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Polysaccharide fraction of the hemi-parasitic mistletoe, *Dendrophthoe falcata* (L) Ettingsh leaves enhances innate immune responses and disease resistance in *Oreochromis niloticus* (Linnaeus)

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ABSTRACT

In aquaculture, diseases and disease-outbreak are a major factor limiting its sustainability. Immunostimulants are an effective alternative to antibiotics, chemotherapeutics and perhaps even vaccines. Immunostimulants are a promising and safe prophylactic measure against infectious diseases. In this study, the immunostimulatory effect of the neutral water-soluble polysaccharide fraction (PF) isolated from the leaves of the hemi-parasitic mistletoe, *Dendrophthoe falcata* (DF) was assessed in Nile tilapia, *Oreochromis niloticus*. Fish weighing 40±5 g were injected intraperitoneally with *Dendrophthoe falcata* polysaccharide fraction (DFPF) at a dose of 2, 20 or 200 mgkg^{-1}. An untreated control group and a positive control group (MacroGard™, 20 mgkg^{-1}) were also maintained. The serum lysozyme, myeloperoxidase, antiprotease and bactericidal activities were significantly enhanced on post-treatment days when compared with that of the untreated control. In the challenge studies, after 7 and 21 days' administration of DFPF, the high dose of 200 mgkg^{-1} caused a significant reduction in percent mortality against *Aeromonas hydrophila* infection. By semi-quantitative gene expression analysis, it was found that there was also a significant upregulation of IL-1β and lysozyme genes. After field trials, DFPF can be applied as an immunostimulant to finfish in aquaculture.

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INTRODUCTION

The aquaculture industry is an important food-producing sector that caters for the growing demand for food fish. For sustainable aquaculture, a safe and promising alternate prophylactic approach is the use of plant-derived immunostimulants since they are active against a range of pathogens, economical and are biodegradable (Awad and Awaad 2017).

Dendrophthoe falcata (L) Ettingsh is a hemi-parasitic mistletoe commonly found in India. Pattanayak and Mazumder (2011) reported the immunostimulatory activity in terms of phagocytic activity, antibody response etc. of hydroalcoholic extract of *D. falcata* in the rat. Dashora, Sodde *et al.,* (2011) have shown its *in vivo* anticancer activity in Swiss albino mice.

All parts of *D. falcata* are used in the traditional Indian system of medicine as a cooling, astringent, aphrodisiac, narcotic and diuretic agent (Aleykutty, Srinivasan *et al.,* 1993). *D. falcata* is known to ameliorate pulmonary tuberculosis,

asthma, menstrual disorder, wound-healing, paralysis, immunomodulation, ulcers, renal and vesicle

calculi (Pattanayak and Sunita 2008; Sinoriya, Sharma *et al.,* 2011).

The structural complexity of polysaccharides warrants different isolation and extraction procedures. Polysaccharides are isolated from fungi, algae, plants, mushroom, lichens, and seaweeds, but among these sources, a few only have been extensively studied. Polysaccharides were reported to have anti-tumour, anti-viral, hypoglycemic effects, anti-inflammatory and anti-complementary effects (Shi 2016) Interestingly, most of these bioactivities are related to the immune system (Xie, Jin *et al.,* 2016). Plant polysaccharides are relatively non-toxic, and they are potent activators of macrophages, in terms of reactive oxygen/nitrogen species production and increased secretion of cytokines, IL-1β and TNF–α (Schepetkin and Quinn 2006).

Nile Tilapia (*Oreochromis niloticus*) is the second largest farmed finfish species after carps (Liu, Zhu *et al.,* 2016). In freshwater aquaculture, *Aeromonas hydrophila*, the motile gram-negative bacteria is the causative agent of haemorrhagic septicaemia, tail and fin rot diseases (Austin and Austin 2016).

The present study reports the possible immunostimulating properties of intraperitoneally administered neutral, water-soluble polysaccharide fraction from the leaves of *D. falcata* (DFPF), in terms of the non-specific immune mechanisms, disease resistance tests and the expression of immune-related genes in *O. niloticus*.

