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Polysaccharide fraction from the Indian mistletoe, *Dendrophthoe falcata* (L.f.) Ettingsh enhances innate immunity and disease resistance in *Oreochromis niloticus* (Linn.)

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ABSTRACT

The polysaccharide fraction (PF) isolated from the hemiparasitic mistletoe, *Dendrophthoe falcata* (L.f.) Ettingsh (DF) leaves was tested for its immunostimulatory properties in *Oreochromis niloticus* (Linn.). Different groups of experimental fish were fed for 1, 2 or 3 weeks with three different doses [low (0.01%), mid (0.1%), or high (1%)] of *D. falcata* polysaccharide fraction (DFPF) - supplemented diet. After every feeding regimen, the fish were assessed for non-specific immunological parameters, immune related gene expression and disease protection. The DFPF treated groups showed significant ($P < 0.05$) enhancement of non-specific immune parameters. Significant ($P < 0.05$) upregulation of lysozyme and TNF-α gene expression was observed in DFPF treated groups. In pathogen challenge studies using *Aeromonas hydrophila*, the DFPF treated groups displayed significant ($P < 0.05$) decrease in percentage mortality and the consequent increase in relative percent survival (RPS). Supplementation of 1% DFPF in the feed for a week conferred the maximum protection against the virulent pathogen challenge, recording a RPS of 100. These results suggest that DFPF has the potential to be used as an immunostimulating feed additive in finfish aquaculture.

1. Introduction

Aquaculture is one of the major food producing industries in the world. The intensification of aquaculture practices, leads to overcrowding, periodic handling and transport of fishes and thus pose severe stress on the immune system of fish ultimately resulting in susceptibility to infectious diseases. Infectious diseases and the consequent loss in aquaculture production affects the economic viability of the industry and acts as a major limiting factor for sustainable aquaculture [1]. The approaches to control diseases include application of antimicrobial agents [2] and vaccines [3] in the farm conditions. Of these methods, antibiotics may have negative impact on the, fish's health, also its consumers' health and natural environment. Similarly, vaccines are putatively expensive and protect fish against a particular disease [4]. The alternative approach is the use of plant-derived immunostimulants which in the current scenario, is considered to be the most promising immunoprophylactic method as they are biodegradable, biocompatible (less side effects), effective in protecting against wide range of diseases and cost-effective [5]. The positive impact of immunostimulants in aquaculture and fish immune system has been reviewed by many investigators [6–9]. Polysaccharides derived from various natural sources are being investigated in basic research and also in therapeutics because of their potential to profoundly modulate the immune system [10–12]. The immunostimulatory effects of the purified polysaccharides such as chitin and chitosan [13], β-glucan [14] and crude polysaccharides from plants like *Ficus carica, Radix isatidis* and *Schisandra chinensis* [15] in fishes have been reported elsewhere.

Nile tilapia (*Oreochromis niloticus*), referred to as “aquatic chicken” is an important aquaculture species worldwide and an ideal experimental model because it quickly adapts to laboratory conditions and manipulations [16]. Nile Tilapia feeds at low trophic levels, tastes good and safer for human consumption than the predatory fishes which can accumulate more heavy metals such as mercury [17]. The Gram negative bacterium, *Aeromonas hydrophila* is an ubiquitous pathogen in fresh water habitats [18]. *A. hydrophila*, considered to be an opportunistic pathogen, is commonly found in fresh waters, is the causative agent of haemorrhagic septicaemia, ulcers, dropsy, tail rot and fin rot in various fish species including Nile tilapia, leading to massive mortality

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Dendrophthoe falcata (L.F.) Ettingsh is a hemiparasitic mistletoe, belongs to family Loranthaceae of the order Santalales. The mistletoe is found in India, Sri Lanka, Thailand, Myanmar, Bangladesh, Malaysia, China and Australia. There are 20 species belonging to the genus *Dendrophthoe* of which 7 species are found in India [22]. The medicinal properties of this plant is known to the tribal people of South India and they have used the plant for curing many diseases [23,24]. It is applied for treating skin infections, renal and vesical calculi, impotency, pulmonary tuberculosis, asthma, ulcers, etc. [25]. Administration of *D. falcata* extract resulted in increase in leukocytes in BALB/c mice. The hydroalcoholic extracts of *D. falcata* aerial parts were shown to have immunomodulatory activities in rat [26]. These extracts are safe to use for therapeutic purpose as the experimental Wistar rats did not suffer from any abnormalities after the oral administration [27]. The whole plant (*D. falcata*) has been claimed to have a variety of medicinal properties such as antioxidant, hepatoprotective, contraceptive, antidiabetic, antimicrobial, anthelmintic, antifertility, *anti*-hyperlipidaemia, anticancer activities etc. [25].

