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Pathogenicity and control of *Aeromonas hydrophila* and *A. veronii* in Indian major carps (*Catla-catla*) by the effect of herbal supplement of *Andrographis paniculata* (Lamiales: Acanthaceae)

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Abstract
Plant extracts are focused for their potential as immunostimulants against the infectious diseases of fish. The present study was designed to evaluate the acquired immunity stimulated by methanolic extract of *Andrographis paniculata* against the pathogens, *Aeromonas hydrophila* and *A. veronii* in *Catla-catla.* The fishes were fed with diet incorporated with A. *paniculata* extract. Post 14 days, serum parameter analyses revealed an increase in total haemoglobin (10-11.5 g %), erythrocyte (34.7 10^5 mm^{-3}) and leukocyte count (3.9 × 10^4 $cell.mm^{-3}$). The phagocytic index of infected fishes treated with extracts increased in a dose dependent manner (31.25%), exhibiting 100 % Relative Percentage Survival. Adverse changes in the disease symptoms were also observed post treatment with extracts. The extracts of *A. paniculata* were able to inhibit the pathogens even at low concentration (50 µl). This study hence proves that the extracts of *A. paniculata* can be used as an immunostimulants.

Keywords: Immunostimulant, acquired immunity, hemoglobin, leucocyte, phagocytic index

1. Introduction
Aquaculture is one of the fastest food supplying sector and aquaculture products produce a higher nutritive food source of animal protein (FAO, 2018) [20]. The past 20 years, aquaculture sector has shown a rare improvement and human needs of world. Aquaculture product constitutes twenty percentage of food needs in developing countries (Bene *et al*., 2007) [8]. Among 50% of protein and minerals intake of South Asia and Africa are supplied by fish (Richardson *et al*., 2011) [59]. Several *Aeromonas spp.* are causing major problem in aquaculture, however, these motile aeromonads also compose as a part of the normal intestinal microflora of healthy fish (Karunasagar *et al*., 1991) [45]. Diseases such as bacterial haemorrhagic septicaemia, caused by motile aeromonads are reported to cause major health problems in fishes, thereby resulting severe losses of the species in freshwater (Roberts *et al*., 1989; Lio-po *et al*., 1992) [60, 46]. *Aeromonas hydrophila* is relatively more abundant in water with a high organic load than in unpolluted water (Jeney Z and Jeney G, 1995) [37]. The adequate development of natural immunostimulants in fish culture for the prevention of disease is a promising novel strategy (Anderson *et al*., 1992; Sakai, 1999) [2, 63]. Immune system can be stimulated naturally with the absorption of herbal extract. These herbal extracts are not only biodegradable but also bio-compatible to the human health. In this research we used herbs to give advance activation to the non-specific immune system of the fish. Several herbal extract has a very good immunostimulant capacity to the fishes by their ability to amplify specific immune response by injection and feeding method (Hardi *et al*., 2017; Galindo-Villegas and Hosokawa, 2004) [31, 23]. Recently, the immune stimulation process in aquaculture is achieved by the application of herbal extracts. The mixture of Chinese herbs in shrimp and Tilapia were reported to improve the non-specific immunity such as bacteriolytic activity and leucocyte function in shrimp and Tilapia (Luo R, 1997; Chansue *et al*., 2000). [49, 12] Some medicinal plants are actively used against several fish diseases. This plant-based therapeutics is very effective, nontoxic as well as eco-friendly.

Different parts of *Azadirachata indica* (Neem) was studied by Chitmanat *et al*., (2005) [14] in this aspect. The Indian almond (*Terminalia catappa*) and Garlic (*Allium sativum*) have been said as alternatives to chemicals to treat fish ectoparasites. However, immunostimulant effect of aqueous extract, *Eclipta alba* (*Bhangra*) leaves (oral administration as feed supplement) in Tilapia fish (O*reochromis mossambicus*) observed by Christybapita *et al*., (2007) [15]. Recent studies have proved that plant based medicinal additives have the potential to enhance fish growth while protecting them from diseases. Nya and Austin, (2009) [54] observed the control of *A. hydrophila* infection after feeding rainbow trout fish (*Oncorhynchus mykiss; walbum*) with *A. sataivam*. Only little work has been done on the medicinal plant supplement fed to the fish in aquaculture study. Therefore, this study was aimed to provide the aqua culturists with a promising management tool for control of fish disease and to give more focus on the potential of medicinal plant as a good alternative to antibiotics in aquaculture.

2. Materials and Methods

2.1 Fish Sample Collection

The fish sample (fingerlings) weighed 15.5 ± 2.6 g (mean ± SD) were collected from fresh water fish breeding centre (Govt. Fish Seed Form, Manimuthar Dam), Manimuthar, Tirunelveli District, Tamil Nadu. Sample fishes were collected and transported by sterile plastic bag with oxygen fill-up. Before going to challenge test, the fishes were kept in the laboratory and grown under lab condition for 7 days. The fishes were allowed to feed with the commercial feed pellets twice a day in a range of 3% / body weight.

2.2 Bacterial Isolation

Aeromonas spp were isolated from local polluted water sample (Kaper *et al*., 1979; Popoff, 1984) [39, 56]. The bacteria isolation was done by serial dilution and streaked on SB-SA medium (this is a selective isolation media of *A. hydrophila* and *A veronii*) and incubated at 35 °C for 24 hours. The bacteria grown were identified using standard manuals (Dubey and Maheshwari, 2012) [17]. The isolated culture was re streaked and pure colony of *A. Veronii* (Rahman *et al*., 2002) [58] and *A. hydrophila* were maintained in nutrient agar slants (-20 °C) for future analyses.