MATERIALS AND METHODS

Experimental animal

Male *Oreochromis niloticus* weighing 40±5 g (n=500) were procured from a tilapia farm (Svara Biotechnovations, Madurai, Tamil Nadu) and acclimated for 2 weeks. Fibre reinforced plastic tanks (150 L) fitted with external canister filters (Eheim, Deizaisau, Germany) to remove ammonia and to provide continuous aeration was used to maintain fish. Daily, the faecal matter and uneaten feed remains were syphoned out, and half of the fish tank water was replaced with fresh water. Fish were maintained in an uncontrolled ambient temperature (28 ± 1°C) under natural photoperiodicity. Water quality parameters including pH at 7.5 and dissolved oxygen at 5 ppm were maintained. Fish were fed *ad libitum* at twice a day with commercial pellet feed 'Grobest' (Growel feeds, Andhra Pradesh, India).

Plant material and polysaccharide preparation

D. falcata parasitised on *Azadirachta indica* was collected from Madurai Kamaraj University campus, Madurai, Tamil Nadu. The collected plant material was identified as *D. falcata* by Dr Stephen, Department of Botany, The American College, Madurai, Tamil Nadu, India. A specimen (No. CFI MP5) of collected *D. falcata*, was submitted to herbarium of School of Life Sciences, Vels Institute of Science Technology and Advanced Studies, Chennai. The leaves were briefly washed with distilled water followed by two days of shade drying. Fractionation of *D. falcata* using Harborne (1998) method with minor modifications of Yengkhom, Shalini *et al.,* (2018) was done.

Experimental design

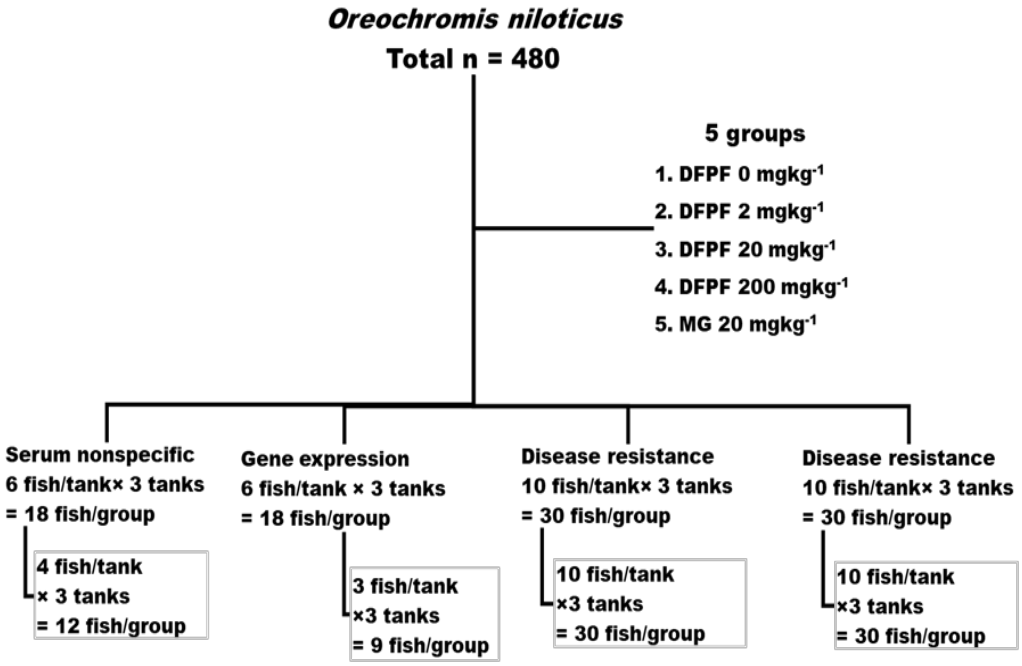

Figure 1: Experimental design

Five groups of fish in triplicates were used for the experiment. Three groups were intraperitoneally administered with 2, 20 and 200 $mgkg^{-1}$ DFPF. The other two groups were injected with sterile distilled water (untreated control, DFPF 0 $mgkg^{-1}$) and yeast-derived immunostimulant, MacroGard™ (positive control, 20 $mgkg^{-1}$) respectively. Sterile distilled water was used to prepare the doses and was passed through 0.45 µm syringe filters (HiMedia, India) before administration. The experimental setup and number of fish used were schematically represented below:

Repetitive bleeding and serum Separation

Common cardinal vein bleeding of fish (Michael 1994) was done repetitively at an interval of 5 days. Before bleeding, the fish were anaesthetised by keeping them in 100ppm of 2-Phenoxyethanol (HiMedia, India) for 5 minutes. Serum was separated according to our earlier procedures (Yengkhom, Shalini *et al.,* 2018).

