Environmental Science and Pollution Research (2020) 27:23420–23436
https://doi.org/10.1007/s11356-019-05957-7

POLLUTANT REMOVAL AND THE HEALTH EFFECTS OF ENVIRONMENTAL POLLUTION

Efficacy of *Andrographis paniculata* supplements induce a non-specific immune system against the pathogenicity of *Aeromonas hydrophila* infection in Indian major carp (*Labeo rohita*)

Radhakrishnan Palanikani[1] · Kanagaraj Muthu-Pandian Chanthini[1] · Ramaiah Soranam[1] · Annamalai Thanigaivel[1] · Sengodan Karthi[1] · Sengottayan Senthil-Nathan[1] · Arunachalam Ganesan Murugesan[1]

Received: 29 November 2018 / Accepted: 11 July 2019 / Published online: 30 July 2019

Abstract

Aeromonas hydrophila, an opportunistic fish pathogen, which causes several major diseases including skin ulcer and haemorrhagic septicemia, contributes considerably to the lethality in aquaculture. Chemical and antibiotic treatment employed against *A. hydrophila* for disease management are expensive and consequently prompted the advent of drug resistance among the pathogens. To overcome these draw backs, alternative aquatic disease control methods using conventional plant-based medicines are focussed. Our present study aimed to augment the fish non-specific immune system with the implementation of methanolic crude extracts of *Andrographis paniculata to Labeo rohita*, for evaluating their efficacy against *A. hydrophila.* Histology of major organs of *A. hydrophila*-infected fish such as the gills and liver displayed severe tissue damage. *A. paniculata* extracts exhibited the strong antibacterial activity against *A. hydrophila* even at lower concentrations (50 μl). The extracts also altered the haematological profile of treated infected fishes by increasing the levels of haemoglobin and total erythrocyte-leucocyte counts, along with the phagocytic index. The extracts also had a significant impact on modifying the anatomy and swimming pattern of infected fish, post treatment with the extracts. Also, *A. paniculata* treated infected fishes in all the plant extract administration methods, viz. injection, oral feeding and diffusion, and reduced the cumulative mortality rate to less than 30%. Even lower concentrations of *A. paniculata* extracts (50 μl) resulted in maximum relative percentage survival of treated fishes. Therefore, our findings suggest that *A. paniculata* was effective against *A. hydrophila* infection in aquaculture, thereby maintaining a healthy status of these fishes in aquaculture.

Keywords Skin ulcer · Mortality · Haematology · Histology · Survival

Introduction

Fish, an aquaculture food product stands a very valuable source of animal protein besides essential nutrients. Over the former times, aquaculture has stayed one among the fastest food-generating areas in the world (Bilen et al. 2016a; FAO 2016). Aquaculture product including fish constitutes 20% of food consumption in developing countries (Béné et al. 2007). Fish contributes to near 50% of protein and minerals in the diet to the people of South Africa and Africa (Richardson et al. 2011). Due to the increasing demand of fish supply, aquaculture of economically important fishes is being established globally. However, cultivated fish are vulnerable to many diseases and an upsurge of disease epidemics has been reported owing to the escalation, resulting in above partial or full loss of production. Fish production from aquaculture is hindered by several limiting factors, for instance overcrowding, meagre water value, poor nutritional status and handling subsidize to physiological variations in fish such as trauma or immunosuppression and, therefore, amplify vulnerability to infection (Bilen et al. 2016b). Moreover, high concentrations of fish

Responsible editor: Philippe Garrigues

✉ Sengottayan Senthil-Nathan
senthil@msuniv.ac.in; senthilkalaidr@hotmail.com

[1] Sri Paramakalyani Centre for Excellence in Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi, Tirunelveli, Tamil Nadu 627 412, India

besides the deficiency of hygienic barriers ease the propagation of pathogens, creating high mortality varieties (Naylor et al. 2000; Cabello 2006; Quesada et al. 2013). Primary and opportunistic pathogens that propagate as a consequence of environmental stress are the common causes of diseases prevailing in aquaculture units in Asia (Lategan and Gibson 2003; Fang et al. 2004).

Aeromonas hydrophila is an opportunistic pathogen and is relatively more abundant in aquatic zones with a high organic load than in unpolluted water (Vasantha-Srinivasan et al. 2019a, 2019b). The Indian major carps (IMC) contribute between 70 and 75% of the entire freshwater fish production (Nandeesha et al. 2013; Edwin et al. 2016a, 2016b). Rohu (*Labeo rohita*), is a fresh water IMC that is widely cultivated in India. This species is survives easily and are cultured in a limited area, having a high demand besides being considered to be highly economical (FAO, STAT 2012; Nandeesha et al. 2013). However, this fish species is susceptible to *A. hydrophila* infection which causes severe damage, leading to 80–100% of the death rate within a week (Shankar et al. 2000).

The aquaculture farmers apply various antibiotics like ampicillin, tetracycline and chloroampiphinicol (Harikrishnan et al. 2011; Kamaraj et al. 2018), to control the *A. hydrophila* bacterial disease. But this bacterium is a heterogeneous species, having various antigens, so that vaccine development is extremely complex (Yin et al. 2009). The constant practice of using antibiotics can lead to the advance of antibiotic-resistant pathogens; the presence of these chemicals, left as residues in the fish, is dangerous for human consumption also causing harmful bioaccumulation impacts (Hidayat et al. 2018). Recently, herbal immunostimulants such as organic compounds from plants have been commonly used as an antibacterial and immunostimulant to control fish diseases (Cuesta et al. 2004; Ratnawati et al. 2013; Karina et al. 2015; Hardi et al. 2016a; Karina et al. 2016).

Increased interest in the stimulation of the fish non-specific immune system against pathogens, using botanicals, is developing (Alambra et al. 2012; Elkamel and Mosaad 2012; Harikrishnan et al. 2011; Menanteau-Ledouble et al. 2015; Misra et al. 2006; Satyantini et al. 2014; Zokaeifar et al. 2012) Non-specific defence mechanisms play a vital role in fish disease management throughout their life cycle. Fishes are comparatively more dependent on non-specific immune reactions for combating harmful pathogens than mammals (Harikrishnan and Balasundaram 2005). Herbal immunostimulants such as tetra *Cotinus coggyria* powder and extract (Bilen et al. 2011; Bilen et al. 2013; Bilen et al. 2014; Senthil-Nathan et al. 2005**),** *Capperis spinose* extract (Bilen et al. 2016a), *Lactuca* extract (Harikrishnan et al. 2011) and *Urtica dioica* extract (Bilen et al. 2016b) have been found effective against important fish pathogens. The dietary supplementation of *P. guajava* leaf extract significantly reduced the mortality and increased the disease resistance of *Oreochromis mossambicus* against *A. hydrophila* infection (Gobi et al. 2016). Several studies report the immunomodulatory effect of *Bosenbergia Pandruta*, *Solanum ferox* and *Zingiber Zumbet* methanol extracts by suppressing the growth of *A. hydrophila*, thereby enhancing disease resistance abilities (Hardi et al. 2016a, 2016b).

