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DIETARY INCLUSION OF GARLIC (*Allium sativum*) EXTRACT ENHANCES GROWTH AND RESISTANCE OF ROHU (*Labeo rohita*) AGAINST MOTILE *Aeromonas* SEPTICAEMIA

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Abstract

Motile *Aeromonas* septicaemia (MAS) caused by *Aeromonas* spp. is one of the major fish diseases that causes substantial losses in the aquaculture industry. The present study was conducted to screen the *in vitro* inhibitory effects of garlic extracts on *Aeromonas veronii* isolated from MAS, evaluate the effects of dietary inclusion of garlic extracts on growth and prevention of MAS in *Labeo rohita*. *In vitro* antibacterial activities of garlic aqueous and organic solvent extracts (ethyl acetate, methanol, ethanol, and acetone) were screened by disc diffusion assay. The minimum inhibitory concentrations (MICs) of ethyl acetate and methanol extracts were determined by using a quantitative bioassay method. Four groups of fish were fed garlic ethyl acetate extract at the rate of 0 (T_1, control), 6.25 (T_2), 12.50 (T_3), and 25.00 (T_4) mg/kg feed with three replications for 90 days. The fish fed with different concentrations of garlic extracts were artificially challenged with the high virulent *A. veronii* strain B55. In this study, ethyl acetate extract of garlic inhibited all the *A. veronii* strains (A22, B7, B9, B19, B27, B36, B55, F143, K743, and L1324) with the maximum inhibition zones. The MICs of ethyl acetate and methanol extracts were obtained 31.25 and 62.5 µg/ml, respectively. The final weight gain of *L. rohita* was obtained 28.21±0.51, 31.33±0.76, 40.05±0.76, and 34.82±0.51 g in the treatments T_1, T_2, T_3, and T_4, respectively. The growth of ethyl acetate extracts-fed fish were significantly ($p < 0.05$) higher compared to the control. The specific growth rate was also found significantly higher in the T_3, T_4, and T_2 relative to control. The fish fed garlic extracts enriched feed at 12.5, and 25 mg/kg developed resistance against MAS, while all the control fish died with expressions of distinct disease symptoms. Therefore, garlic ethyl acetate extracts could be used for growth enhancement and prevention of MAS in *L. rohita.*

Keywords: Garlic extract, growth, disease resistance, virulent *Aeromonas veronii*.

Introduction

Bangladesh is the second aquaculture-producing country in the world (FAO, 2020). The total fish production of the country was 4.38 million metric tons in the year 2018-19 (FAO, 2020). During the last decade, the fish production of the country became almost double due to the rapid expansion and intensification of freshwater aquaculture. However, with the intensification of aquaculture, fish disease has become one of the major impediments in aquaculture

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production of the country. The major fish diseases frequently occurred in the country are epizootic ulcerative syndrome (EUS), motile *Aeromonas* septicaemia, tail and fin rot, bacterial gill rot, dropsy, streptococcal infection, fungal diseases, parasitic diseases, etc. (Chowdhury, 1998). Among these, motile *Aeromonas* septicaemia (MAS) is one of the most important diseases in carp fishes in Bangladesh (Rahman, 2004). The disease is characterized by superficial lesions, haemorrhages, ulcerations, abscesses, exophthalmia, ascetic fluid, and liver and kidney lesions, etc. (Garcia *et al*., 2007). It is caused by different motile species *viz*., *A. hydrophila*, *A. caviae*, *A. sobria,* and *A. veronii* (Hassan *et al*., 2017; Stratev and Odeyemi, 2016; Cai *et al*., 2012; Rahman, 2004). Globally, at least 23 species of commercial and ornamental fish species have been reported to be susceptible to MAS (Jagoda *et al*., 2014).