2.2.1 Bacterial inoculum preparation for disease

The isolated bacterial culture was grown on nutrient agar plates for use pathological experiments. Serially diluted 24 hr old culture (density 10^5 CFU/ml^{-1}) (Ben-David and Davidson, 2014) [7] were then centrifuged at 3000g/10 min and the supernatant was removed, the pellet were washed with phosphate buffer saline (PBS) and the LD_{50} density of the two bacteria were adjusted up to 10^5 cfu/ml$^-$ (Chelladurai *et al*., 2014; Hardi *et al*., 2014) [13, 26].

2.3 Plant Extract Preparation

Andrographis paniculata were obtained from Southern Western Ghats, Tamil Nadu. The extraction process was followed by the standard manner of (Harikrishnan and Balasundaran, 2005; Hardi *et al*., (2014 & 2016) [32, 27, 28]. The plant material was sliced and washed, air dried at 37° C for 96 hours in oven and were powdered in a blender, <50mm uniform particle size (Kumar *et al*, 2014) [44]. Dry plant powder (100 g) was added to 100 ml of 70% ethanol to make a slurry form (1:1 ratio) and incubated for 96 hours at room temperature. (Trusheva and Trunkova, 2007) [69].Slurry was filtered with the help of what-man filter paper (No.1) and centrifuged (6000 rpm / 3-5 min). Finally, Crude extract was used to prepare a stock solution (Senthil-Nathan, 2006) [66]. Standard stock solutions were prepared by dissolving the residues in the ethanol.

2.4 Antimicrobial assay

Varying concentrations of plant extracts (50, 100, 150 and 200 µl) were used for antibacterial analysis (Hardi *et al*., 2016) [28] using well diffusion method. Minimum inhibitory concentration (MIC) were tested using broth dilution method.

2.5 Design of experimental challenge test

This study was conducted to identify the test concentration from each extract for modulating the fish nonspecific-immune system against *A. hydrophila* and *A. veronii.* This is done by three experimental treatments, as follows: (i) the fish was injected neither with the extract nor with the pathogen. This treatment served as a control (no extract, no pathogen); (ii) the fish was injected with 50*µl* of *A. paniculata* extract and challenged with *A. hydrophila* (*A. paniculata* 50*µl, A. hydrophila*); (v) the fish was injected with PBS solution and challenged with *A. hydrophila* (no extract, *A. hydrophila* and *A. veronii*). All treatments were performed in triplicates. About 0.1 ml of extract was administrated to the fishes via intraperitoneal injection (Cipriano, 1982) [16].

Blood drawing and preparation

About 0.2 ml of the blood was drawn from the caudal vein using a syringe containing a pinch of EDTA anticoagulant. Blood test including erythrocyte, haemoglobin, and leucocyte analyses were checked on a day 0, 7 and 14 (pre-injection, injecting time and post injecting time).

2.5.1 Total erythrocyte count

Total erythrocyte analysis was done by the method of Blaxhall and Daisley (1973) [9]. The blood sample was drawn using 0.5 scale pipette, following by drawing Hymen solution until it reached the scale 101. The mixture was homogenised by shaking the sample with a motion resembling the form of number 8 and the cells were counted in a haemocytometer viewed under fluorescent microscope (40X; Optika, Japan).

2.5.2 Haemoglobin test (Hb)

The haemoglobin was tested by the procedure following the method of (Wedemeyer and Yasutake, 1977) [71]. A salinometer was filled with 0.1 N HCL until it reached the bottom most scale marks of the salinometer (scale 10). The tube was then placed in between the two tubes with standard colour. Fish blood (0.2 ml) was drawn from the micro tube using sahli pipette and then put in to the sahli tube and left for 3 minutes, and the pipette tip was cleaned beforehand. Distilled water was added in to the tube pinch by pinch and, at the same time stirred until colour was changed exactly the same as the standard colour. The haemoglobin concentration was cited as g%.

2.5.3 Total leukocyte count

Total leucocyte was counted by the method of Anderson and Siwicki, 1995 [3]. The blood sample was drawn using 0.5 scale pipette (a special pipette for leukocyte analysis), and Then turk's solution was drawn until it reached the scale of 11 of the pipette. The first droplet was contained on the haemocytometer and covered with a coverslip. The counting was done and the cells were counted in a haemocytometer

viewed under fluorescent microscope (40X; Optika, Japan).

2.5.4 Phagocytic index analysis (PI)
The procedure followed the method of Anderson and Siwicki, 1995 [4]. About 50µl blood in a micro tube was added with 50µl of *A. hydrophila* and *A. veronii* suspension (10^5 cell/ml^{-1}). The mixture was homogenised and incubated at room temperature for 20 minutes. Henceforward, a smear preparation of the mixture was prepared on the glass slide and then air dried. The smear preparation was stained by immersion in Giemsa staining for 15 minutes, washed in slowly running water, and then dried using tissue papers. The slide was observed under a microscope. The amount of cell showing phagocytosis per 100 observed cells was counted.