Andrographis paniculata is otherwise called Kalmegh (King of Bitters; Family Acanthaceae), native to Sri Lanka and India. Andrographolide ($C_{20}H_{30}O_5$, melting point 230–239 °C) is a medicinal compound which is present in the foliage and root of the *A. paniculata* plant. The compound can be easily isolated from *A. paniculata* foliage as a crude extract (Rajani et al. 2000; Edwin et al. 2016a, 2016b). Andrographolide possess a multi-hepatoprotective property (Xu et al. 2006; Lin et al. 2009; Zhang et al. 2009; Wang et al. 2010; Chao et al. 2010; Jutti Levita et al. 2010; Tan et al. 2010; Zhou et al. 2010). Lately, the stimulation of immune function with the help of some medicinal plants (eco-friendly) in the aquaculture industry is gaining attention (Karthi et al. 2018; Chellappandian et al. 2018; Vivekanandhan et al. 2018a; Senthil-Nathan 2015; Vasantha-Srinivasan et al. 2019a, 2019b). This present study was performed to investigate the immune modulatory effect of *A. paniculata* on the non-specific immune parameters and its resistance to *A. hydrophila* pathogen in *Labeo rohita.*

Materials and method

Fish sample collection

Fingerlings of *L. rohita*, weighed 15.5±2.6 g (mean ± SD) were collected from a freshwater fish breeding centre (Govt Fish Seed Farm, Manimuthar Dam, Manimuthar, Tirunelveli District, Tamil Nadu (08° 36′ 40.3″ N 75° 23′ 20.7″ E) (Fig. 1). Sample fishes were secured and transported in oxygen-filled sterile plastic bags. Prior to the challenge test, the fishes were acclimatized to be grown under lab conditions for 7 days. The fishes are fed with commercial feed pellets two times a day (3% body weight).

Collection of plant materials

Aerial parts of healthy *A. paniculata* plants were collected during morning hours in Southern Western Ghats (Kallar site, (altitude 'M'-140 latitude 08° 48′ 09.0″ N). The collected plant material was identified by taxonomists from the Department of Plant Conservation and Biotechnology, Sri Paramakalyani Centre for Excellence in Environmental Sciences, Manonmanium Sundaranar University, Alwarkurichi, Tirunelveli, and the voucher specimen no. 1040 was submitted to the herbarium (Fig. 2). The samples

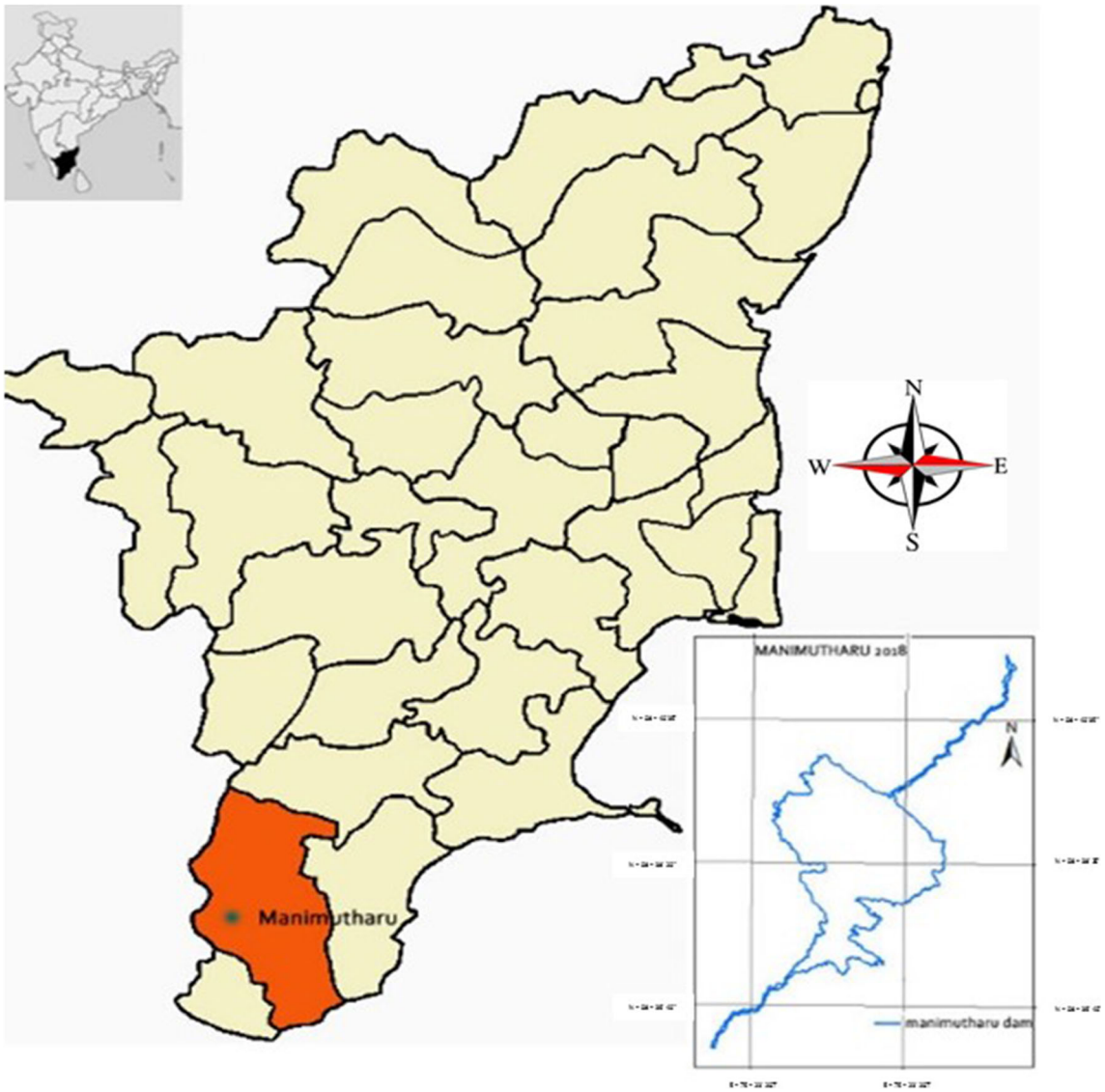

Fig. 1 Collection site of fish fingerling

were poised and washed initially with tap water and then with sterile water.

Preparation of *A. paniculata* crude extract

The collected plants were shade dried for 14 days at room temperature (27–32 °C) until they became brittle, then pulverized to powder in order to prevent photolysis and thermal degradation. Crude extracts were obtained by following standard extraction procedures (Harikrishnan and Balasundaram 2005; Hardi et al. 2014, 2016a). Dried plants were severed into minor fragments and ground to a coarse powder in an electrical stainless steel blender (Kumar et al. 2014). The powdered material (100 g) was subjected to Soxhlet extraction with methanol (500 ml) for 48 h, 45–80 °C (Umamaheswari and Prince 2007. The residue was concentrated using a rotary vacuum evaporator to produce the crude extract (Senthil-Nathan 2007) and stored at 4 °C. The crude extract was dissolved in 1% DMSO to facilitate complete solubilization in fish-containing tank water in diffusion method.

Bacterial sample collection

Aeromonas hydrophila culture was purchased from MTCC (MTCC-1739).

Bacterial inoculum preparation for disease

The bacterial culture was grown on nutrient agar plates and was used for pathological experiments. Serially diluted 24-h old culture (10^8 CFU/ml) was centrifuged at 3000*g*/10 min. The pellet was washed with phosphate buffer saline (PBS) and

Fig. 2 Morphology of *Andrographis paniculata*

the LD_{50} density of the bacteria was adjusted up to 10^8 CFU/ml to be used for bioassays (Ben-David and Davidson 2014; Chelladurai et al. 2017; Hardi et al. 2014).

