centrifuged at 1000 g for 5 min to separate the plasma. 0.1 mL of plasma was placed in a polypropylene serum vial and mixed with 0.1 mL of 12% polyethylene glycol suspended in deionized water. The incubation period was 2 h at room temperature with constant mixing. After centrifugation for 10 min at 5000 g, the supernatant was removed and the protein content was determined. This value was subtracted from the total serum protein concentration to arrive at the amount of immunoglobulin present in the plasma.

2.13 Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY). Results are presented as mean ± standard error (SE)

with a sample size of 5. Tukey's test, One-way analysis of variance (ANOVA) was used to examine the data. A significance level of $P \leq .05$ was set to determine statistical significance.

3. RESULTS AND DISCUSSION

3.1 Growth Performance

The growth parameters of the *C. punctata* in all four groups are presented in Table 2. With the increase of supplementation of *Arnebia* extract from 0 mg/kg (basal diet) to 40 mg/kg (AE_{40}), weight gain (WG) and specific growth rate (SGR) were considerably enhanced ($P \leq .05$) (Table 2; Fig. 1).

3.2 Immune Response

The impact of the AE supplementation on certain immunological markers is presented in Table 3. As compared to the control group (fed on basal diet), all immunological parameters under study viz. IgM, serum globulins, lysozyme activity, C3 and C4 concentrations increased significantly ($P \leq .05$) in all treatment groups.

IgM content in AE_{40} group exhibited a significant increase ($P \leq .05$) in comparison to basal diet, AE_{10} and AE_{20} experimental groups. On the other hand, serum IgM content did not change significantly among fish fed with AE_{10} and AE_{20} diets as compared to fish fed on basal diet. All supplemented groups had significantly higher serum C4 concentrations than the control group. However, the increase in C4 concentration of AE_{40} was not significantly higher than those in AE_{10} and AE_{20} fed groups ($P \geq .05$).

3.3 Determination of LD_{50}

After 96 hours, the mortality percentage of *C. punctata* were calculated accordingly. The findings were calculated using Probit analysis for *F. columnare* to obtain $LD_{50\text{-}96h}$ value which was found to be 2.1×10^4 cfu/mL.

Table 2. Growth performance of *C. punctata* fed on different experimental diets

Diet Type	IW (gm)	FW (gm)	WG (gm)	SGR
Basal Diet	50.67 ± 0.8	157.20 ± 0.4	310.24 ± 6.4	2.70 ± 0.1
AE_{10}	51.23 ± 0.4	180.23 ± 0.8	351.80 ± 7.1	2.99 ± 0.2
AE_{20}	50.80 ± 0.4	209.21 ± 0.7	411.83 ± 5.2	3.37 ± 0.2
AE_{40}	49.93 ± 0.5	218.17 ± 0.9	436.95 ± 9.1	3.51 ± 0.1