2.5.5 Resistance to bacteria
In addition to blood analyses, another parameter observed in this study were the swimming pattern which includes gasping for air, weak movement, and aggressive behaviour including gill skin ulcer. The observation was done by monitoring fish swimming pattern for 30 minutes. Changes in the anatomic pathological symptoms of external organ were observed, including changes in body darkening, changes in fin, loss of scales and exophthalmos. The observation was done with live and dead fishes also suffering such symptoms. The symptomatic changes coding was categorised in to four levels: Normal, low, medium and high. Normal meant that there was no change in the swimming pattern nor the anatomic pathology of the fish; low meant that the number of fish undergoes changes <20%; medium >20-50%; and high >50% from the total number of fish in each treatment. The observation of the changes in fish swimming pattern and anatomic pathology was conducted on day 14 of treatment.
Fish death after the injection with pathogenic bacteria was monitored every day (0 to 14). To ensure that the fish death was caused by the pathogenic bacterial infection, re-isolation of bacteria from the fish's liver and kidney was done in Rimler-shotts agar medium (a selective medium for culturing *A. hydrophila* and *A. veronii*). In addition, relative percentage of survival study (RPS) was noted by using the Ellis formula, (1988) [19].

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

2.6 Statistical analysis
Data (mean ±value) was calculated by using one-way-analysis of variance (ANOVA) followed by Tukey's post hoc test (SPSS) by comparing each treatment ($P<0.05$).

3. Results
3.1 Antimicrobial assay
The plant extracts produced a dose dependent increase in the zone of inhibition from 50µl concentration and MIC was recorded as 15µl against both pathogens.

3.2 Fish blood profile
The fishes exposed to *A. hydrophila* and *A. veronii* showed a decrease in Hb levels from 10 to 6 and 7g% respectively after 14 days. There was an increase in the level of haemoglobin (11.5g %) after the application of *A. paniculata* extracts (Table 1). However, the total erythrocyte of the control fish was 32.0×10^5 cell.mm^{-3}. The total erythrocyte count of the fish injected with pathogen that was not treated with any of the plant extracts decreased to 24.5-25 cell mm^{-3} pathogen. The total erythrocyte (Table 1) of the fish treated with extract was higher than that of control (value from 32.0-34.7 cell. mm^{-3}).

3.3 Total leukocyte count (TLC)
The total leukocyte count(TLC) of the fish supplemented with different dosage of *A. paniculata* extract increased and differed significantly when compared with that of the fish with no treatment but infected with the two bacteria($P<0.05$). The normal Catla fish have a TLC range of from 2.5 to 2.7×10^4 cell.mm^{-3}. In contrast the fish supplemented with extracts of different concentrations and infected with the pathogen showed an increase in TLC range ($3.2\text{-}3.9 \times 10^4$ cell.mm^{-3}). Meantime, the fish that was without injected with any extracts, but infected with the bacteria showed a lower TLC of $2.8\text{-}3.0 \times 10^4$ cell.mm^{-3} (Figure. 1). Total leucocyte of the fish supplemented with the extracts at day 14 differed significantly ($P<0.05$) from that of the fish that was not treated with any of the plant extracts. The above test is the evident of the immune stimulation (TLC increase) was occurred on the fish via plant extracts of *A. paniculata* at the end of this test. However, there was no significant differences in TLC at different concentrations of extracts.

Table 1: Blood profile of the *Catla catla* fish treated with extracts for prevention of *A. hydrophila* and *A. veronii* from 0-14 day

Treatments	Haemoglobin (g %)		Total erythrocyte (10^5 cells mm^{-3})	
	D0	D14	D0	D14
No extract no pathogen	9	10	29.7	32.0
Andrographis paniculata 50µl, A. hydrophila	10	11	29.9	33.8
Andrographis paniculata 100µl, A. hydrophila	9	11.5	19.6	34.0
Andrographis paniculata 150µl, A. hydrophila	9	11	20.5	34.3
Andrographis paniculata 200µl, A. hydrophila	10	10	30.1	34.7
Andrographis paniculata 50µl, A. veronii	9	12	29.3	32.0
Andrographis paniculata 100µl, A. veronii	9	11	29.2	32.1
Andrographis paniculata 150µl, A. veronii	8	11.5	30.1	32.0
Andrographis paniculata 200µl, A. veronii	10	11	29.5	32.2
A. hydrophila only	9	6	28.5	25.0
A. veronii only	10	7	30.0	24.5

Note: D 0:0 day; D 14: day 14

3.4 Phagocytosis index (PI)

The phagocytosis index of the *C. catla* at day 14 increased after being fed with different concentrations of *A. paniculata* extract supplemented with feed. The treatment of fishes with extracts increased PI from 20.53-23.15% to 51-54.10% ($P<0.05$) at 200µl extract concentration post 6 days (Figure. 2). PI value of *C. catla* treated with 50µl of *A. paniculata* extract was 34.57-31.25%. A slower increase in fish PI also occurred in the dose treated with 100µl *A. paniculata* (39-44.70%) and 50 - 55.50% at 150µl.