The overall fish health management practices are very poor in Bangladesh. Most of the fish farmers have little or no knowledge of fish health management to respond effectively to a fish disease problem. They indiscriminately use different antibiotics and synthetic drugs with little or no success at all. However, prolonged and careless use of antibiotics resulted in the development of antibiotic resistance in pathogens and accumulation of residual effects in fish which are a major health concern worldwide (Hannan *et al*., 2019). Medicinal plant extracts possess potential antimicrobial substances that have long been studied as an alternative to commercial drugs (Awad and Awaad, 2017). Garlic (*Allium sativum*) is an important medicinal plant that is reported to promote growth, survival, immune responses, and modulation of the gut microbiome of tilapia (*Oreochromis niloticus*) (Foysal *et al*., 2019), rainbow trout (*Oncorhynchus mykiss*) (Büyükdeveci, *et al*., 2018), African catfish (*Clarias gariepinus*) (Eirna-liza *et al*., 2016), Asian sea bass (*Lates calcarifer*) (Talpur and Ikhwanuddin, 2012). It contributes to control pathogens, especially bacteria and fungi and enhances the health status of fish (Foysal *et al.*, 2019; Rahman *et al*., 2017).

Rohu (*Labeo rohita*) is one of the most important species of Indian major carp which is a very popular commercial fish in Bangladesh, India, Myanmar, Nepal, etc. The fish is frequently reported to be susceptible to motile *Aeromonas* septicaemia in aquaculture systems in our country (Chowdhury, 1998). However, studies on the beneficial effects of garlic extracts on growth and disease resistance in *L. rohita* against MAS caused by *A. veronii* is yet to be reported. This study aimed to find out *in vitro* antimicrobial activity of garlic extracts on *Aeromonas veronii*, and evaluate the effects of dietary inclusion of garlic extracts on growth, and disease prevention efficacy against motile *Aeromonas* septicaemia in *L. rohita*.

Materials and Methods

In vitro inhibitory effects of garlic extracts on *A. veronii*

Fifty grams of garlic bulb was washed with sterile distilled water, cut into small pieces, and pasted by using mortal-pastel. Twenty-five grams of the garlic paste were separately dissolved in 250 ml of different types of solvents *viz.,* sterile distilled water, methanol, ethanol, ethyl acetate, and acetone to make 100mg/ml stock solutions. The extracts

were evaporated at 45°C for 1h in a rotary evaporator. Then the extracts were dissolved in 1ml of respective solvents. Ten microliters of concentrated crude extracts were soaked into sterilized filter paper discs prepared from Whatman filter paper (Sigma-Aldrich, Germany) and evaporated overnight in a Laminar Air Flow Cabinet (Esco, Singapore) to make the final concentration 125 µg/disc. In this study, 10 fish pathogenic *A. veronii* strains (A22, B7, B9, B19, B27, B36, B55, F143, K743, and L1324) preserved in the Laboratory of IBGE were used. Thirty microliters of overnight broth culture (10^5 CFU/ml) of fish pathogenic *A. veronii* strains were separately spread on Mueller Hinton Agar plates using an L-shaped glass rod, then different garlic extract discs were placed on culture plates of individual strains. The plates were incubated at 37°C for 24h in an incubator (Hannan *et al.*, 2019). Inhibitory activity of the crude garlic extracts was evaluated after Rahman *et al*. (2017).

Determination of minimum inhibition concentrations

The minimum inhibition concentrations (MICs) of garlic extracts against a high virulent strain of *A. veronii* (strain B55) were determined by serial two-fold dilution method after Rahman *et al.* (2017). The ethyl acetate and methanol extracts were used in this study as most of the *A. veronii* strains were inhibited by these extracts. Briefly, dilutions of ethyl acetate and methanol extract were adjusted at 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 µg/ml (w/v) and the discs with these dilutions were prepared as described earlier. Three replications were used for each dilution. Thirty microliters of bacterial culture having a concentration of 10^5 CFU/ml were inoculated in each culture plate and the discs were aseptically placed on the culture. The plates were incubated at 37°C for 24 h in an incubator. The growth of bacteria that were decreased in the next dilution was considered as MIC value.

Preparation of garlic extract containing feeds

Commercial feed pellets (Paragon Feed Ltd.) were used in this study. The feed contained approximately 26% protein, 10% carbohydrate, 7% lipid, and 27% ash. A stock of garlic ethyl acetate extract (25 mg/ml) was prepared. Then, ethyl acetate (199 ml) was added to the stock solution to obtain a working solution of 200 ml at 125 µg/ml (4×MIC) concentration. Two and four-fold dilution of the stock solution was mixed with ethyl acetate to obtain 200 ml working solutions of 31.25 (1×MIC), and 62.6 µg/ml (2×MIC) concentrations. Then ethyl acetate extracts (200 ml) were mixed with 1 kg commercial feed, dried at room temperature, and stored in a cool and dry place.