Antimicrobial assay

Antibacterial assays were performed using crude extracts of different concentrations (50, 100, 150 and 200 μl) using the well diffusion method. The broth dilution method was used to estimate the minimum inhibitory concentration (MIC) (Devillers et al. 1989; Hardi et al. 2016c).

Design of experimental challenge test

This analysis was done by using different experimental treatments, (Table 1). After a 2-day acclimatization period, the fishes were further conditioned for challenge tests. The fingerlings were transferred to tanks containing clean water for 1 week (27 ± 1 °C, pH 7.2 ± 0.4, Salinity 10 ± 3 and DO > 6.5 mg^{-1}). The feed was changed from commercial feed pellets to rice bran and groundnut oil cake. The bioassays were carried out by administrating 0.1 ml of extract to the fishes via intraperitoneal injection, oral feeding and diffusion methods (Table 1). All treatments were repeated thrice.

In the injection method, 0.1 ml of the extract was administered by intraperitoneal injection. In the oral feeding method, the extract (0.1 ml) was administrated to the fish by supplementing with feed (Hardi et al. 2017); In the diffusion method, the fish were allowed to swim in the water tanks added with 0.1 ml of crude extract for 30 min (Cipriano et al. 1984; Hardi et al. 2017). The same day after treatments, the fish was challenged with *A. hydrophila* (10^8 CFU/ml^{-1}) inoculated via intramuscular injection. The treated fishes were observed for 28 days.

Blood drawing and preparation

Blood (0.2 ml) was drawn from the fish caudal vein by means of a syringe containing a pinch of anticoagulant (EDTA). Blood parameters such as erythrocyte-leucocyte counts and haemoglobin level were analysed by drawing blood on days 0, 7, 14 and 28 (pre-injection, injection time and post-injection time, respectively).

Total erythrocyte count

Total erythrocyte count (TEC) was done by drawing blood (0.5-scale pipette) and using Hayman solution (till scale 101). The combination was mixed well by shaking with a motion resembling the form of number 8. The cells were counted in a haemocytometer viewed under a fluorescent microscope (× 40; Optika, Japan; Blaxhall and Daisley 1973).

Table 1 Treatments profile of the Rohu (*Labeo rohita*) fish

S. no.	Treatments	*A. hydrophila* (10^8 CFU/ml^{-1})	*A. paniculata* Extract
1	(i) Control	–	–
2	(ii)	0.1 ml	50 μl
3	(iii)	0.1 ml	50 μl
4	(iv)	0.1 ml	100 μl
5	(v)	0.1 ml	200 μl
6	(vi)	0.1 ml	PBS 50 μl

PBS, phosphate buffer saline

Haemoglobin test

The haemoglobin level was tested using a salinometer (Wedemeyer and Yasutake 1977). Distilled water was used to dilute the drawn fish blood colour to resemble the standard colour in the salinometer. The haemoglobin concentration was cited as g%.

Total leukocyte count

Total leucocyte was counted by drawing the blood sample on a 0.5-scale pipette (Anderson and Siwicki 1995a). The first droplet was added to the haemocytometer and covered with a coverslip. The cells were counted by viewing the haemocytometer under a fluorescent microscope (× 40; Optika, Japan).

Phagocytic index analysis

The effect of *A. hydrophila* on fish blood was observed by analysing the phagocytic index. Fish blood was mixed with *A. hydrophila* suspension (10^5 cells/ml) and extracts (50 ppm) and incubated at room temperature for 20 min. The suspension was carried forward for estimating the phagocytosis index (PI) by placing few drops on a slide and observed under a microscope (× 40; Optika, Japan). The number of cells showing phagocytosis was counted and expressed PI was as % (Anderson and Siwicki et al. 1995b).

Resistance to bacteria

The fishes were observed for changes in swimming patterns and anatomy post bacterial infection as well as after treatments with *A. panniculata* extracts on both live and dead fishes on the 14th day. The symptomatic change coding was categorized into four levels and rated on a scale (Table 2).

Fish injected with *A. hydrophila* was monitored for mortality every day (0 to 28). *A. hydrophila* was re-isolated from the liver and kidney of the dead fish, cultured in Rimler-Shotts agar medium to confirm the pathogenicity of *A. hydrophila*. Additionally, the relative percentage of survival (RPS) study was calculated by using the Ellis (1988) formula.

Table 2 Calculation of infection index for Rohu

S. No	Infection index	Percentage	Indicator
1	Normal	0	○
2	Low	< 20	✓
3	Medium	< 20–50	✓✓
4	High	< 50	✓✓✓

$$\mathrm{RPS} = 1 - \frac{(\text{Percent mortality in treated group})}{\text{Percent mortality in control group}} \times 100$$

Histology

The gill and liver of infected fish were isolated for histological studies (Roberts and Rodger 2012). Gill and liver samples (1 cm^3) were taken aseptically from the infected fish and were fixed with 10% buffered formalin. Then, the samples were subjected to trimming and processed in an automatic tissue processor for dehydration, clearing, and infiltration for 21 h. The samples were sectioned and stained with haematoxylin and eosin (Nahar et al. 2016). They were then allowed to dry overnight and the cell structures were observed by using a light microscope (Nikon, Eclipse, E 800).

Statistical analysis

The experimental data were analysed using ANOVA. Differences between the treatments were determined by Tukey's test ($P = 0.05$) (Snedecor and William 1989).

Results

Minimum inhibitory concentration

The methanolic extracts of *Andrographis paniculata* exhibited antibacterial activity against *A. hydrophila,* with an MIC of 50 µl, in a 2-h time duration (Table 3).

TEC and Heamoglobin levels

The application of *A. paniculata* extracts (50 mg/l, 100 mg/l, 150 mg/l and 200 mg/l) enhanced the total erythrocyte count and the normal haemoglobin levels in the injection (Table 4), oral feeding (Table 5) and diffusion methods (Table 6).

The fish infected with *A. hydrophila* displayed Hb and TEC levels significantly lower than those of the control, 4–5 g% and 22–23 cells mm^{-3} respectively (Table 4, 5, and 6). The fish treated with plant extracts increased the haemoglobin and TEC levels from 9–9.5 g% to 9–13 g% and 29–30.8 to 32–37.7 × 10^5 cells mm^{-3},respectively, in all the treatment methods ($P < 0.005$). Among the three treatment methods, there was a significant rise in Hb (13 g %) and TEC levels (37.7 × 10^5 cells mm^{-3}).

Total leucocyte

Total leucocyte count (TLC) of the fish supplemented with different dosages of *A.paniculata* extracts increased and differed significantly, compared with that of the control on the

Table 3 MIC of the methanol extract of herbal plants against *A. hydrophila*

	A. hydrophila + different concentrations of extract (μg/ml)					
Plant extracts	0	10	25	50	100	200
Andrographis paniculata	+	+	+	–	–	–

'0' control, ('+' growth), ('– no growth)

28th day (P<0.0005). *A. hydrophila* infection reduced the TLC levels (Fig. 3). The plant extracts increased the TLC levels of *A. hydrophila*-infected fishes from 2.6–3.0 to 3.2–4.9 × 10^5 cells mm^{-3}.

This result indicated that *A.paniculata* extracts were able to increase the production of leucocyte significantly as a defence mechanism against pathogenic bacteria infection at the end of the treatment. The TLC levels increased with treatments in a dose-dependent manner. However, at higher concentrations, 150 and 200 μl, the TLC levels did not differ significantly ($P > 0.005$). TLC values of treated infected fishes were significantly higher in the injection method, which did not differ significantly with that of the oral feeding method (4.9 × 10^5 cells mm^{-3}) ($P > 0.005$).