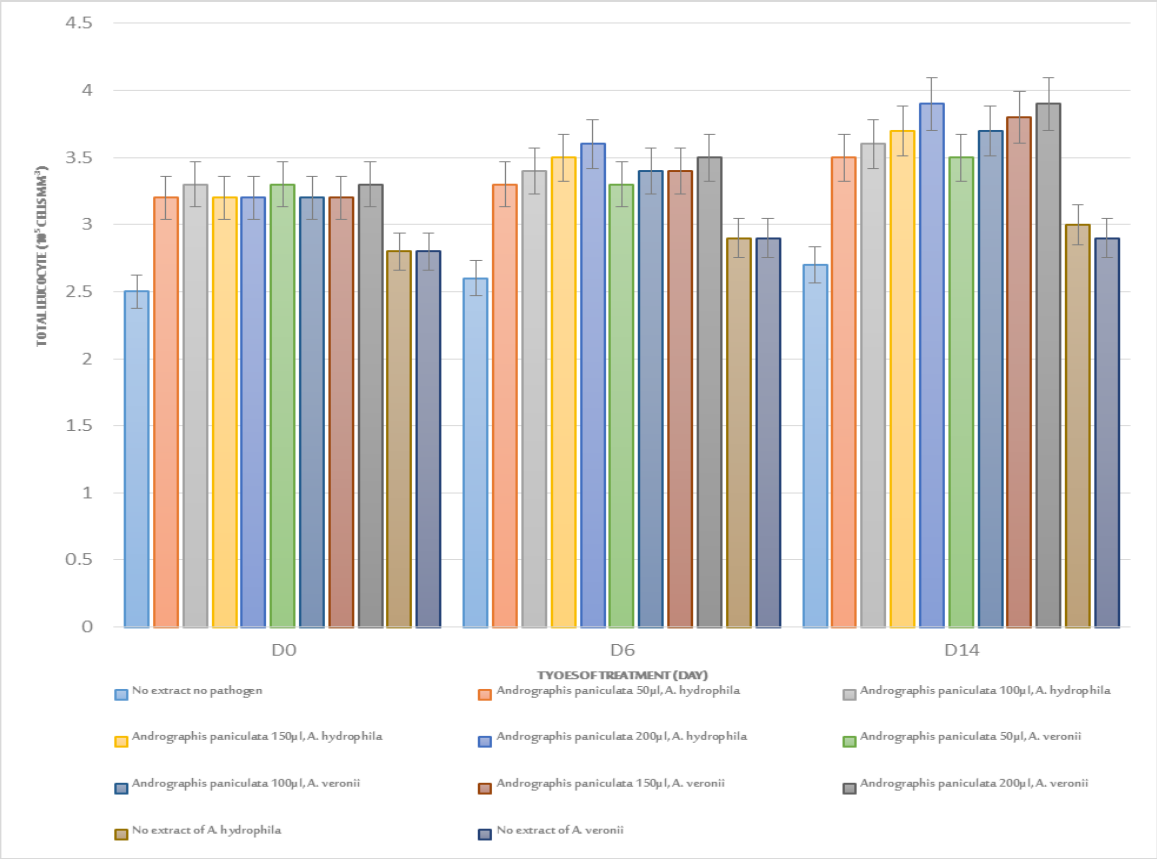

Fig 1: Effect of different dosage value of *A. paniculata* extracts to total leucocyte of the *Catla catla* (Catla)

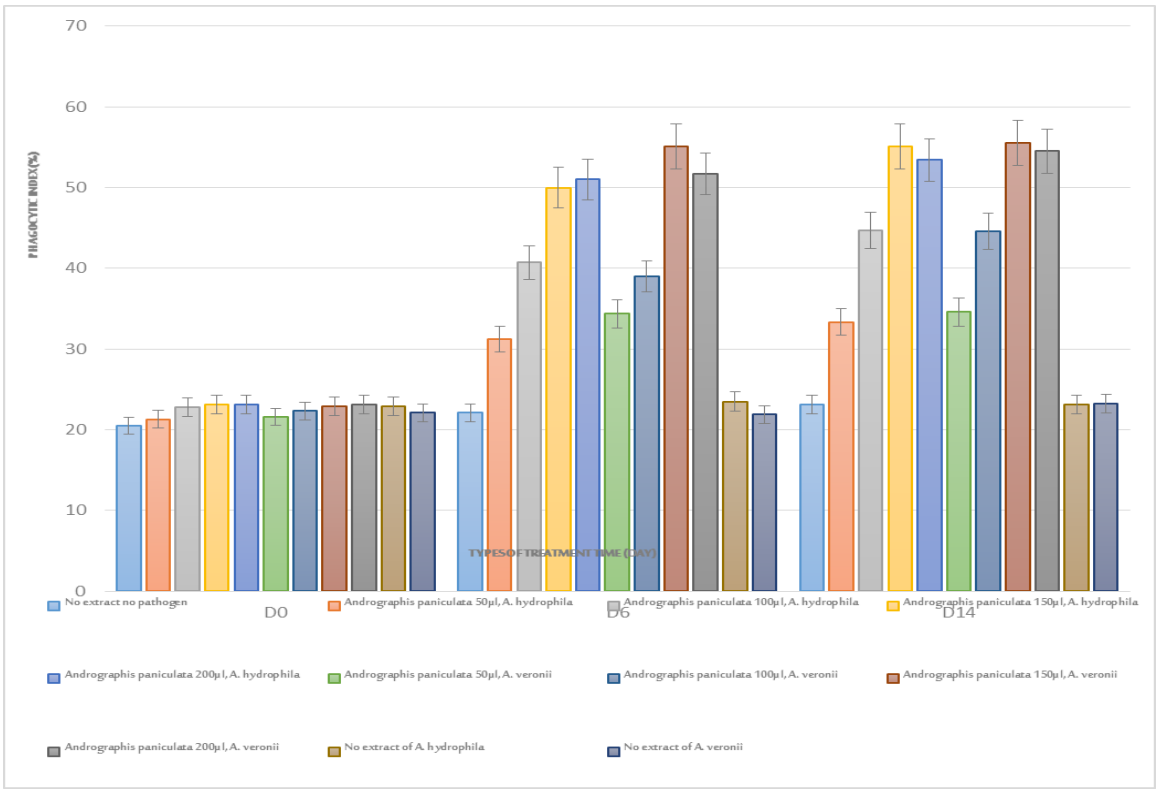

Fig 2: Effect of different dosage value of *A. hydrophila* extracts to phagocytic index of the *Catla catla* (Catla)