Experimental design for feeding trial

To assess the effects of dietary inclusion of garlic extracts, a total of 120 uniform-sized (15.38±0.20g) fingerlings of *L. rohita* were randomly distributed in 12 plastic water tanks (10 fish in each) of 300L capacity. The tanks were divided into four treatments such as T_1 (control) (0), T_2 (6.25 mg garlic extract/kg feed), T_3 (12.5 mg garlic extract/kg feed), and T_4 (25 mg garlic extract/kg feed) with three replicates following a completely randomized design.

Growth performance of *L. rohita* by dietary garlic ethyl acetate extracts

After acclimatization in the laboratory (10 days), the fish for treatment T_1 were fed with the commercial basal diet (without garlic extract) and treatments T_2, T_3, and T_4 were fed with the garlic ethyl acetate extract containing feed for 90 days. The fish were fed twice a day at a saturation level. Water was exchanged (approximately 75%) every two days interval. Aeration was maintained throughout the experimental period. Water temperature and pH during the experimental period were recorded within the range of 28-30°C and 7.5-8.0, respectively. The growth of *L. rohita* was evaluated in terms of weight gain, and specific growth rate (SGR). Sampling was performed every 15 days interval.

The weight gain was calculated by using the formula:

Total weight gain (g) = Mean final weight-mean initial weight.

The specific growth rate was calculated by using the formula:

$$SGR\ (\%\ bw/day) = \frac{(\ln W_2 - \ln W_1)}{([Duration\ of\ the\ experiment\ (day)])} \times 100$$

Where, W_1= The initial live body weight (g)

W_2= The final live body weight (g)

***In vivo* infection challenge of garlic ethyl acetate extracts treated fish**

To know whether the garlic ethyl acetate extracts are effective in the prevention of motile *Aeromonas* septicemia caused by *A. veronii*, an *in vivo* bioassay was carried out. A high virulent laboratory strain of *A. veronii* (B55) identified by Hossain (2016) from a fish suffering from motile *Aeromonas* septicaemia was used in this study. The bacteria were inoculated into the nutrient broth and incubated at 28°C for 24h in an orbital shaker. Bacterial pellets were harvested by centrifugation at 5000 rpm for 10 minutes. Then the pellets were suspended in a sterile physiological saline solution. Ten fish from each aquarium were collected after the 90 days of feeding (no fish died during the experiment) with garlic ethyl acetate extracts. The fish were anesthetized by using 60mg/L solution of MS-222 (Sigma-Aldrich, USA) (Foysal *et al*., 2019) and 100 µl of culture suspension (10^7 CFU/ml) of *A. veronii* strain B55 was intramuscularly injected at the dorsal side of each fish. The fish were kept in 12 different aquariums (80L) in a confined room at 15-17°C temperature and observed for 21 days. The experiment was conducted at 15-17°C temperature since MAS usually occurs at low temperatures. The fish were supplied with the commercial basal diet at saturation level. During this period, fish were observed for the expression of any external disease symptoms and abnormal behavior. Aeration was maintained throughout the experiment. Around 50% of water from the aquarium was exchanged in two days intervals.

Statistical analysis

All the data during the study period were statistically analyzed using a one-way analysis of variance (ANOVA) to test the significant results ($p < 0.05$) between the means. The standard error (±SE) was calculated to identify the range of means. All statistical analyses were performed with the aid of the computer software Statistix 10.0 version.

Results and Discussion

In vitro inhibitory effects of garlic extracts

Garlic is known as a medicinal panacea that possesses a wide range of antimicrobial activity against bacteria, fungi, protozoa, and viruses (Ankri and Mirelman, 1999). Numerous solvents, including methanol, ethanol, acetone, and water are frequently used for extracting bioactive compounds from different plant materials (Truong *et al*., 2019). In this study, both aqueous and polar extracts of garlic were screened *in vitro* to assess their inhibitory effects on fish pathogenic *A. veronii* strains. Among these, the polar extracts (methanol, ethanol, ethyl acetate, and acetone extracts) inhibited the growth of different fish pathogenic *A. veronii* (Table 1). However, the ethyl acetate extracts inhibited all of the *A. veronii* strain in disk diffusion assay with bactericidal effects. The methanol extract of garlic also inhibited the growth of all strains except A22. The zone of inhibitions for any individual strain was also found higher for the ethyl acetate extracts compared to other solvent extracts of garlic. It might be due to the better solubility of the active compounds of garlic in ethyl acetate than other solvents. Ajanal *et al*. (2012) and Mahdi-Pour *et al.* (2012) stated that the plant materials contain diverse bioactive compounds and their solubility properties differ in different solvents which is consistent with our findings.