There are no reports regarding the polysaccharide fraction of *D. falcata* leaves (DFPF) on fish immunity. Hence, the present work was done to test the effect of oral administration of DFPF as a feed supplement on non-specific, specific immune mechanisms, including the protection on experimental challenge with *A. hydrophila* in *O. niloticus*.

2. Materials and methods

2.1. Experimental animal and facilities

Monosex male Nile Tilapia of 50 ± 5 g size were procured from Svara Biotechnovations, Madurai and used for all the experiments. After acclamation in 500 L fibre reinforced plastic (FRP) tanks for a couple of weeks, the fish were kept in 150 L FRP tanks with attached canister external filter (Eheim, Deizisau, Germany) for water recirculation, oxygenation and removal of ammonia. Water quality criteria including pH – 7.5, dissolved oxygen – 5 ppm, TDS – 400–450 ppm were monitored and maintained in the tanks throughout the experimental period. All the experiments were carried out in ambient photoperiod and water temperature (28 ± 2 °C). Excess feed and faecal waste were siphoned out daily and a quarter portion of water in the tank is replaced with fresh water on alternate days. All the experimental procedures complied with Jenkins group's 'guidelines for use of fish in research' [28].

2.2. Preparation of DFPF and supplemented feed

D. falcata that has parasitized neem tree, *Azadirachita indica* in the premises of Madurai Kamaraj University, Madurai (9.9404°N, 78.0105°E) were collected and its taxonomy was confirmed by Dr. D. Stephen (Assistant Professor, Department of Botany, The American College, Madurai, India). A sample of the mistletoe (specimen no: CFI MP5) was deposited in the herbarium of the Department of Botany, Lady Doak College, Madurai.

D. falcata leaves were cleansed with distilled water, shade-dried for 10days, and then hand-crushed. DFPF was prepared as described by Harborne [29] with minor modifications. Briefly, 100 g of *D. falcata* leaf flakes were immersed in 1 L methanol: distilled water (4:1) and kept at 15 °C for 7 days with intermittent mixing. Using sterilized muslin cloth (grade 50, 28 × 24 threads $inch^{-1}$, 1 mm pore size), the contents were filtered to collect the residue. The residue was taken in a fresh beaker and about 500 mL boiling ethanol (90 °C) was added till it became colourless. The residue was treated with 1 L ethyl acetate for 20 min and filtered. After discarding the residue, the filtrate was boiled (90 °C) in 1% sodium chloride solution (800 mL). To this mixture 1000–1500 mL of ethanol was added till the neutral, water-soluble polysaccharides were precipitated from the solution. This was again transferred to a rotary vacuum evaporator (Buchi, Flawil, Switzerland) for concentrating the solution. Concentrated extracts were later air dried in small glass Petri plates to fine powder of DFPF and was stored at −20 °C until used further.

The composition of basal diet is groundnut oil cake 25%, wheat flour 15%, fish meal 42%, tapioca flour 15%, minerals and vitamins mixture 3%. Required amount of the basal diet, after its preparation, was supplemented with DFPF or MacroGard™ (MG, a yeast derived β-glucan, kindly gifted by Dr. P.K. Sahoo, Central Institute for Freshwater Aquaculture, Bhubaneswar, India). All the ingredients were mixed thoroughly using a blender, passed through the presser and the long slender threads made were air dried and stored in 4 °C. Analysis of feed showed that it contains lipid-11% and ash-9%, carbohydrate-24%, protein-39% [30].

2.3. Experimental design

The experiments were carried out according to the scheme outlined below:

In brief, fish were randomly separated into five groups namely an untreated control group, three experimental groups (treated with different doses (0.01%, 0.1% and 1%) of DFPF feed supplement and a positive control (0.1% MacroGard™ (MG) - treated) group. Each group was maintained in triplicates in all experiments.

2.4. Collection of serum

Twelve fish (four fish from each tank) were randomly caught from each group and were bled at the end of each feeding schedule. For bleeding, the fish were anaesthetized by keeping them in water containing 100 ppm 2-phenoxyethanol (HiMedia, Mumbai, India) for 5–10 min till they are motionless. Blood (250 μL) was collected from common cardinal vein of tilapia using 1 mL tuberculin syringe fitted with a 24 gauge needle [31] and transferred into sterile serological tubes. Blood was allowed to clot for 30 min at room temperature and kept overnight in a refrigerator. Next day the tubes were centrifuged for 10 min at 400 × *g* for serum separation. The serum separated was stored at −20 °C until used for experiments.