IW- Initial weight; FW- Final weight; WG- Weight gain; SGR- Specific growth rate

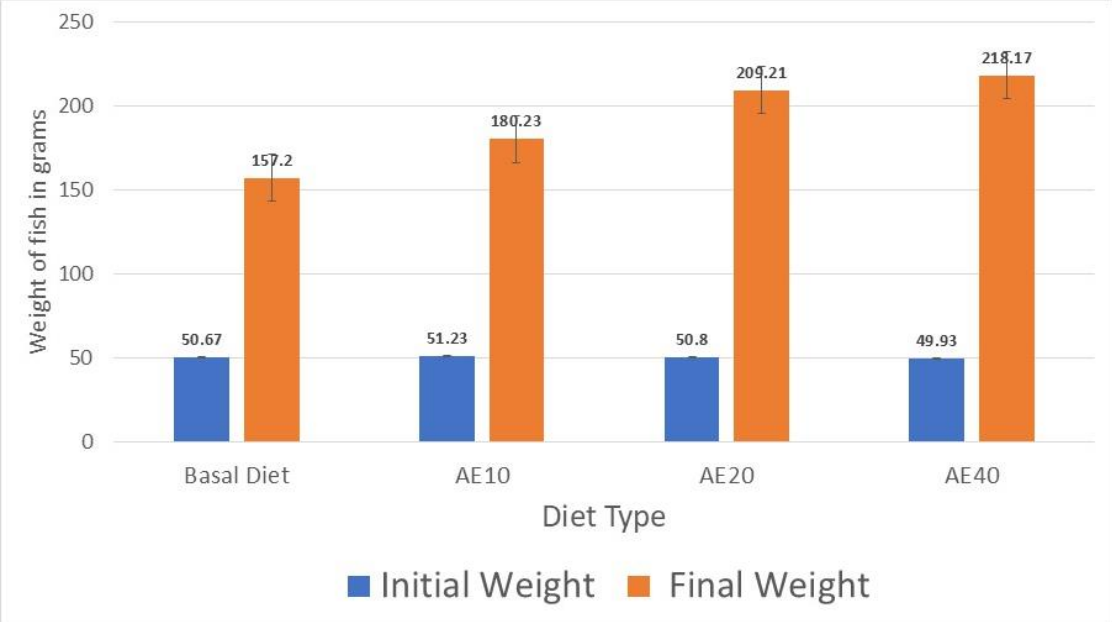

Fig. 1. Effect of AE supplementation on growth of *C. punctata*

Table 3. Immune response of *C. punctata* in different experimental groups

Immune Response Index	Unit	Exposure Time	Basal Diet	AE_{10}	AE_{20}	AE_{40}
Lysozyme Activity	mg/ml	7 days	60.23 ± 0.75^{a}	65.51 ± 0.57^{a}	68.30 ± 0.71^{a}	75.17 ± 0.40^{a}
		21 days	64.56 ± 0.74^{b}	69.50 ± 0.65^{b}	70.33 ± 0.93^{a}	79.72 ± 0.64^{b}
		42 days	63.17 ± 0.63^{b}	72.42 ± 0.53^{c}	76.10 ± 0.86^{b}	82.32 ± 0.52^{c}
Ig M	mg/dl	7 days	0.78 ± 0.07^{a}	0.89 ± 0.01^{a}	0.93 ± 0.01^{a}	0.99 ± 0.01^{a}
		21 days	0.82 ± 0.08^{b}	0.94 ± 0.07^{b}	1.04 ± 0.01^{b}	1.30 ± 0.05^{b}
		42 days	0.81 ± 0.08^{a}	1.00 ± 0.08^{c}	1.11 ± 0.01^{c}	1.45 ± 0.01^{c}
C3	mg/l	7 days	45.22 ± 0.53^{a}	50.48 ± 0.42^{a}	69.35 ± 0.49^{a}	68.38 ± 0.26^{a}
		21 days	45.52 ± 0.46^{a}	54.56 ± 0.44^{b}	74.55 ± 0.48^{b}	69.24 ± 0.88^{a}
		42 days	44.5 ± 0.54^{a}	59.89 ± 0.54^{c}	70.46 ± 0.52^{a}	74.89 ± 0.6^{b}
C4	mg/l	7 days	30.53 ± 0.74^{a}	37.62 ± 0.85^{a}	38.26 ± 0.43^{a}	39.40 ± 0.53^{a}
		21 days	32.42 ± 1.01^{a}	39.7 ± 0.93^{ab}	40.60 ± 0.37^{b}	45.38 ± 1.04^{b}
		42 days	31.19 ± 0.83^{a}	42.25 ± 1.0^{b}	42.17 ± 0.61^{b}	45.51 ± 0.61^{b}
Serum globulin	g/dl	7 days	7.44 ± 0.42^{a}	8.25 ± 0.64^{a}	8.05 ± 0.19^{a}	8.25 ± 0.57^{a}
		21 days	8.08 ± 0.32^{ab}	8.32 ± 0.37^{a}	9.36 ± 0.51^{ab}	9.16 ± 0.31^{a}
		42 days	9.40 ± 0.38^{b}	8.52 ± 0.51^{a}	9.88 ± 0.35^{a}	9.87 ± 0.44^{a}

The data (5 replicates) represent the mean value ± SEM (standard error mean) and were found to be statistically operative and significant according to Tukey's test at P ≤ .05. Mean ± SEM followed by the different letters within each column are significantly different according to Tukey's test (one-way ANOVA) at P ≤ .05

3.4 Disease Resistance

Challenge test with LD_{50} of *F. columnare* was conducted after 42 day feeding trial for a period of 10 days. The results showed that compared to the control group where survival percentage is only 15% in *F. columnare* infection, the survival rate in AE supplemented diet groups were significantly higher ($P < .05$) (Table 4).

Many therapeutic plants and their derivatives have been widely researched in recent years [29]. *A. benthamii* has been found to be effective in treating cardiac issues and has anti-pyretic properties [30]. The herb has also been used for centuries to heal ailments of the tongue and throat [31]. Secondary metabolites produced from this genus include Arnebin 1 and Arnebin 3, which have been demonstrated to have anti-cancer effects [32]. Hydroxy-isovalerylshikonin, acetylshikonin and deoxyshikonin, as well as arnebinone, arnebin-7, stigmasterol, and naphthoquinones have all been isolated from *Arnebia* species. Its roots are used in dressing of wounds as antibacterial and antiseptic agent [33]. Investigations on the antioxidant activity and DNA protection properties from the ethyl acetate extract of roots of *A. benthamii* showed that the shikonin contained in the root extract was efficient in scavenging hydroxyl radicals thereby protecting the DNA. These studies have also shown that complete plants of *A. benthamii* have cytotoxic and antioxidant capabilities [17]. Antifungal, antibacterial, and anti-inflammatory effects make up the majority of the uses of the commercial medications prepared from *A. benthamii* [31]. Plant-extracts also show impact on growth performance. Studies on supplementing the diet of *Oreochromis niloticus* with ginseng extract, tribulus extract, and date palm pollen grains, have shown improvement in growth performance [24].