Phagocytosis index

Phagocytosis index of Rohu fishes treated with plant extracts increased in a dose-dependent manner at a day 14 and day 28. On days 0, 7 and 14, no significant difference in PI values of treated and untreated fishes was observed. However, the PI values on day 28 increased and differed significantly with that of control fishes ($P < 0.005$). The *A. paniculata* treatment by the injection method increased the PI of fishes reaching 61.40% from 20.30–21.30% (Fig. 4) after 14 days. The extracts also increased the PI of infected fishes to 47.40 and 61.40% at respective 100 and 200 μl treatment concentrations ($P < 0.005$). However, the PI increase at dosage treatments 150 and 200 μl was not significantly different ($P > 0.005$).

Anatomy and physiological changes

Fishes infected with the *A. hydrophila* pathogen displayed changes in its external and internal organs such as fin rot, skin ulcer, damage in gill, loss of scale, haemorrhagic septicaemia (Fig. 5b). The treatment of infected fishes with *A. paniculata* started to disappear gradually from day 14 and completely vanished on day 28. Among the treatment methods, oral feeding was very effective followed by diffusion and injection methods (Tables 7 and 8).

Histology analysis of gill

Histopathological changes were observed in the gill of the infected Rohu fishes. The infected fishes exhibited haemorrhages at the base of the fins, erosion of the pectoral fins, ulcers on the skin surfaces and fin rot after 72 h of infection. Normally, the surface of the gill lamellae (GF) was covered with epithelial cells running in a parallel position (Fig. 6a). Primary lamellae (PL), secondary lamellae (SL), nucleated erythrocyte (Er—black arrow), gill of the infected fishes after 24 h completely collapsed with GF lamellar aneurysm (L), damaged primary lamellae (DPL) and damaged secondary lamellae (DSL) (Fig. 6b). Completely detached filament degraded secondary lamellae (shown as a yellow arrow) and clusters of bacterial cells (BC) in the filament were also observed.

Histology analysis of liver

The liver is the largest extramural organ that contains heatopoietic tissue and macrophage aggregates. Overall, the control group showed normal hepatocytes (NH), blood vessels (BV) and blood sinusoids (BS) (Fig. 6a). Blood sinusoids were lined with reticulo-endothelial cells, which were surrounded by hepatocytes. After infection with *A. hydrophila*, the hepatocyte cells were completely damaged and an influx of blood cells in capillaries occurred (Fig. 6b).

Table 4 Blood profile of the Rohu (*Labeo rohita*) fish treated with extracts for prevention of *A. hydrophila* from 0 to 14 and 28 days

Treatments	Haemoglobin (g%)			Total erythrocyte (10^5 cells mm^{-3})		
	D0	D14	D28	D0	D14	D28
No extract, no pathogen	9	9	9.5	29.7	30	30.8
A. paniculata 50 μl, *A. hydrophila*	9	10.2	11.5	27	31.5	32
A. paniculata 100 μl, *A. hydrophila*	10	11	12	21	31	33
A. paniculata 150 μl, *A. hydrophila*	9	11	12.5	25	32.3	34.7
A. paniculata 200 μl, *A. hydrophila*	10	11.5	13	29	32.2	37.7
No extract only *A. hydrophila*	9	6	4	28.1	24	23

D0, day 0; *D14*, day 14; *D28*, day 28

Table 5 Blood profile of the Rohu (*Labeo rohita*) fish treated with extracts for prevention of *A. hydrophila* from 0 to 14 and 28 days

Treatments	Haemoglobin (g%)			Total erythrocyte (10^5 cells mm^{-3})		
	D0	D14	D28	D0	D14	D28
No extract, no pathogen	9	9	9.5	29.7	30	30.8
A. paniculata 50 μl, *A. hydrophila*	10	10.2	11.5	27.5	31.5	32
A. paniculata 100 μl, *A. hydrophila*	9	10.5	12	21.7	31	33.0
A. paniculata 150 μl, *A. hydrophila*	9	11	12.5	25.4	32	34.5
A. paniculata 200 μl, *A. hydrophila*	10	11.5	13	29	32.2	37.1
No extract only *A. hydrophila*	9	7	5	28.1	24	23

D0, day 0; *D14*, day 14; *D28*, day 28

Blood congestion in the sinusoids and hypertrophy (H) of hepatocytes were observed.

Changes in swimming pattern

A. hydrophila-infected fishes displayed changes in swimming patterns and behaviours that were evident by visual observations. The fishes exhibited unstable swimming patterns by swimming up to the surface water, gasping and settling down in the fish tank. They also displayed signs of weakness and aggressive behaviour when touched.

However, after treatment, the fish exhibited ordinary behaviour. Interestingly, gasping and aggressive response were not observed in fish treated with the extracts and only a few of the fish displayed debilitated swimming behaviour and stayed at the bottom of the aquarium (Table 9).

Cumulative mortality and death rate

The fishes infected with *A. hydrophila* displayed higher cumulative mortality rate (CMR) and cumulative death rate (CDR) that were significantly different from control and treatment rates (Figs. 7 and 8). The fishes treated with the extracts of *A. paniculata* displayed CMR less than 30% (Fig. 7). The CMR was lower in fishes treated with lower doses of extracts.

The CDR of fishes infected with *A. hydrophila* was significantly higher compared with that of the control and treated fishes ($P < 0.005$). Moreover, the CDR of infected fishes treated with 50 μl *A. paniculata* extracts in all the treatment methods and control were not significantly different, implying the safety and efficacy of the extracts in fishes against *A. hydrophila* infection (Fig. 9).

Relative percentage survival

Fishes treated with a lower dose of 50 μl *A. paniculata* extract displayed RPS rates that were significantly higher than those of the control ($P < 0.005$) (Fig. 9). However, at higher treatment dosages, the infected fishes exhibited lower RPS rates compared with those of the control implicating the toxicity of extracts at concentrations higher than 50 μl in all the three treatment concentrations.

Discussion

As an important sector of the food industry, aquaculture is a rapidly developing trade in terms of production and revenue. In order to meet high demands, fish farmers are intensifying fish cultures globally. This has resulted in several ill effects such as overstressing as well as disease outbreaks leading to

Table 6 Blood profile of the Rohu (*Labeo rohita*) fish treated with extracts for prevention of *A. hydrophila* from 0 to 14 and 28 days

Treatments	Haemoglobin (g%)			Total erythrocyte (10^5 cells mm^{-3})		
	D0	D14	D28	D0	D14	D28
No extract, no pathogen	9	9	9.5	29	30	30.8
A. paniculata 50 μl, *A. hydrophila*	10	10.2	11	27	31	32
A. paniculata 100 μl, *A. hydrophila*	9	11	11	21.8	31.8	33
A. paniculata 150 μl, *A. hydrophila*	9	11	12	25.6	32.5	34.5
A. paniculata 200 μl, *A. hydrophila*	10	11.5	12.5	29	33.6	36
No extract only *A. hydrophila*	10	7	5	28.5	26	22