2.5. Serum lysozyme activity

Serum lysozyme activity was quantified by the turbidimetric method of Parry, Chandan [32] with microplate modification of Hutchinson and Manning [33]. In a 96-well "U" bottom microtitre plate, 10 μL serum/well was added followed by addition of 250 μL *Micrococcus lysodeikticus* (0.3 $mgmL^{-1}$; Sigma-Aldrich, St. Louis, USA) cell suspension in 0.05 M sodium phosphate buffer, pH 6.2. After incubation of plates at 28 °C for 0.5 and 4.5 min, the reduction in absorbance at 490 nm in a microplate reader (Bio-Rad, Hercules, USA) was noted. One unit of lysozyme activity was defined as the reduction in absorbance units by 0.001 min^{-1} [34].

2.6. Serum antiprotease activity

Serum antiprotease activity was measured following the method of Bowden, Butler [35]. Sera from control and treated fish (10 μL) were incubated with 20 μL 0.1% trypsin (HiMedia, Mumbai, India) in 0.01 M Tris HCl (pH 8.2) for 5 min in 1.5 mL tubes. After incubation, 500 μL of the substrate, sodium-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA, SRL, Mumbai, India) was added to each tube. Following the incubation for 25 min at 22 °C, 470 μL 0.1 M Tris HCL (pH 8.2) was added to each tube to make up to 1 mL. Addition of 150 μL 30% acetic acid to the tubes ceased the reaction. Finally, 200 μL of this yellow coloured reaction mixture was transferred to a 96 well microplate and optical density at 415 nm was measured. The inhibitory capacity of antiprotease activity was calculated regarding the percentage of trypsin inhibition as reported by Ref. [36].

$$\text{Percentage trypsin inhibition} = \frac{\text{trypsin blank OD} - \text{test sample OD} \times 100}{\text{Trypsin blank OD}}$$

2.7. Leukocytes separation from peripheral blood

To assess the cellular immune parameters, the leukocytes from peripheral blood were collected according to the procedure of Miller and Mc Kinney [37] with a few changes. After anaesthetizing the fish with 2-phenoxyethanol, fish were bled (500 μL) from the common cardinal vein using 5 mL syringe (24 gauge needle) loaded with 2 mL blood collecting medium (RPMI-1640 (HiMedia, Mumbai, India) with 50,000 IUL^{-1}sodium heparin, 1,00,000 IUL^{-1} penicillin and 100mgL^{-1} streptomycin) and was immediately overlaid on 1 mL Ficoll (HiSep, HiMedia, Mumbai, India). The tubes were spun at 400 × *g* for 20 min at 4 °C. The leukocytes in the interface were collected and transferred to a fresh centrifuge tube and were washed twice with 2 mL wash medium (RPMI-1640 supplemented with 10,000 IUL^{-1} sodium heparin, 1,00,000 IUL^{-1} penicillin and 100 mgL^{-1} streptomycin) and finally suspended in 1 mL of cell culture medium (RPMI-1640 with 3% of pooled Tilapia serum, 4 mM Glutamine 1,00,000 IUL^{-1} penicillin and 100 mgL^{-1} streptomycin). The number of live cells were counted using 0.25% (v/v) trypan blue (HiMedia, Mumbai, India) exclusion method using a haemocytometer and by using cell culture medium, adjusted to 4×10^7 $cellsmL^{-1}$

2.8. Measurement of reactive oxygen species (ROS)

The intracellular respiratory burst activity was quantified according to the method of Secombes [38] with minor modifications. Peripheral blood leucocytes (10^6 cells) in 175 μL cell culture medium were transferred to microtitre wells in triplicates and treated for 2 h at 28 °C with 25 μL of nitroblue tetrazolium (NBT,$1gL^{-1}$; HiMedia, Mumbai, India). After the treatment, cells were fixed by adding 200 μL of 100% methanol to each well and leaving the plate for 5 min at room temperature. Then, the fixed cells were washed two times with 125 μL of 70% methanol. The plates were left to air-dry overnight in order to remove methanol completely. The NBT in the reduced form appearing as purple formazan precipitate was dissolved with 125 μL 2 N potassium hydroxide and 150 μL dimethyl sulphoxide to measure OD at 650 nm in a microplate reader.