3.5 Anatomic and swimming pattern of catla

The fish infected with bacteria *A. hydrophila* and *A. veronii* underwent some changes such as rotting, skin ulcer, damage in gill, loss of scale, and gasping, weakness as well as changes in the swimming patterns. The application of each dose *A. paniculata* extracts effectively prevented *A. hydrophila* and *A. veronii*, which was indicated by the absence of the symptoms at day 14. Meantime, only few fish have observed, gasping and weakened (fish to take rest on the bottom of the aquarium) after treatment of *A. paniculata* extract treatments (Table 2).

Table 2: Catla (*Catla catla*) – anatomic and physiological and swimming pattern changes occurred after challenging test to *A. hydrphila* and *A. veronii*.

Treatments	Anatomic and Swimming pattern changes (symptoms)					
	Fin rot	Skin ulcer	Damage in gill	Loss of scale	Gasping	Weakness
No extract no pathogen	○	○	○	○	○	○
Andrographis paniculata 50µl, A. hydrophila	○	✓	✓	○	○	○
Andrographis paniculata 100µl, A. hydrophila	○	✓	○	○	○	○
Andrographis paniculata 150µl, A. hydrophila	○	✓	○	○	○	○
Andrographis paniculata 200µl, A. hydrophila	○	○	○	○	○	○
Andrographis paniculata 50µl, A. veronii	○	✓	○	✓	○	○
Andrographis paniculata 100µl, A. veronii	○	○	○	○	○	○
Andrographis paniculata 150µl, A. veronii	○	○	○	○	○	○
Andrographis paniculata 200µl, A. veronii	○	○	○	○	○	○
No extract of A. hydrophila	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓✓
No extract of A. veronii	✓	✓	✓	✓	✓	

Note; normal (○), low (✓). Medium (✓✓), high (✓✓✓)

3.6 Cumulative mortality of fish

The cumulative mortality of fish shows a several death has been occurred on time of test. *C. catla* infected with *A. hydrophila* and *A. veronii*, when injected with 100, 150 and 200 µl of *A. paniculata* extracts, exhibited 15, 30 and 40% as well as 10, 25 and 35% cumulative mortality when injected with 100, 150 and 200 µl of *A. paniculata* extracts respectively after 6 days (Figure 3).