Minimum inhibition concentration (MIC) of garlic extracts

The minimum inhibition concentrations (MIC) of ethyl acetate and methanol extracts were determined against a high virulent laboratory strain of *A. veronii* (strain B55) by using a quantitative bioassay method since these extracts exhibited inhibitions against most of the fish pathogenic *A. veronii* strains. Bioassay revealed that the MIC of ethyl acetate and methanol extracts of *A. sativum* were 31.25, and 62.5µg/ml, respectively against the strain (Table 2, Fig. 1). As the ethyl acetate extract showed a lower MIC than methanol extract, the ethyl acetate extract was used for further studies.

Growth performance of *L. rohita* by dietary garlic ethyl acetate extracts

In order to evaluate the effects of ethyl acetate extracts of garlic on the growth of *L. rohita*, the

Table 1. *In vitro* inhibitory activity of different extracts of garlic on different strains of *Aeromonas veronii*

Type of extracts	Inhibition zone (mm)									
	A22	B7	B9	B19	B27	B36	B55	F143	K743	L1324
Aqueous	–	10±0.47	–	–	9±0.47	–	–	–	–	–
Methanol	–	30.67±0.47	17.33±0.51	23.83±0.37	23.67±0.47	18.33±0.47	16±0.0	20.83±0.37	19±0.0	21.67±0.47
Ethanol	–	11.83±0.37	–	–	16.83±3.67	14±0.0	–	–	–	–
Ethyl-acetate	19.33±0.47	28.67±0.47	29.33±0.47	33±0.57	22±0.0	24.17±0.37	37.33±0.47	33.67±0.47	24±0.57	24.33±0.47
Acetone	15.83±0.37	–	–	–	23.17±0.37	–	11.67±0.47	–	–	–

Note: No inhibition zone, data presented as mean±SE (n=3). A22, B7, B9, B19, B27, B36, B55, F143, K743, and L1324: Different strains of *A. veronii*.

Table 2. Minimum inhibition concentration of garlic extracts

Garlic Extracts	Zone of inhibition (in mm) at different concentration of garlic extracts							MIC (µg/ml)
	1000µg/ml	500µg/ml	250µg/ml	125µg/ml	62.5µg/ml	31.25µg/ml	15.625µg/ml	
Ethyl acetate	37.17±0.47	30.00±0.41	28.16±0.24	13.83±0.24	10.17±0.23	7.00±0.00	–	31.25
Methanol	16.50±0.41	14.33±0.24	12.17±0.24	9.00±0.00	7.17±0.24	–	–	62.50

Note: No inhibition zone, data presented as mean±SE (n=3)

fish were fed dietary garlic extracts containing feed. At the end of the 90 days feeding trial, the final weight gain was recorded 28.21±0.51, 31.33±0.76, 40.05±0.76, and 34.82±0.51 g in the treatments T_1, T_2, T_3, and T_4, respectively (Table 3). Growth of all of the treatment group fish fed the ethyl acetate extracts of garlic was found significantly ($p < 0.05$) higher than the control group fish. The highest body weight gain was obtained in fish fed with garlic extracts supplemented feed at the rate of 12.5 mg garlic extract/kg feed, (T_3) at day 90 which was statistically significant ($p < 0.05$) compared to other treatments.

In this study, the specific growth rate of the fish was obtained 0.94±0.02, 1.00±0.01, 1.15±0.01, and 1.06±0.02 % in the treatments T_1, T_2, T_3, and T_4, respectively (Table 3). The specific growth rate of all of the treatment group fish was also found significantly ($p < 0.05$) higher than the untreated control group fish. The highest specific growth rate was obtained in T_3 followed by T_4.