2.9. Production of reactive nitrogen species (RNI)

RNI released by peripheral blood leucocytes in the medium was quantified using Griess reagent [39]. In a 96 well flat bottom plate, peripheral blood leucocytes (10^6 cells per well, in triplicates) were plated using 175 μL culture medium and incubated at 28 °C for 96 h. After 96 h, 50 μL culture supernatant was transferred to a new plate. To this, equal volume of Griess reagent (1% sulphanilamide, 2.5%phosphoric acid 0.1% N-naphthyl-ethylenediamine) was added. After incubating for 10 min, OD was read at 570 nm. Molar concentration of nitrite in culture medium was then read from a standard curve plotted earlier using a range of known $NaNO_2$ concentration.

2.10. Myeloperoxidase activity (MPO) in peripheral blood leucocytes

The total peroxidase content in peripheral blood leucocytes was quantified following the method described by Palić, Andreasen [40] with minor modifications. In a 96-well flat bottom plate, 25 μL cell suspension (10^6 cells per well in triplicate) were taken and treated for 20 min with 125 μL of 0.02% (w/v) cetyltrimethyl ammonium bromide (HiMedia, Mumbai, India). After the treatment, 50 μL 3, 3′, 5, 5’, -tetra methyl benzidine hydrochloride – H_2O_2 (Sigma, Bengaluru, India) was added. Plates were left undisturbed for 2 min in order to develop blue colour. To terminate colour development, 50 μL 2 M sulphuric acid (Merck, Bengaluru, India) was added. Plates were centrifuged for 15 min at 600 × *g* and 200 μL of supernatant was taken in a new microtitre plate and OD was read at 450 nm.

2.11. Immune gene expression

To study the modulation of TNF-α and lysozyme gene expression by DFPF in Nile tilapia by RT-PCR, 3 fish in each group were sacrificed using an overdose of 2-phenoxyethanol and spleen was excised aseptically. Excised spleens were collected separately in 1.5 mL tubes containing RNALater (Sigma, Bengaluru, India) and immediately stored at −20 °C till RNA extraction.

Total RNA was extracted from the spleen using Trizol reagent (Sigma-Aldrich, USA) based on the manufacturer's guidelines. cDNA was synthesised from the total RNA using Omniscript® reverse transcription kit as per the manufacturer's protocol (Qiagen, Germany). Briefly, in 0.2 mL sterile PCR tube, 3 μL total RNA, 2 μL oligo-dT primer (10 μM), 2 μL 5 mM dNTP mix, 2 μL 10X RT buffer and 1 μL RT enzyme were added and volume was made to 20 μL with sterile water. The reaction mixture was incubated for 1 h at 37 °C in a thermocycler (Eppendorf Mastercycler®Nexus, Hamburg, Germany) and cDNA was synthesised. The synthesised cDNA (3 μL) was then amplified with 1 μL respective primers (25 pM) using 10 μL REDTaq®ReadyMix™ PCR reaction mix (Sigma-Aldrich, USA). PCR program was initial denaturation: 95 °C 5 min, 40 cycles of 95 °C 15 s, annealing temperature 60 s, 72 °C 30 s and final extension: 72 °C for 5 min. The primer sequences and annealing temperatures were given in Table 1 β-actin was employed as internal reference gene for PCR reaction. The amplicons were loaded in 1.5% agarose gel stained with ethidium bromide (10 μgmL^{-1}) and the bands were photographed using a gel-documentation unit (AlphaImager, Protein Simple, New Delhi, India). The intensity of bands was analysed using ImageJ software [41]. Data were then presented as ratio of expression (relative expression) of gene of interest to that of the expression of β-actin [20].

2.12. Disease resistance test

At the end of 1, 2 or 3 weeks feeding, all the groups were challenged with pre-determined challenge dose (LD_{50} dose) of live virulent *A. hydrophila*. The cumulative mortality of fish in each triplicate group was noted up to 15 days post challenge. Sample of peritoneal wash from the dead fish added in Aeromonas selective, Rimler-Shott's medium (HiMedia, India) indicated the presence of *A. hydrophila.* The percentage mortality and relative percentage survival (RPS) were then

Table 1
List of primer sequences used in this study.

