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 (T₁, control), 6.25 (T₂), 12.50 (T₃), and 25.00 (T₄) 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 T₁, T₂, T₃, and T₄, 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 T₃, T₄, and T₂ 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.
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 (10⁵ 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 10⁵ 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 T₁ (control) (0), T₂ (6.25 mg garlic extract/kg feed), T₃ (12.5 mg garlic extract/kg feed), and T₄ (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 T₁ were fed with the commercial basal diet (without garlic extract) and treatments T₂, T₃, and T₄ 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:

\[
\text{Total weight gain (g) = Mean final weight - mean initial weight.}
\]

The specific growth rate was calculated by using the formula:

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

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* septicemia 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±0.67 | 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.
Garlic extract against *Aeromonas* septicaemia in *Labeo rohita*

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$_4$.

### 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)

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.
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 T3 and T4 treatment groups, no mortality was found in the challenged fish. Only, 23.33±4.71% mortality was observed in the treatment group T2 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.

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

| Parameters          | Treatments |
|---------------------|------------|
|                     | T1         | T2         | T3         | T4         |
| 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), T2: 6.25 mg extract/kg feed, T3: 12.5 mg extract/kg feed, and T4: 25 mg extract/kg feed.
Garlic extract against *Aeromonas* septicaemia in *Labeo rohita*

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...
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), Vibrios 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 allin, 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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