D0, day 0; *D14*, day 14; *D28*, day 28

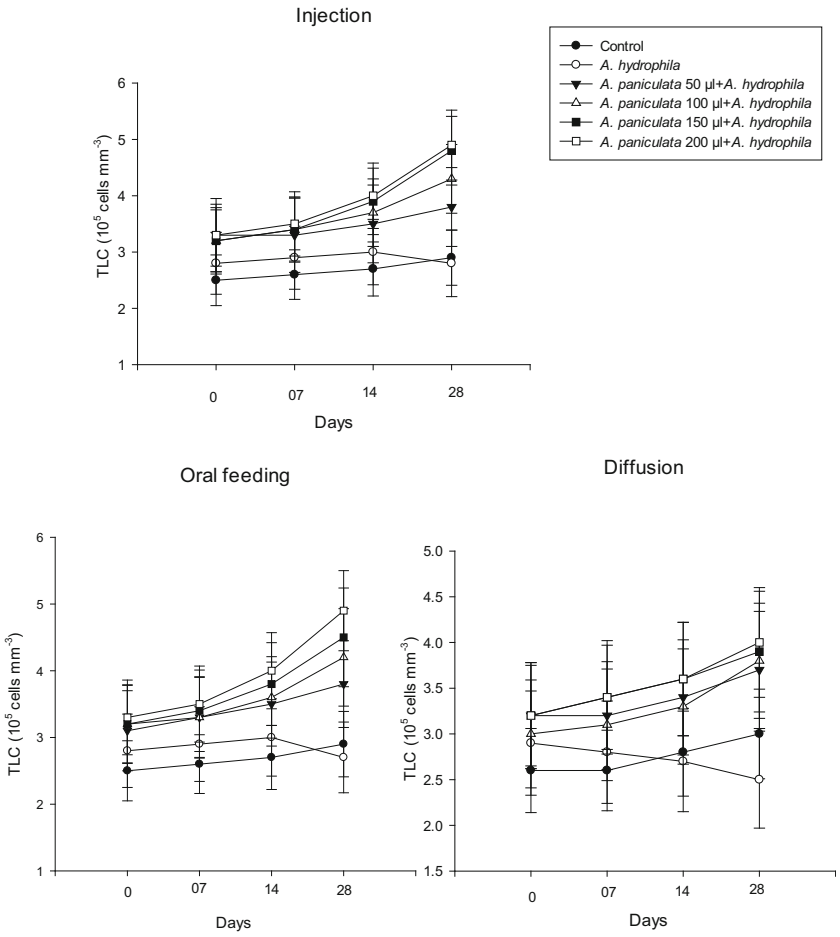

Fig. 3 Total leucocyte count (TLC) of Rohu in the injection, oral feeding and diffusion methods. **a** *A. paniculata* 50 µl, *A. hydrophila*. **b** *A. paniculata* 100 µl, *A. hydrophila.* **c** *A. paniculata* 150 µl, *A. hydrophila.* **d** *A. paniculata* 200 µl, *A. hydrophila*

severe yield loss. Besides the careless and overuse of chemicals for disease management, alternative disease control methods to stimulate fish non-specific immune response to confer disease resistance are being emphasized by ichthyologists worldwide. The usages of natural immunostimulants that are eco-friendly, biodegradable and safe are being preferred for aquaculture disease management lately (Raa et al. 1992; Ortuno et al. 2002; Palanikani et al. 2018).

Amidst the vital fish species cultured in India, Indian carps Catla (*Catla catla*), Rohu (*Labeo rohita*), and Mrigal (*Cirrhinus mrigala)* are of high commercial value (FAO 2016). Disease has caused mass mortalities to the Indian aquaculture industry, with a special regard to motile *Aeromonas* infections (Paniagua et al. 1990; Alain 2009; Govind et al. 2012). The non-specific immune system of Rohu fishes consists of cellular and humoral components. Cellular components include monocytes, lympocytes, neutrophiles and macrophages, while the humoral components include lysozymes and complements (Magnadóttir 2006). Phagocytosis in fish plays a pivotal role in eliminating disease-causing pathogens. Plant extracts as immunostimulants are regarded as the good alternatives for synthetic chemicals. In addition, some probiotics are also used to manage bacterial diseases, yet the probiotics only suppress the growth of pathogenic bacteria rather than eradicating the pathogen (Hardi et al. 2016c; Ponsankar et al. 2019).

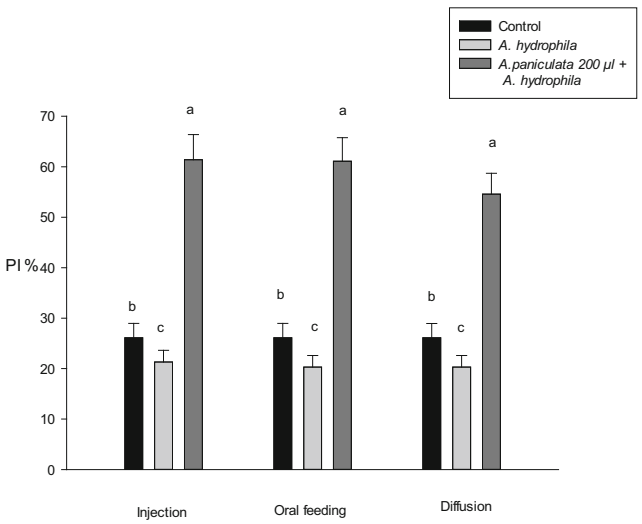

Fig. 4 Phagocytosis index of Rohu in the injection, oral feeding and diffusion methods

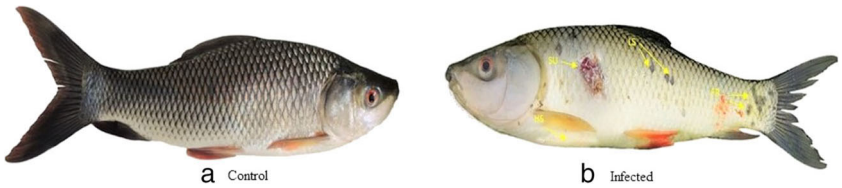

Fig. 5 **a** Control (IMC) *Labeo rohita.* **b** Infected (IMC) *Labeo rohita*. SU, skin ulcer; LS, loss of scale; HS, haemorrhagic septicaemia; FN, fin rot

With vaccines only targeting a specific type of bacteria (Hardi et al. 2013) and the necessity of the application of a booster dose for controlling the fish infections leading to the development of resistance among the pathogens, plant-based disease management strategies were focussed Kesarcodi-Watson et al. 2008; Nugroho and Fotedar 2013). Some plant extracts are reported with immunostimulant and antibacterial activities that can provide effective disease control (Vivekanandhan et al. 2018a, 2018b; Venkatalakshmi and Michael 2001; Hardi et al. 2016a, 2016b) and some viral growth (Saptiani et al. 2016a, 2016b). Hence, the study was focused on controlling the *Aeromonas* infection through stimulating the non-specific immune response of Rohu by the application of *A. paniculata* extracts.

The pathogenicity of *A. hydrophila* was determined by exposing the pathogen to Rohu fishes via the injection method. All the Rohu fishes developed rapid changes in their anatomy, physiology and swimming patterns post challenge with the pathogen. The disease symptoms intensified with time due to pathogen proliferation and additional entry into the fish facilitated by some injuries as Rohu fishes do not have protection scales and their sharp fins can easily tear the skin of another fish within the immediate vicinity. Similar symptoms on fish infected with *A. hydrophila* as well as *Pseudomonas* sp. were also observed (Cipriano et al. 1984; Afifi and Guerrero 2000 Al-Faragi and Alsaphar 2012; Mhaisen et al. 2013; Stratev et al. 2015; Kumar et al. 2016; Abdelhamed et al. 2017).