No.	Gene Name	Primer sequence (5′ to 3′)	Annealing temperature (°C)	Product size (bp)	Reference
1.	β-actin	F:CCACACAGTGCCCATCTACGA R:CCACGCTCTGTCAGGATCTTCA	60	110	[84]
2.	Lysozyme	F: TTGGGAGTGTTCAACAGTGG R: GCCTCTGACAGCATTTGACA	60	300	This study
3.	TNF-α	F: CCTGGCTGTAGACGAAGT R: TAGAAGGCAGCGACTCAA	55	124	[85]

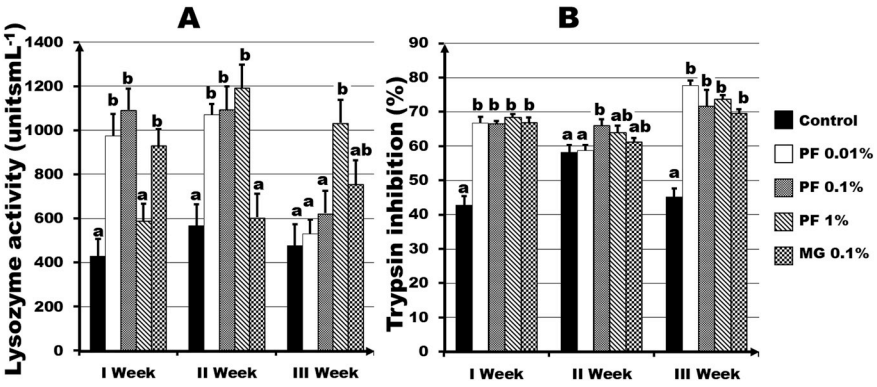

Fig. 1. Effect of 1, 2 and 3 weeks feeding with diet supplemented with DFPF on serum lysozyme activity**(A)** and antiprotease activity **(B)**. Data represents mean ± SEM of 12 fish. Different alphabets represent significant difference ($P < 0.05$) between means as assessed by one-way ANOVA with aposteriori Tukey's comparison of control and treated groups.

determined. The formula for RPS is

$$\text{RPS} = 1 - \left\{\frac{\text{Percentage mortality in treated group}}{\text{Percentage mortality in control group}}\right\} \times 100$$

2.13. Statistical analysis

Data were presented as mean ± SEM. Means were compared by one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison aposteriori test. Means were considered statistically different if $P < 0.05$. For statistical analyses, Sigmaplot v11.0 (Systat Software, San Jose, CA) was employed.

3. Results

3.1. Serum lysozyme activity

After 1 week feeding, there was a significant ($P < 0.05$) increase in serum lysozyme activity in the groups fed with 0.01%, 0.1% DFPF or (0.1%) MG (Fig. 1A). In groups fed for 2 weeks, while all the doses of DFPF significantly ($P < 0.05$) increased lysozyme activity, the positive control, MG did not enhance the activity. In 3 weeks, among the experimental groups and the positive control, only the group fed with 1% DFPF caused a significant ($P < 0.05$) increase in lysozyme activity.

3.2. Serum antiprotease activity

Trypsin inhibitory activity was significantly ($P < 0.05$) increased in all the groups fed with DFPF supplemented diets after 1 or 3 weeks (Fig. 1B). However, after 2 weeks feeding, only 1% DFPF caused an increase in antiprotease activity. The positive control MG recorded significant ($p < 0.05$) trypsin inhibition upon 1 and 3 weeks feeding.

3.3. ROS production

ROS production by peripheral blood leukocytes was significantly ($P < 0.05$) enhanced in the groups orally administered with 1% DFPF or MG for 1, 2 or 3 weeks. However, 0.1% DFPF fed group recorded significant ($P < 0.05$) increase of ROS production only after 1 or 2 weeks' administration of DFPF. The low dose of 0.01% DFPF could significantly ($P < 0.05$) enhance ROS production only after two weeks administration of DFPF (Fig. 2A).

3.4. RNI production

The groups fed with 0.1% and 1% DFPF and MG, recorded an increase ($P < 0.05$) in RNI production on after first week feeding. At the end of the second week, there was a significant ($P < 0.05$) increase in production of RNI by all the treated groups including MG. But on the end of third week, only 1% DFPF recorded enhanced ($P < 0.05$) RNI production (Fig. 2B).

3.5. MPO activity

As shown in Fig. 2C, after 1 and 3 weeks feeding, all the treated groups including the positive control showed a significant increase in peripheral blood leukocyte MPO activity ($P < 0.05$). However, after two weeks treatment, the increase ($P < 0.05$) was seen only in the 0.1% DFPF group and even positive control, MG did not enhance the enzyme activity after 2 weeks of feeding.