In this study, the growth parameters (WG and SGR) in all dosages of AE-treated groups were significantly enhanced. In addition to this, it was also found that the AE supplementation also increased the survival rate of *C. punctata.*

The mechanisms underlying the growth-promoting and immunomodulatory effects of *A. benthamii* extract may involve antioxidant activity exhibited by Shikonin and other bioactive compounds that scavenge free radicals thereby reducing oxidative stress and promoting cellular health [17]. These bioactive compounds may stimulate the innate immune system, enhancing the production of immune molecules [34].

The innate immune system protects the health of fish by acting as a first line of defense against harmful bacteria [26]. Lysozyme is a nonspecific immunologic component present in fish serum, mucus, and eggs that activates complement system and phagocytes in addition to lysing pathogens [35]. IgM is a critical biomarker for determining the health and humoral immune status of fish [36]. Another essential innate immunological characteristic for fish is complement activity, which may efficiently eliminate harmful bacteria and stimulate inflammatory responses [37]. Complement activity has been considered as a suitable indication of fish immunocompetence since complement is down-regulated in certain stress conditions. Oral immunostimulants are widely acknowledged as having the potential to improve this function [38].

Table 4. Effect of AE supplementation on disease resistance of *C. punctata* to *Flexibacter columnare*

Exposure Time	Fish Deaths in Each Diet Type			
	Basal Diet	**AE_{10}**	**AE_{20}**	**AE_{40}**
2 days	6 ± 0.04	4 ± 0.08	3 ± 0.01	2 ± 0.20
4 days	4 ± 0.08	2 ± 0.06	1 ± 0.20	1 ± 0.04
6 days	4 ± 0.20	2 ± 0.05	2 ± 0.04	0
8 days	2 ± 0.06	1 ± 0.20	1 ± 0.06	1 ± 0.2
10 days	1 ± 0.04	0	0	0

Table 5. Mortality rate of *C. punctata* after 10 days of exposure to *Flexibacter columnare*

Parameter	Basal Diet	AE_{10}	AE_{20}	AE_{40}
Mortality rate after 10 days of exposure time	85%	45%	35%	20%
Survival rate after 10 days of exposure time	15%	55%	65%	80%

The findings of this study demonstrate that the levels of IgM, lysozyme, and serum globulins are considerably higher in all AE-treated experimental groups as compared to the control group. Several plant extracts have also been shown to enhance levels of lysozyme and IgM in fish serum, including soybean isoflavones [39] and Eryngii mushroom polysaccharides [40]. In comparison to the control group, all *A. benthamii*-supplemented experimental groups demonstrated a propensity to increase in serum C3 and C4 concentrations. These findings corroborate the findings of Zhou et al. [39] which show that diets supplemented with herbal extracts like soybean isoflavones tend to raise C4 concentration. Complement activation that lasts a short time is typically helpful to fish [41]. Therefore, improved complement levels in the *A. benthamii* experimental sets may contribute to the sturdiness exhibited by the fish to *F. columnare* infection. Thus, the elevated levels of C3, C4, IgM, and lysozyme in the *A. benthamii* supplemented experimental groups point to a boost in immunological response leading to better protection against infection.

4. CONCLUSION

Thus, during this study, *A. benthamii* extract was found to significantly improve *C. punctata* survival rate, when challenged with *F. columnare. A. benthamii* administration may also bring about modulation of immune response, thereby contributing to the enhancement of disease resistance in *C. punctata.*

The findings of this study suggest that *A. benthamii* extract may be a valuable supplement in aquaculture diets, enhancing growth performance and disease resistance. Herbal immunostimulants like *A. benthamii* extract offer a sustainable and eco-friendly alternative to synthetic additives. However, this study needs to be strengthened further by employing a larger sample size to yield more robust results. Efforts are ongoing to increase the study duration for more comprehensive assessment of growth performance and immunological response.

Further research of our group intends to investigate the optimal dosage and duration of *A. benthamii* extract supplementation and explore the effects of *A. benthamii* extract on other aquatic species. Further studies need to be carried out to elucidate the molecular mechanisms underlying the immunomodulatory effects of *A. benthamii* extract.

The current study is the first to show that a diet supplemented with *A. benthamii* extract boosted growth, immunity, and disease resistance of fish *C. punctata.* Furthermore, supplementing the food with 40 mg/Kg of *A. benthamii* offered remarkable protection against the *F. columnare* challenge. In light of the findings the authors propose that *AE*-supplemented diet has tremendous potential to improve productivity of fish farms.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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