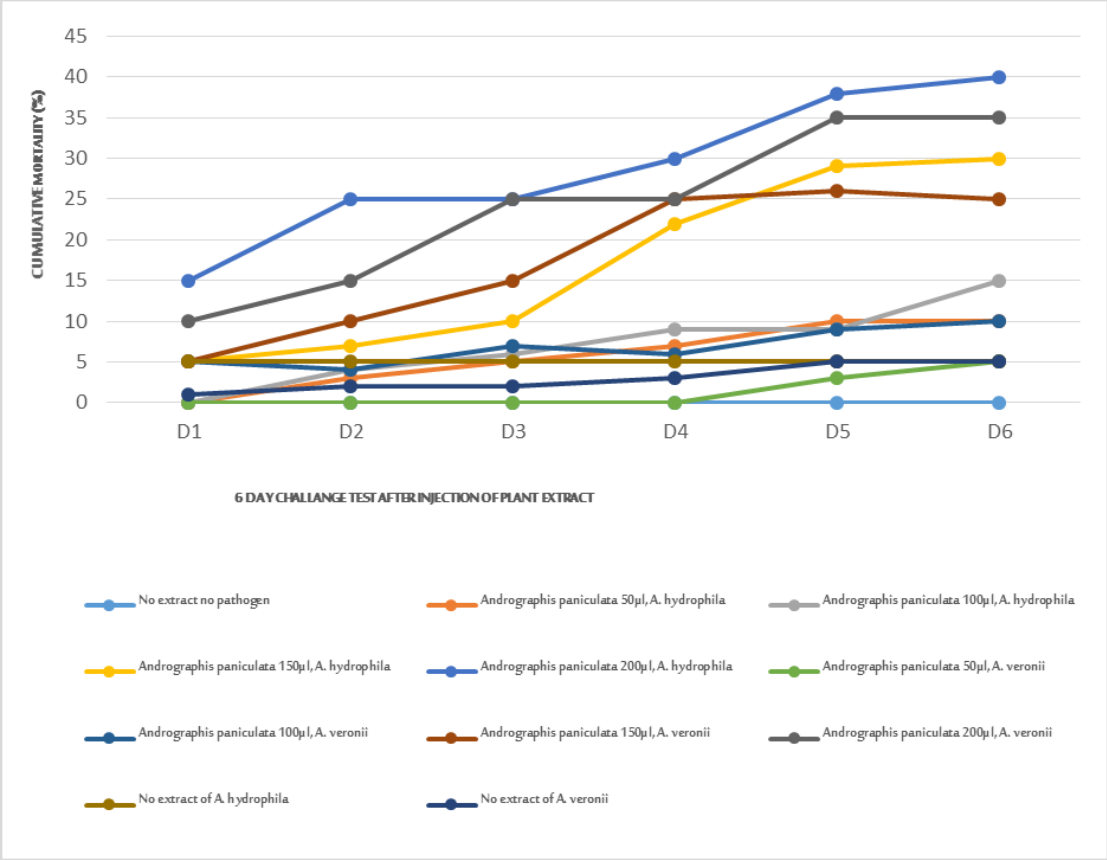

Fig 3: Effects of different doses *A. paniculata* extract cumulative mortality level (%) in Catla after 6 days.

Catla infected with *A. hydrophila* and *A. veronii* exhibited the lowest mortality rate (10 and 5%) post treatment with 50µl of *A. paniculata* extract.

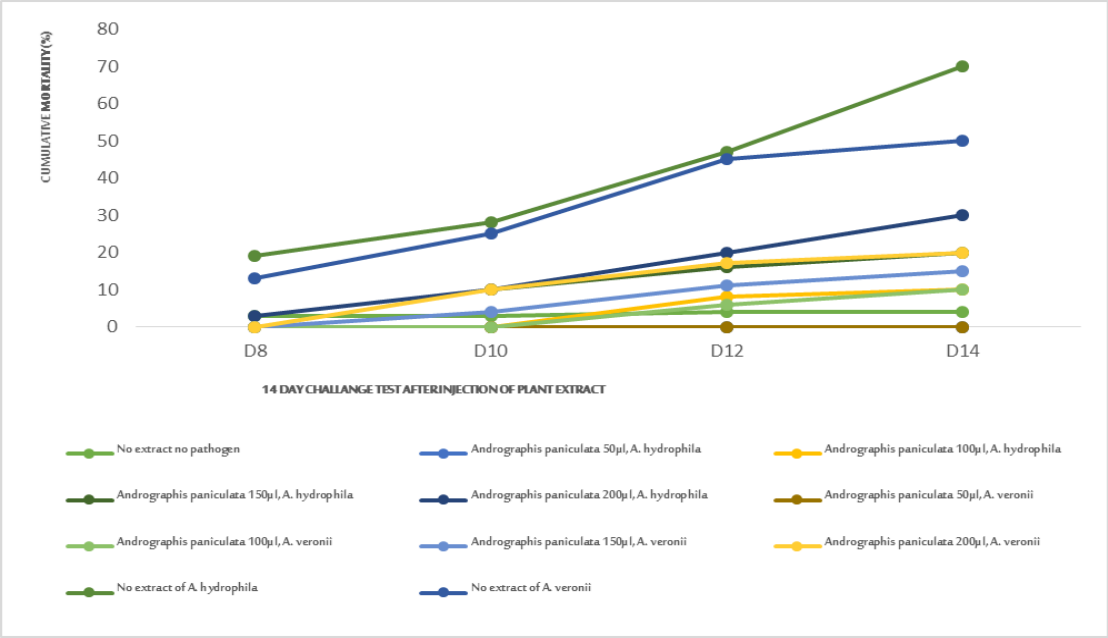

Fig 4: Effects of different doses *A. paniculata* extract cumulative death level (%) in Catla after 14 days.

Higher mortality rates of 70 and 60% was observed in fishes challenged with *A. hydrophila* and *A. veronii* after 14 days. Infected Catla treated with extracts displayed mortality rates that were significantly different from that which were not treated ($P<0.05$). There were no significant differences in death rates of fishes treated with different concentrations of extracts.

C. catla infected with *A. hydrophila* and *A. veronii*, when injected with 100, 150 and 200 µl of *A. paniculata* extracts, exhibited 0, 10 and 20% as well as 10, 15 and 25% cumulative mortality when injected with 100, 150 and 200 µl of *A. paniculata* extracts respectively after 14 days (Figure 4).

3.7. Relative percentage survival (RPS)

Catla exhibited higher RPS rate (90%). The fishes exposed to both the pathogens exhibited 100% RPS when treated with 50 µl *A. paniculata* extract. However, the RPS (Figure.5) rates decreased on increase of extract concentration higher than 50 µl, *viz.*, 85, 75 and 67 % as well as 87, 75 and 69% when infected with *A. hydrophila* and *A. veronii* respectively.

4. Discussion

Fish are vulnerable to numerous bacterial infections, primarily when nurtured in high densities. Diseases epidemics are accountable for raised death rates and reduction of the throughput competence, producing high economic fatalities to the aquaculture industry (Figueiredo *et al*., 2006) [21]. *A. hydrophila* and *A. veronii* are major fish pathogens known to infect variety of fishes, predominantly present in freshwaters. *A. hydrophila* infection in *Oreochromis niloticus* have caused severe disease outbreaks causing 60% mortality (Hardi *et al*, 2017) [31]. Also, *A. hydrophila* and *A. veronii* have been reported as major pathogens for Indian major Carps (Karunasagar *et al*., 1986; Karunasagar *et al*., 1989; Pradhan *et al*., 1991) [40, 42, 57]. *A. hydrophila* are responsible for cases of skin infections, septicemia and gastroenteritis in fish and human (Yu *et al*., 2007) [72]. *A. veronii* causes severe haemorrhage and skin ulcer disease. (Cai *et al*., 2012; Eissa *et al*., 2015) [11, 18] among various fish species worldwide (Sreedharan *et al*., 2011; Nawaz *et al*., 2006) [68, 52].