3.6. Gene expression studies by RT-PCR

Expression of lysozyme gene was significantly higher ($P < 0.05$) in all the treated groups including positive control group after 1 and 3 weeks feeding. However, after 2 weeks feeding, only 1% DFPF and MG treated groups recorded significant ($P < 0.05$) increment in the lysozyme gene expression (Fig. 3A). Regarding TNF-α gene expression, while fish fed with 1% DFPF displayed significant ($P < 0.05$) increment irrespective of the feeding duration, the MG treated group exhibited enhanced expression only after3 weeks feeding. The rest of the experimental groups did not display any significant increase in TNF-α expression in any of the feeding regimens (Fig. 3B).

3.7. Disease resistance test

In disease resistance test (Fig. 4A), dose dependent decrease of percentage mortality was observed in the DFPF-treated groups. Fig. 4B shows that 1% DFPF treated groups recorded maximum RPS values of 100, 61 and 50 in 1, 2 and 3weeks feeding regimen respectively. The group fed with 0.1% DFPF achieved RPS values of 92, 46, 29 and 0.01% DFPF caused an RPS of 71, 38, 15 in 1, 2 and 3 weeks feeding respectively. MG (0.1%) though significantly reduced the percent mortality; it could achieve 'not so impressive' RPS values of 35, 53 and 36 after 1, 2 and 3 weeks respectively.

4. Discussion

The route of delivering immunostimulants in fish can be parenteral or oral. Between these, oral route of delivery of immunostimulant as feed supplement is considered to be the best method because it is not labour intensive and can be applied to large number of fishes [8]. Hence, in the present study, DFPF were incorporated in the fish diet to assess its immunostimulatory effect in Nile Tilapia.

There are various methods of polysaccharides isolation [42] and the extraction method applied is crucial in determining the immunomodulatory effect of the plant derived extract. For example, aqueous and alcoholic extracts of *Panax ginseng* deactivate and activate toll like receptors respectively [43]. In the present study, polysaccharide separation was carried out according to the method of

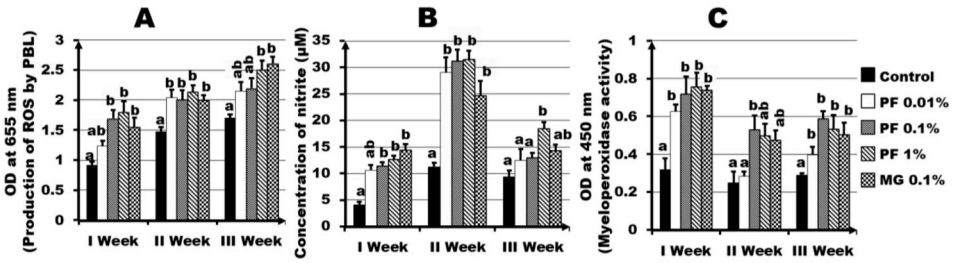

Fig. 2. Effect of 1, 2 and 3 weeks feeding with DFPF supplemented feed on ROS **(A)**,RNI**(B)** production and leukocyte MPO activity **(C)**by peripheral blood leukocytes. Data represents Mean ± SEM of 12 fish. Different alphabets represents significant difference ($P < 0.05$) between means as assessed by one-way ANOVA with aposteriori Tukey's comparison of control and treated groups.

Harborne [29] with minor modifications as has been adopted earlier in this laboratory for its separation from brown alga, *Padina gymnospora* [44] and chlorophycean macroalga, *Caulerpa scalpelliformis* [45]. As with our earlier studies, DFPF extracted by Harborne method showed excellent immunostimulating and disease resistance properties.

Lysozyme is one of the prevalent bactericidal enzymes and is the first line of defence against bacterial infections. With multifaceted activities including anti-inflammatory, anti-viral and phagocytic stimulating activities, lysozyme plays a crucial role in innate immunity [46]. The present study reports significant enhancement of serum lysozyme activity by DFPF. This finding is in line with other studies where administration of *Ulva rigida* polysaccharides in grey mullet *Mughil cephalus* [47], β-glucan in *Pangasianodon hypophthalmus* [14], yeast derived β-glucan in Persian Sturgeon [48], resulted in enhanced lysozyme activity. β-glucan from various sources also increased lysozyme activity in other fishes like *Pseudosciaena crocea* [49], *Dicentrarchus labrax* [50], and *Salmo salar* [51]. Phagocytic cells are the major producers of lysozyme [52].