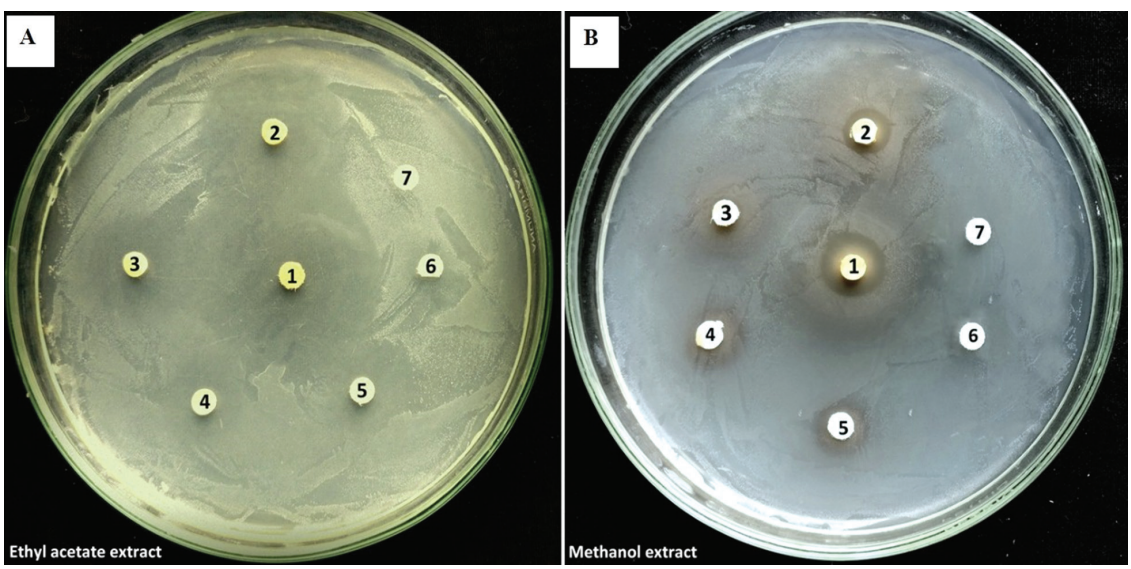

Fig. 1. ***In vitro*** **antibacterial activity of discs containing different concentrations of ethyl acetate (A) and methanol extracts (B) of garlic (*A. sativum*) against fish pathogenic *A. veronii* strain B55. Concentration of extracts at disc 1: 1000 µg/ml; disc 2: 500 µg/ml; disc 3: 250 µg/ml; disc 4: 125 µg/ml; disc 5: 62.5 µg/ml; disc 6: 31.25 µg/ml and disc 7: 15.625 µg/ml.**

Table 3. Growth parameters and mortality rate of rohu (*L. rohita*) fed different concentrations of ethyl acetate extract of garlic supplemented diets.

Parameters	Treatments			
	T_1	T_2	T_3	T_4
Weight gain (g)	28.21±0.51	31.33±0.76	40.05±0.76	34.82±0.51
SGR (%bw/day)	0.94±0.02	1.00±0.01	1.15±0.01	1.06±0.02
Mortality rate (%)	100.00±0.00	23.33±4.71	0.00±0.00	0.00±0.00

Note: control (basal diet), T_2: 6.25 mg extract/kg feed, T_3: 12.5 mg extract/kg feed, and T_4: 25 mg extract/kg feed.

Dietary inclusion of garlic has been reported to enhance the growth, survival, and feed utilization in different fish species (Akbary *et al*., 2016; Lee *et al*., 2014). Shalaby *et al*. (2006) reported that the final weight and SGR of Nile tilapia (*Oreochromis niloticus*) significantly increased with the increasing level of garlic in the feed. Aly *et al*. (2008) and Aly and Mohamed (2010) studied the growth rates of Nile tilapia after feeding with garlic (10 and 20g/kg diet feed) and found statistically non-significant increases after 1 and 2 months, but a significant increase only after 8 months. Dietary supplementation of garlic is reported to improve feed conversion and protein efficiency (Agbebi *et al*., 2013; Nya and Austin, 2009) that significantly contribute to the growth of fish. Garlic specifically its active ingredient allicin modulates the total bacterial counts, and status of beneficial bacteria in the fish gut, kill various pathogenic bacteria, enhance immunocompetence, improve gastrointestinal motility, and modulate the secretion of various enzymes to improve digestion, nutrient absorption, and enhance the energy utilization, resulting in improved growth in fish (Foysal *et al*., 2019; Büyükdeveci *et al*., 2018; Lee and Gao, 2012). These reports support our findings.