Since the deleterious effect caused by chemical pesticides, plant-based pesticides that are eco-friendly and effective are being looked upon. This study is focussed on the potential of *A. paniculata* extracts against *A. hydrophila* and *A. veronii* infections in *C. catla*. This study reveals that the effective disease control was obtained through injection method it systematically reduced the mortality rate of Catla after being infected by *A. hydrophila* and *A. veronii*.

Normally enhancement of fish immune system is done by the administration of immunostimulants, that boost the immune system (Logambal, 2001) [48] and vaccines, that can induce the specific immune system (Pasnik *et al*., 2005) [55]. Vaccine may be only target a specific type of bacteria (Hardi *et al*., 2013 & 2016) [24, 30] and also it is necessary for booster dose to be applied. The nonspecific immune system of fish consists of a humoral and cellular component (monocyte, lymphocyte, neutrophils, macrophages etc). Humoral compounds like lysozyme and other components (Secombes and Fletcher, 1992; Magnadottir 2006) [65, 50]. Besides, usage of antibiotics could induce to pathogenic resistance to fish while being harmful to the environment (Kesarcodi-Watson *et al*., 2008; Nugroho Fotedar, 2013) [43, 53]. Some plant extracts have an ability of immunostimulant and antibacterial activities that can enhance the nonspecific immunity and thereby inhibits bacterial and viral growth (Joseph and Carnahan, 1994; Venkatalakshmi and Michael, 2001; Hardi *et al*., 2016; Saptiani *et al*., 2016) [38, 70, 29, 64].

A. paniculate, a plant of herbaceous family, Acanthaceae, native to India and Sri Lanka, widely cultivated in South Asia, are used as antibiotics and antidotes (Roopavathy, 2011) [61]. Andrographolide, an active component isolated from *A. paniculate* is being evaluated for various bioactivities such as antimicrobial, antioxidants, antidiabetic, antitumor and immunomodulator properties (Basha *et al*., 2013) [6]. The potential of *A. paniculate* as immune stimulants in *C. catla* was evaluated.

The extracts of *A. paniculate* were effective against the pathogens *A. hydrophila* and *A. veronii*, exhibiting a significant antibacterial activity at a concentration of 50µl. The extracts were able to inhibit the pathogens at a minimum concentration of 15µl.

The fishes were exposed to the pathogens by intramuscular

injection method. The pathogenesis of the bacteria were effective by injection method (Hasan, 2007) [35] and the extracts were dissolved in the water tank where the fishes were allowed to swim.

Bacterial infection has a direct effect on an organism's haematology and serum parameters by enzymatic digestion of the erythrocytes, that leads to reduced TLC and Hb levels (Harikrishnan *et al*., 2010; Hardi *et al*., 2014) [34,25]. As a consequence, *C. catla* infected with *A. hydrophila* and *A. veronii* displayed decreased levels of haemoglobin (6 to 7 g %), TEC (24.5-25 cell mm^{-3}) and TLC (2.8-3.0 × 10^4 $cell.mm^{-3}$). An increase in the Hb (10-11.5 g %), TEC (32.0-34.7 $cell.mm^{-3}$) and TLC (3.2-3.9 × 10^4 $cell.mm^{-3}$) levels in fishes treated with A. *paniculate* extracts after 14 days. This was also in agreement with the results reported by (Hardi *et al*., 2017) [31]. The potential of the extracts to increase oxygen transportation throughout the body of the fish has resulted in an increase in TEC, while increased levels of TLC is as a result of increased phagocytic activity (Bridle *et al*., 2011; Zokaeifar *et al*., 2012; Balasundaram and Harikrishnan, 2009) [10,74,5]. Hence, it is evident that the extract was acting as an immunostimulant, conferring protection against the infections and thereby having a positive impact on the health of the fish.

Phagocytosis is a process of eliminating pathogens and an increase in PI indicates higher rates of nonspecific immune response. A variety of agents including microorganisms (bacteria, fungi, virus) products (Lamas and Ellis, 1994; Solem ST, *et al*., 1995) [45, 67] stimulate phagocytic cells such as monocytes, neutrophils, and lymphocytes. Although the PI increases with the incidence of infection, the defence mechanisms generated subsequently are futile in offering effective protection. As a result, the *C. catla* treated with the pathogens displayed 87% mortality. The increase in the phagocytic index of fishes treated with extracts after 6 days, 51-54.10% at 200µl concentration, is a clear evidence that *A. paniculate* extracts are able to stimulate the fish immune system. This was also proved by (Basha *et al*., 2013) [6] and also by several other researchers (Jeney and Anderson, 1993; Sahu, 2004; Harikrishnan *et al*., 2009,, Mastan 2015; Zhang *et al*., 2009) [36, 62, 33, 51, 73] also proved an increased PI in *Epinephelus tauvina* treates with Chineese herbs.

Apart from acting as immunostimulants, the fishes fed with the extracts exhibited higher RPS (80%) at a concentration of 50 µl, while conferring complete protection from the pathogens, *A. hydrophila* and *A. veronii*.