Antiprotease namely α1-antitrypsin, α2-antiplasmin,α-2-macroglobulin inhibit the proteases produced by pathogenic bacteria that break down the host tissues for accessing nutrients and invades the host cell to grow in vivo [35,53]. Host antiprotease do not show any toxicity towards the self but they act specifically against the bacterial proteases [54]. Production of α-2-macroglobulin resistant protease by *Aeromonas salmonicida* was considered to be one of its important pathogenic adaptation [55]. In the present study, DFPF supplemented diet significantly increased serum antiprotease activity against the test protease, trypsin in the fish after one and three weeks feeding regimens. The result is in agreement with other findings, such as dietary administration of chitin and chitosan in *Epinephelus bruneus* [56], intraperitoneal administration of *C. scalpelliformis* polysaccharides in *O. niloticus* [57] that showed enhanced serum antiprotease activity.

During phagocytosis, activated neutrophils and macrophages produce ROS namely singlet oxygen, hydrogen peroxide, superoxide anion [58] by intense uptake of oxygen and the process is called respiratory burst activity. These ROS are toxic products that limit the growth of bacterial pathogens in fish [59,60]. The results showed that there was significant increase of ROS production irrespective of the dose of DFPF and duration of its administration. This enhanced production might be due to the proliferation of leukocytes as observed in rainbow trout, *Oncorhynchus mykiss*, in which β-glucan feeding for 4 weeks showed increased levels of phagocytosis and ROS production [61]. In rainbow trout, due to immunostimulant treatment, NBT-positive cells in blood were steadily increased [62]. Hence, it could be suggested that DFPF could have activated the phagocytic cells resulting in enhanced free radicals production. ROS production by phagocytic cells has been shown to be an important marker for disease resistance in fish [63]. Hence, increased production of ROS by DFPF could have increased the disease resistance resulting in higher RPS.

Macrophages and neutrophils also produce RNI and they show potent cytotoxicity against bacterial and protozoan pathogens [64]. DFPF treated groups showed significant increase in RNI production after 1 and 2 weeks treatments. Similarly, the acidic polysaccharides from *U. rigida* have been shown to cause macrophages producing more amount of nitrite [65]. *Tinospora cordifolia* leaf extracts in *O. mossambicus* [66] and an α-D-glucan isolated from the same plant caused an increase in nitrite production [67]. However, in another study, the supplementation of Astragalus polysaccharides, did not have any effect on NO production in Nile Tilapia [68].

Fish neutrophils contain MPO enzyme, a haeme-containing lysosomal glycoprotein in their azurophilic granules. MPO enzyme upon activation catalyses hydrogen peroxide conversion to hypohalous acids which are detrimental to the survival of pathogens [69]. Deficiency of MPO enzyme has deteriorated the host immunity against the pathogen challenge suggesting its anti-inflammatory role [70]. Dietary administration of DFPF resulted in significant enhancement of leukocyte MPO activity in this study. Similar enhancement of MPO activity was also observed in this laboratory on dietary administration of polysaccharides of *C. scalpelliformis* (unpublished).

The ultimate aim of using immunostimulants is protection against pathogens and it can be tested by the relative percent survival of the treated fish after their experimental infection with live virulent pathogen [71,72]. The fish fed with DFPF supplemented diet decreased the percentage mortality significantly compared to that of the untreated

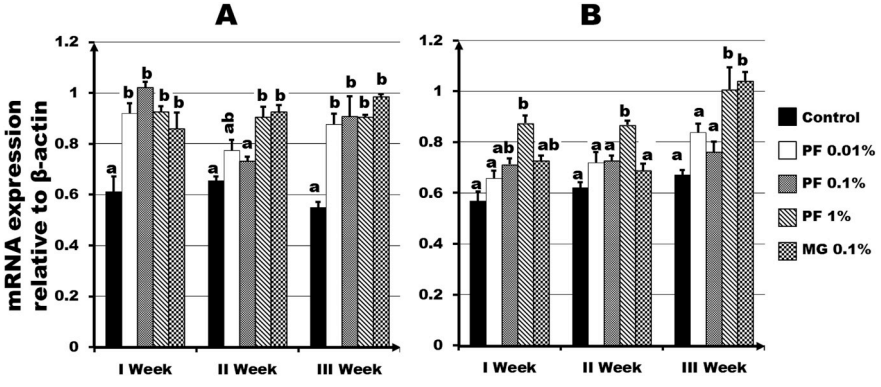

Fig. 3. Effect of 1, 2 and3 weeks feeding with DFPF supplemented diet with the gene expression of lysozyme **(A)** and TNF-α **(B)** Data represents Mean ± SEM of 3 fish. Different alphabets represents significant difference ($P < 0.05$) as calculated by one-way ANOVA with Tukey's aposteriori test.