In vivo disease prevention efficacy of garlic ethyl acetate extracts

In this study, the fish (*L. rohita*) fed with different concentrations of garlic extracts enriched feed for 90 days was found to develop resistance against MAS when artificially challenged with the *A. veronii* strain B55. In the T_3 and T_4 treatment groups, no mortality was found in the challenged fish. Only, 23.33±4.71% mortality was observed in the treatment group T_2 while 100% of the control group fish died with the expression of distinct external disease symptoms (Table 3). The disease symptoms observed in the control group fish were: excess mucus secretion, large ulceration adjacent to the injection site followed by tissue necrosis, haemorrhages in the skin, and at the base of fins, erosion of tail and fins (Fig. 2). However, in some of the garlic extract-fed fish, mild hemorrhages were observed after 1-2 days of the intramuscular injection of the bacterial pathogen but, the fish recovered these hemorrhages with 7-21 days (Fig. 3). The survived fish were also maintained in the same aquarium for an additional 30 days with the basal feed. Within this period, no external disease symptoms or abnormalities were observed in the post challenged fish.

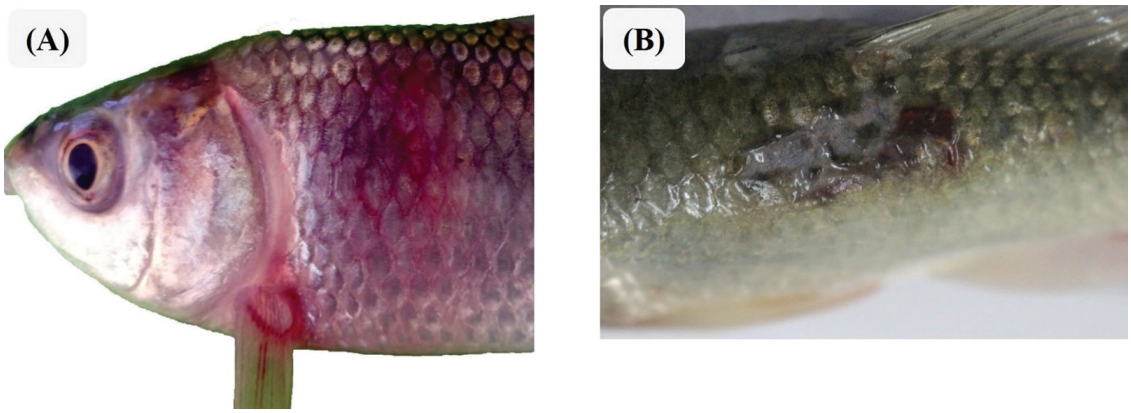

Fig. 2. External symptoms of motile *Aeromonas* septicaemia infection in control (T_1) fish (*L. rohita*) challenged with *Aeromonas veronii* strain B55. (A) hemorrhages at the dorsal region and at the base of pectoral fins; (B) large necrotic lesion at the injection site.