Rohu fishes infected with *A. hydrophila* displayed symptoms in varied levels of severity. Those treated with *A. paniculata* extracts exhibited moderate symptoms. It was also reported that the severity of symptoms depends upon the immunological status of the fishes. The higher the immnumological efficiency, the lower the infection. Subsequently, the lower infection levels caused only medium levels of external symptoms. This was also reported by Sheeba (2012) and Hardi et al. (2017), who reported anatomical changes in Nile tsilapia challenged with *A. hydrophila* as well as *Pseudomonas* sp.

Fishes infected with pathogens exhibit clinical signs such as alterations in swimming patterns (El-Ghany and Alla 2008). Besides the external symptoms, the infected fishes also displayed changes in swimming patterns and behaviours such as gasping and weakness along with vigorous movements and settling at the bottom of the tank. The infected fishes also displayed haemorrhage at the base of the fins, erosion of the pectoral fins, skin ulcer of the surface and septicaemia. The appearance of these symptoms and anatomical-physiological changes confirms the pathogenicity of *A. hydrophila* by comparing with previously reported works of Yambot and Inglis (1994), who reported acute mortality among Nile tilapia caused by *A. hydrophila*.

Horne and Baxendale (1983) and Kanno et al. (1989) also reported the adherence of bacteria to the intestine, gill and skin. *A. hydrophila* is capable of damaging the host organs such as the gill, liver, intestine and heart. The histology of the gills and liver in infected Rohu showed similar patterns of deterioration. The gills of infected fish collapsed completely after 24 h of infection, exhibiting haemorrhages and erosion at the fin base and pectoral fins respectively and skin ulcer as well as fin rot after 72 h. The lamellar aneurysm was damaged

Table 7 Anatomic, physiological and swimming patterns of Rohu after the challenge test treated with *A. hydrophila* in the injection method

Treatments	Anatomic and wimming pattern changes					
	Fin rot	Skin ulcer	Damage in gill	Loss of scale	Gasping	Weakness
No extract no pathogen	○	○	○	○	○	○
A. paniculata 50 µl, *A. hydrophila*	✓	✓✓	✓	✓	✓	✓
A. paniculata 100 µl, *A. hydrophila*	○	✓	✓	○	✓	✓
A. paniculata 150 µl, *A. hydrophila*	○	○	○	○	○	○
A. paniculata 200 µl, *A. hydrophila*	○	○	○	○	○	○
No extract only *A. hydrophila*	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓✓

D0, day 0; *D14*, day 14; *D28*, day 28

Normal (○), low (✓), medium (✓✓), high (✓✓✓)

Table 8 Anatomic, physiological and swimming pattern of Rohu after the challenge test treated with *A. hydrophila* in the oral feeding method

Treatments	Anatomic and swimming pattern changes					
	Fin rot	Skin ulcer	Damage in gill	Loss of scale	Gasping	Weakness
No extract, no pathogen	○	○	○	○	○	○
A. paniculata 50 µl, *A. hydrophila*	✓	✓	✓	✓	✓	✓✓
A. paniculata 100 µl, *A. hydrophila*	○	✓	✓	✓	✓	✓
A. paniculata 150 µl, *A. hydrophila*	○	○	○	○	○	○
A. paniculata 200 µl, *A. hydrophila*	○	○	○	○	○	○
No extract only *A. hydrophila*	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓✓

Normal (○), low (✓), medium (✓✓), high (✓✓✓)

and the secondary lamellae degraded by detached filament, showing clusters of bacterial cells. After infection with *A. hydrophila*, the hepatocyte cells were completely damaged and the presence of blood cells that have influxed from the capillaries was reported. Additionally, blood congestion in the sinusoids and hypertrophy of hepatocytes were also observed. Similar histological observations were reported by several researchers as a result of *A. hydrophila* in several fish species (Khamees et al. 2013; Stratev et al. 2015; Kumar et al. 2016; Abdelhamed et al. 2017). Based on these observations, the pathogenicity of *A. hydrophila* was confirmed.

Management of *A. hydrophila* in an aquaculture system is extremely difficult and the development of resistance against chemical control methods is further complicating the disease control programs (Wang and Weller 2006). Several plants were recorded with antibacterial activity against *A. hydrophila* (Cipriano 2001; Obi et al. 2007; Mahfuzul Hoque et al. 2007; Al Laham and Al Fadel 2014). Few of the plant bioactive compounds in clove (eugenol) and cinnamon (eugenol and cinnamaldehyde) have been reported with higher antagonistic activities against *A. hydrophila* (Ramena et al. 2018).

In the current research, the methanol extracts of *A. paniculata* were found to be very effective against *A. hydrophila* even at 50 µl (MIC). The derivatives of *A. paniculata* such as isoandrographolide, neoandrographolide, andrographolide and isoandrographolide are reported to poses several bioactivities such as antimalarial, anti-diabetic, anti-viral, anti-inflammatory and immunostimulatory activities (Tajuddin and Tariq 1983; Yu

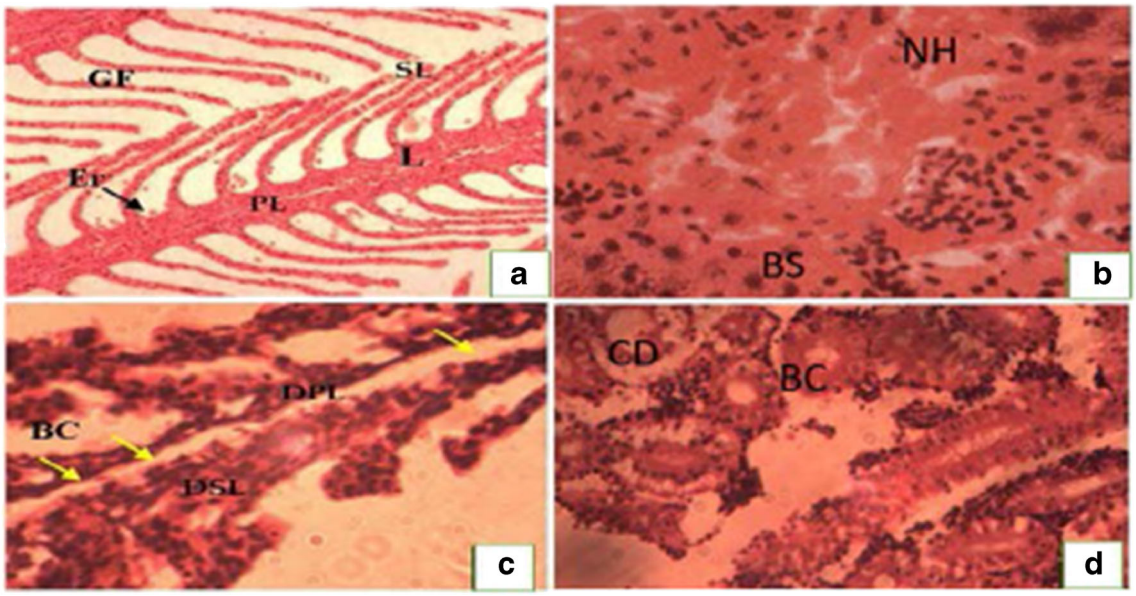

Fig. 6 **a**, **b** Histology analysis of the gill and liver. **a** Control group showing gill filament (GF), secondary lamellae (SL), nucleated erythrocyte (Er). Haematoxylin and eosin × 100 (*Labeo rohita*). **b** The Gills of infected fish after 72 h, completely collapsed GF, damaged primary lamellae (DPL), lamellar aneurysm (SL) and damaged secondary lamellae (DSL); clusters of bacterial cells (BC) in filament (yellow arrow), (*Labeo rohita*). Haematoxylin and eosin × 100. **c** Control group showing normal hepatocyte (HC), blood vessels (BV) and blood sinusoids (BS). Haematoxylin and eosin × 100 (*Labeo rohita*). **d** The liver infected with *A.hydrophila*; cells of hepatocytes are completely damaged with influx of blood cells (BC) in capilliaries and cytoplasmic degeneration (CD). Haematoxylin and eosin × 100 (*Labeo rohita*)