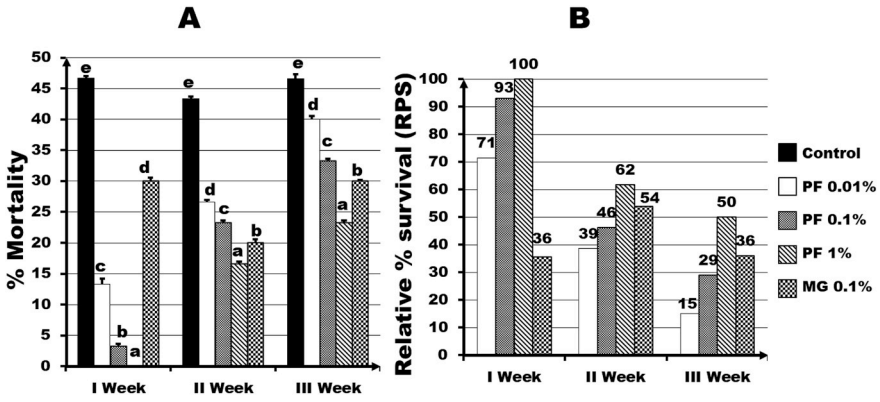

Fig. 4. Effect of 1, 2 and 3 weeks feeding with DFPF supplemented diet on disease resistance in terms of percentage mortality **(A)** and relative percentage survival, RPS **(B)**. Each column represents mean and bars represent S.E of 10 fish in triplicates. Different alphabets represents significant difference ($P < 0.05$) as calculated by one-way ANOVA with Tukey's aposteriori test.

control group. The observed results are in agreement with other reports wherein the polysaccharides of *P. gymnospora* in *Cyprinus carpio* gave protection against *A. hydrophila* and *Edwardsiella tarda* [44] and feeding of β-glucan in *Pangasianodon hypophthalmus* caused improved survival rate against *E. ictaluri* [73]. In another study, dietary microbial levan fed *C. carpio* gave a RPS value of 100 against *A. hydrophila* [74]. In all these studies, relevant nonspecific immune responses were also elevated.

In the present study, DFPF has enhanced expression of lysozyme gene. Dietary administration of polysaccharides of *P. gymnospora* has been shown to increase the expression levels of lysozyme in head kidney cells of *C. carpio* [75]. Intraperitoneal administration of polysaccharides of *C. scalpelliformis* in Nile Tilapia [45]; *Astragalus* polysaccharides in *C. carpio* [76] upregulated the expression of TNF-α and lysozyme in spleen cells. Nile tilapia fed with β-1,3 glucan exhibited enhanced production of cytokines like IL-10, TNF-α, IL-1β, IL-12 in fish plasma [77]. Yang et al. [78] reported that *Ficus carica* polysaccharides as feed supplement augmented the cytokine genes expression. There are many convincing evidences that polysaccharides serve as potent immunostimulants, positively modulating the immune system thereby improving the immune responses and disease resistance in fishes. In the present study, DFPF was responsible for the observed immunostimulatory effects and enhancement of protection of Nile tilapia against *A. hydrophila*. Polysaccharides are directly involved in activation of macrophages [79] and are responsible for the enhancement of non-specific immunity. The immunostimulants can elicit an immune response after binding with their receptors on target cells, eventually leading to the production of antimicrobial molecules [80]. Dectin-1 [81] receptor on natural killer cell, c-type lectin in connection with Toll-like receptor-4, complement receptor-3 involved in innate immunity etc. are suggested to be the receptors for polysaccharides. These receptors upon activation induce various intracellular signalling pathways leading to the synthesis of pro-inflammatory cytokines [82,83]. The immunostimulatory effects of DFPF were perhaps due to the activation of any or some of the above mentioned polysaccharides receptors. More studies in this line are required to delineate the mechanism of action of DFPF.

5. Conclusion

To conclude, in Nile Tilapia, DFPF-supplemented diet has enhanced the innate immune mechanisms which conferred protection or resistance against the experimental challenge with *A. hydrophila*. DFPF augmented TNF-α and lysozyme genes' expression. More detailed studies are required on dose and duration regimens to determine the optimal values and also the efficacy of the candidate immunostimulant should be tested in various culture species against different common fish pathogens before application to finfish aquaculture.

Conflicts of interest

The authors have no conflicts of interest to declare.

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