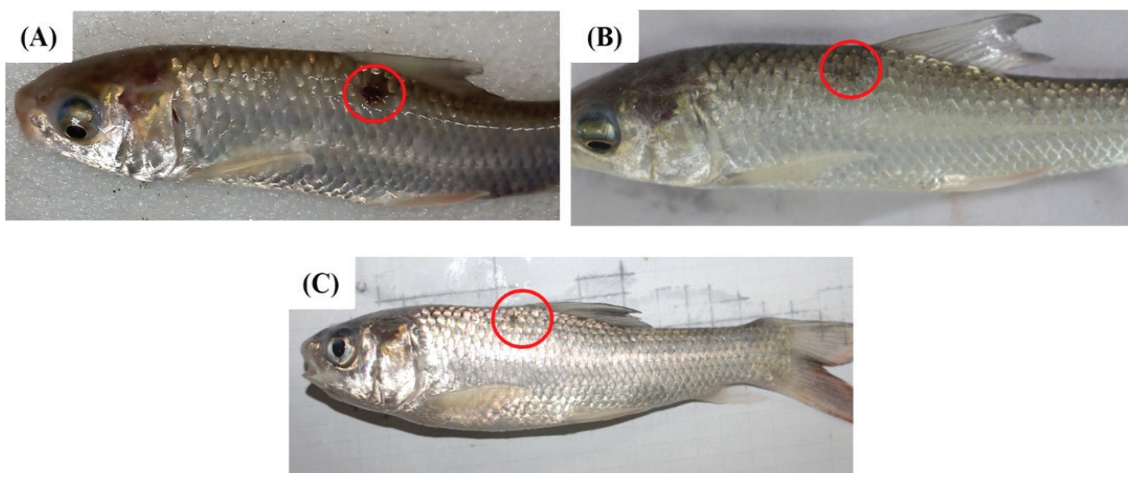

Fig. 3. Recovery of fish fed with garlic ethyl acetate extracts at different post challenge time. (A) reddening (mild haemorrhagic lesion) at the injection site at 2nd day; (B) partial healing of lesion at 10th day; (C) completely healed lesion at 21st day of post challenge with *Aeromonas veronii* strain B55.

The MAS is found in a wide range of freshwater and brackish water fish species and caused substantial economic damage to fish culture operations (Hanson *et al*., 2019; Rahman, 2004). Although *A. hydrophila*, *A. caviae*, *A. sobria,* and *A. veronii* has been recognized as the causative agents (Stratev and Odeyemi, 2016; Cai *et al*., 2012; Rahman, 2004), *A. veronii* is increasingly been reported to be involved in MAS (Hassan *et al*., 2017; Rahman, 2004). Herbal extracts possess antibacterial properties, counteract stress, enhance growth, stimulates the appetite and immune system in farmed fishes (Reverter

et al., 2014). Several reports suggested that medicinal plants can prevent the *A. hydrophila* infection in fishes (Wang *et al.,* 2016; Yin *et al*., 2009). Dietary inclusion of garlic has been reported to enhance the disease resistance in different fish species *viz., Streptococcus iniae* infection in tilapia (Foysal *et al*., 2019), Vibriosis in shrimp (*Penaeus monodon*) (Hannan *et al*., 2019), Enterococcal infection in tilapia (Rahman *et al*., 2017), *Edwardsiella tarda* infection in African catfish (*Clarias gariepinus*) (Abraham and Ritu, 2015), *Neobenedenia* parasitic infection in fish (Militz *et al*., 2013), *Vibrio harveyi* infection in Asian sea bass (*L. calcarifer*) (Talpur and Ikhwanuddin, 2012). Nya and Austin (2009) reported that garlic supplemented with fish feed led to control of experimental infection with *A. hydrophila* in rainbow trout (*O. mykiss*). Sahu *et al.* (2007) claimed that dietary supplementation of *A. sativum* stimulates the immunity and makes *L. rohita* more resistant to infection by *A. hydrophila*. However, this is the first report on the prevention of MAS caused by *A. veronii* in an important species of Indian major carp, *L. rohita*. Garlic contains several bioactive compounds including allicin, alliin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, and S-allyl-cysteine (Shang *et al*., 2019) which might help the fish to develop resistance against the disease. Nya *et al*. (2010) reported that allicin is responsible to prevent disease caused by *A. hydrophila*. The immunostimulating effect of medicinal plants is attributed to the early activation of non-specific defense and boosting of specific immune response (Nithikulworawong, 2012) that helps fish to develop resistance against diseases. Basha *et al*. (2013) determine medicinal plants as an interesting alternative for the treatment of diseases, as they are not expensive, renewable, safe, and easy to prepare.

Conclusion

The ethyl acetate extract of garlic inhibited the growth of all of the laboratory strains of fish pathogenic *A. veronii* in *in vitro* assay with the highest inhibition zones. Dietary inclusion of garlic ethyl acetate extracts significantly enhanced the growth of L. *rohita*. The most important findings of this study is the development of resistance against MAS caused by *A. veronii* in L. *rohita* fed with different concentrations of garlic ethyl acetate extract enriched feed.

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