Table 9 Rohu (*Labeo rohita*)—anatomic and physiological and swimming pattern changes occurred after the challenge test to *A. hydrophila* in the diffusion method

Treatments	Anatomic and swimming pattern changes					
	Fin rot	Skin ulcer	Damage in gill	Loss of scale	Gasping	Weakness
No extract, no pathogen	○	○	○	○	○	○
A. paniculata 50 µl, *A. hydrophila*	✓✓	✓✓	✓	✓✓	✓	✓✓
A. paniculata 100 µl, *A. hydrophila*	○	✓	✓	✓	✓	✓
A. paniculata 150 µl, *A. hydrophila*	○	○	○	○	○	○
A. paniculata 200 µl, *A. hydrophila*	○	○	○	○	○	○
No extract only *A. hydrophila*	✓✓	✓✓✓	✓✓	✓✓✓	✓✓	✓✓✓

Normal (○), low (✓), medium (✓✓), high (✓✓✓)

et al. 2003; Liu et al. 2007; Umamaheswari and Prince 2007; Kumar et al. 2004). Andrographolide is a bicyclic diterpenoid lactone reported with various immune-modulating properties (Hidalgo et al. 2013; Shamsizadeh et al. 2017).

The health condition of a fish can be measured by analysing their haematological parameters (Cataldi et al. 1998; Sheeba 2012). Hence, the effect of *A. paniculata* extracts on haematological parameters of healthy and infected

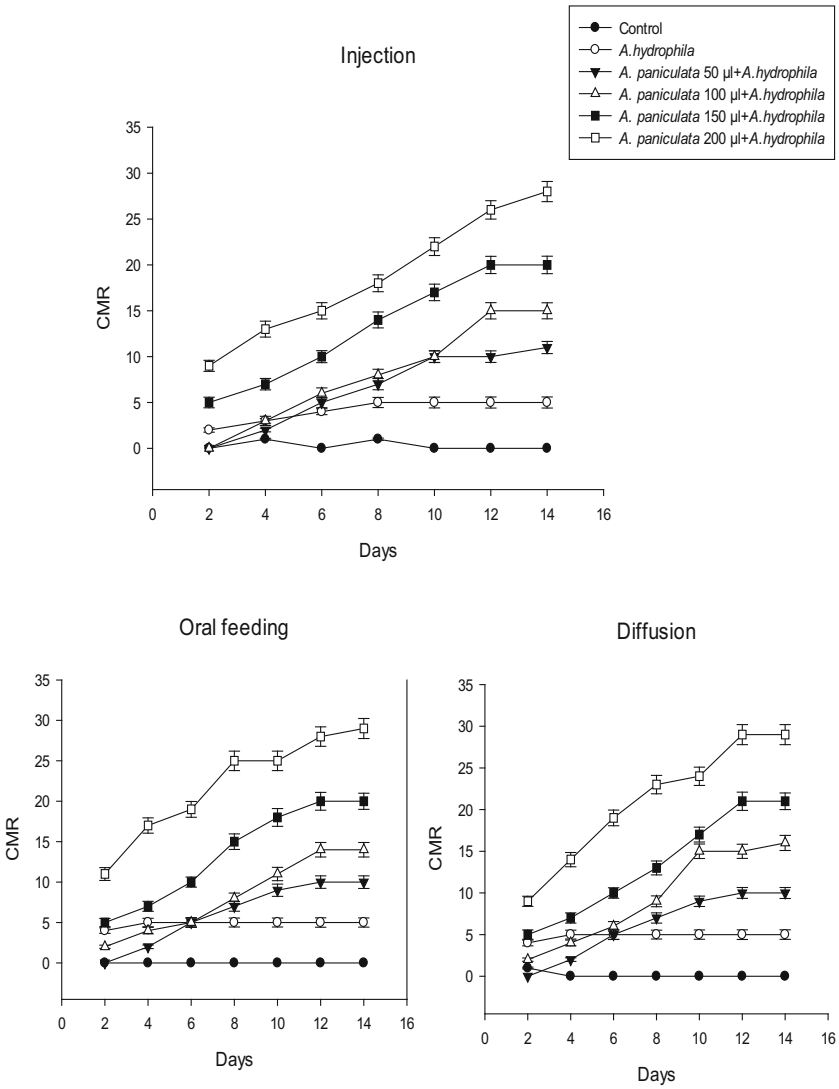

Fig. 7 Cumulative Mortality Rate of (CMR) Rohu in Injection,Oral feeding and Diffusion method. **a** *A. paniculata* 50 µl, *A. hydrophila*. **b** *A. paniculata* 100 µl, *A. hydrophila*. **c** *A. paniculata* 150 µl, *A. hydrophila*. **d** *A. paniculata* 200 µl, *A. hydrophila*