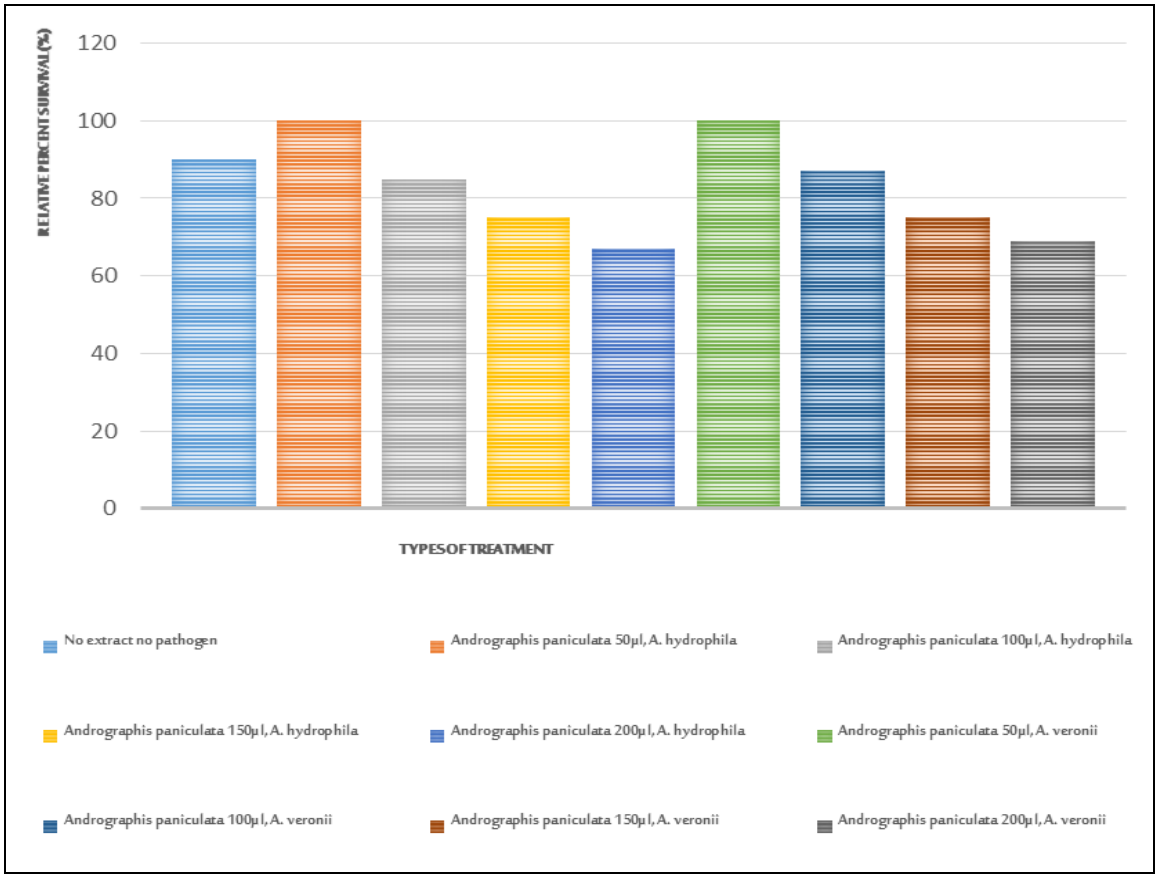

Fig 5: Effect of different doses of *A. hydrophila* extracts to the Relative percentage survival (RPS) of the fish Catla (catla catla)

This was evident by the observation of decreased mortality rates (0 to 30%, post 14 days) in infected *C. catla*, receiving each of the dose of *A. paniculate* (50 to 200 µl). This result was associated with nonspecific immune system performance of the Catla, an important component of the fish immune system. This study was in agreement with the previous test report, in which the injections of *Ocimum sanctum* (Logambal *et al*., 2000) [47] increased *Oreochromis mossambicus* resistance to *A. hydrophila*. The application of *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa* extracts (Balasundaram and Harikrishnan, 2009) [5] to *Carassius auratus* and *Rosmarinus officinalis* (Abutbul *et al*., 2004) [1] extracts enhanced protection against *A. hydrophila* and *Edwardsiella tarda* (Fujiki *et al*., 1994) [22]. The fish *Labeo rohita* gain resistance from the extract of *Achyranthes against* treated with *A. hydrophila* (Joseph and Carnahan, 1994) [38]. However, it was also observed that higher concentrations of *A. paniculate* extracts, more than 50 µl were harmful, that

increased the death rate of *C. catla* to 30% at 200 µl.
C. catla infected with *A. hydrophila* and *A. veronii* exhibited abnormal swimming patterns, including gasping and weakened swimming ability. Remarkably, gasping was not observed in fish treated with the extracts. Some fishes rested on the bottom of the aquarium that exhibited a weakened swimming behaviour.

5. Conclusion
From the results, it is evident that the extracts of *A. paniculata* significantly increased the nonspecific immunity of *C. catla* and was able to inhibit the growth of *A. hydrophila* and *A. veronii.* Besides, the extracts were able to considerably reverse the symptoms of the disease. Hence *A. paniculate* can be proposed as an effective immunostimulant against *A. hydrophila* and *A. veronii*, thereby reducing the ill effects caused by these pathogens in the aquaculture industry.

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