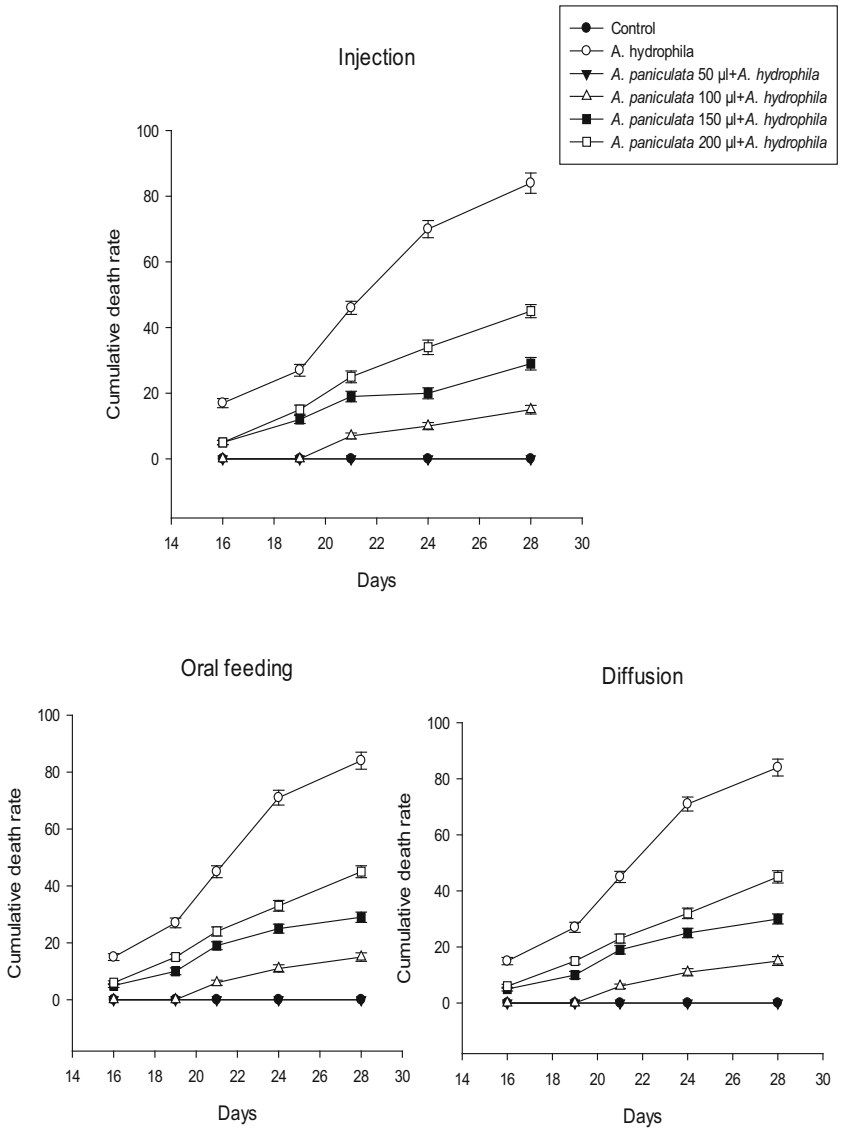

Fig. 8 Cumulative death rate of Rohu in the injection, oral feeding and diffusion methods. **a** *A. paniculata* 50 µl, *A. hydrophila*. **b** *A. paniculata* 100 µl, *A. hydrophila*. **c** *A. paniculata* 150 µl, *A. hydrophila*. **d** *A. paniculata* 200 µl, *A. hydrophila*

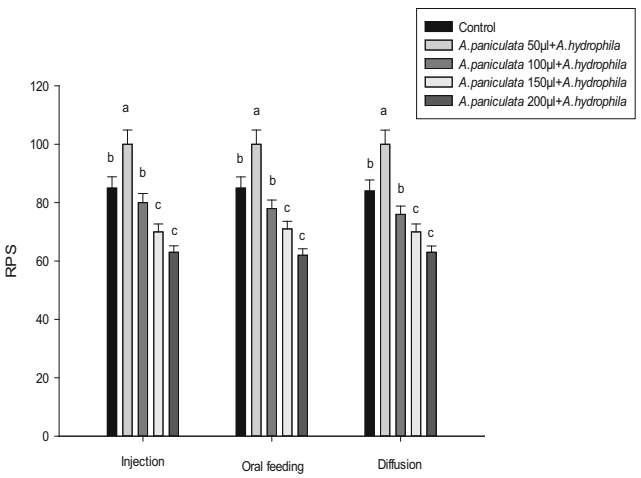

Fig. 9 Relative percentage of survival (RPS) of Rohu in the injection, oral feeding and diffusion methods

fish were determined in terms of TEC, TLC and Hb, even though several immunostimulants did not enhance the haematology status of the exposed organism (Harikrishnan et al. 2010). Infection by a pathogen tends to lower the Hb levels of a fish by the secretion of hemolysin enzyme that degrades RBC resulting in the downfall of TEC and TLC (Sheeba 2012; Hardi et al. 2014). *A. paniculata* extracts increased the haemoglobin (from 4 to 13.5 g%), erythrocyte (from 22 to 37.9×10^5 cells mm-^{3}) and leucocyte (from of 2.7 to 4.9×10^5 cells mm^{-3}) counts at the end of 28 days that was reduced due to *A. hydrophila* infection. The upsurge in TEC level might be a result of oxygen transportation then distribution all over the body of the fish caused by the increase in Hb levels.

The simultaneous increase in TEC corresponds to the increased phagocytosis index of treated infected fish that maximized (62.3%, injection method) after 28 days. Phagocytic cells (monocyte, neutrophil and lymphocyte) act as immune

cells by engulfing and destroying the pathogen, and are vital components of the non-specific immune system. The ability of *A. paniculata* extract to increase the phagocytic index is evidence that the herbal extracts act as immunostimulants (Sahu 2004; Mastan 2015; Pradeepa et al. 2016; Senthil-Nathan 2013). The potential of *A. paniculata* extracts to improve the haematology status of the Rohu thus confers disease resistance. As a consequence, this will improve the overall health of the fish (Bridle et al. 2011; Vasantha-Srinivasan et al. 2017a, 2017b; Zokaeifar et al. 2012; Lawhavinit et al. 2011; Alambra et al. 2012).

Three methods, viz. injection, oral feeding and diffusion methods, were chosen as treatment methods. Among them, the injection method proved to be effective by significantly increasing the haematological status of the fish under study. Harikrishnan et al. (2009) showed that the methanol extract of *Azadirachta indica, Ocimum santum* and *Curcuma longa* supplemented with the fish feed enhanced the phagocytic index of goldfish 2 weeks after treatment. Christybapita et al. (2007) reported the potential of aqueous extract of *Elipta alba* leaves to enhance the non-specific immune response in *Oreochromis mossambicus* and also offering effective disease protection. The phagocytic index will naturally increase upon any pathogenic infection. However, *A. hydrophila* severely reduced the PI of infected fishes lower enough to affect their survival, leading to 86% mortality rate. In the meantime, treatment of infected fishes with *A. paniculata* extracts boosted the phagocytic index of *Labeo rohita* as early as 7 days after administration. An increase in PI before the onset of disease can provide effective disease resistance capabilities due to the readiness of PI cells to combat the incoming pathogens (Satyantini et al. 2014; Thanigaivel et al. 2017; Senthil-Nathan et al. 2008). This study agreed with the previous report of Logambal et al. (2000), in which the injection of *Ocimum sanctum* increased the resistance of *Oreochromis mossambicus* and *Carassius auratus* against *A. hydrophila*. Similarly, Abutbul (Abutbul et al. 2004) testified that augmentation of fish feed with *Rosmarinus officinalis* extract promoted the survivability of *A. hydrophila* infected fish. Likewise, *Achyranthes* extract was also reported to confer increased resistance against *A. hydrophila* infection in *Labeo rohita* (Joseph and Carnahan 1994; Senthil-Nathan 2007; Vasantha-Srinivasan et al. 2017a, 2017b; Fujiki et al. 1994). Basha et al. (2013) also reported the efficiency of andrographoide to stimulate the non-specific immunity and resistance against *A. hydrophila* in *L. rohita.*

The survival rate of the *Labeo rohita* supplemented with *A. paniculata* extracts after *A. hydrophila* infection was higher than that of the fish supplemented without extract. The methanol of the *A. paniculata* plant extract effectively increased the performance of the non-specific immunity in *L. rohita*, constricting the growth of *A. hydrophila,* subsequently increasing their survivability. There was an increase in the cumulative death rate (CDR) of the infected fishes after day 14 and reached a maximum of 85% on day 28. Nevertheless, the *A. paniculata* extract (50 μl) was effective in bringing down the CDR to null, thus displaying 100% relative percentage survival rates in all the methods at the end of 28 days in all the fishes tested. These results indicated that *A.paniculata* applications effectively increased the phagocytosis index of the fishes, thus inhibiting the growth of *A. hydrophila* inside the fish.

Conclusion

The recent study shows the application of plant-based products towards effective and safe disease management is increasing. Additionally, cumulative advantages of these natural products such as being economical, reliable and stable besides the absence of side effects are gaining popularity. This research finally concludes that *A. paniculata* extract can be regarded as a potential immunostimulant that can be effective at very lower concentrations (50 μg/l) against *A. hydrophila* infection. Hence, the use of medicinal plants as a method of treatment in freshwater aquaculture is safe and effective, and can also provide broad-spectrum protection against various infections. Since only limited research has been carried out in the management of *A. hydrophila* infection, our findings might open new dimensions in the application of plant products as effective fish immunostimulants.

Funding information This research was funded by the Department of Biotechnology, Ministry of Science and Technology, grant number BT/IN/Indo-US/Foldscope//39/2015.

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