Serological assays

Turbidimetry based lysozyme assay reported by Parry, Chandan *et al.,* (1965) with the microplate adaptation (Hutchinson and Manning 1996) was used to measure serum lysozyme activity. A decrease in absorbance by 0.001 $unitsmin^{-1}$ is defined as one lysozyme unit (Stolen 1990).

The method of Quade and Roth (1997) improvised by Sahoo, Kumari *et al.,* (2005) was used to measure MPO activity.

Percentage of trypsin inhibition was calculated using the method of Bowden, Butler *et al.,* (1997). The antiprotease activity calculated in terms of percentage of trypsin inhibition using the formula of Zuo and Woo (1997). Serum bactericidal activity was determined by the method of Welker, Lim *et al.,* (2007).

$$\% \text{ Trypsin inhibition} = \frac{\text{Trypsin blank OD-sample OD}}{\text{Trypsin blank OD}} \times 100$$

Gene expression studies by Semi-quantitative PCR

After 24 hours of immunostimulants administration, fish were euthanized using an overdose of 2-phenoxyethanol. Spleen has collected aseptically from the fish and stored in RNAlater (Sigma-Aldrich, USA) at -20^{0}C for further processing.

Total RNA was isolated using Trizol (Sigma-Aldrich, USA), cDNA conversion was done using Omniscript® Reverse transcription kit (Qiagen, Germany) and the resulting cDNA was amplified using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, USA) were performed according to manufacturer's protocols. PCR reaction (20 µl) contained 25 pM primers (Table 1), 5 µl cDNA. Electrophoresis was done according to Yengkhom, Shalini *et al.,* (2018) and analysis was done using ImageJ v1.50b software (Schneider, Rasband *et al.,* 2012) for Windows.

Disease resistance test

Methods described by Yengkhom, Shalini *et al.,* (2018) were used to test the disease resistance efficacy of DFPF administered 7 or 21 days prior to challenge with LD_{50} of *A. hydrophila.*

Fourier transformed - infrared spectrum (FT-IR) of DFPF

DFPF was subjected to FT-IR analysis using Schimadzu FT-IR (Madhura College, Madurai) using standard KBr pellet method.

Statistical analysis

Data are shown as arithmetic mean± standard error and analysed using Sigmaplot v.11. Means were compared by one-way ANOVA with Tukey's pairwise comparison of means. If $P<0.05$, means were considered to be significantly different from each other.

RESULTS

Serum lysozyme activity: Lysozyme activity of the groups treated with low and mid doses of DFPF was significantly higher on all the days tested. High dose of DFPF significantly enhanced lysozyme activity only on 15 and 20 days post-treatment (Fig.1A). MacroGard™ increased lysozyme activity on day 15 and 20 only.

MPO activity

As shown in figure 2B, the low and mid doses of DFPF have significantly enhanced the MPO activity on all days tested. However, the high dose has enhanced the serum MPO activity only on day 5. The positive control, MacroGard™ enhanced the serum MPO activity significantly on all the days tested.

Serum antiprotease activity

As shown in figure 2C, all the DFPF treated groups exhibited a significantly higher antiprotease activity on day 5. On day 10, the mid and high doses showed significantly increased antiprotease activity. On day 15, the mid dose caused the enhancement, but on day 20, no modulation was observed. MacroGard™ has enhanced the antiprotease activity on days 5, 10 and 15.

Serum bactericidal activity

Different doses of DFPF have enhanced the bactericidal activity on different days without any dose showing any persisting pattern. Thus, as shown in figure 2D, while the low dose showing any persisting pattern.

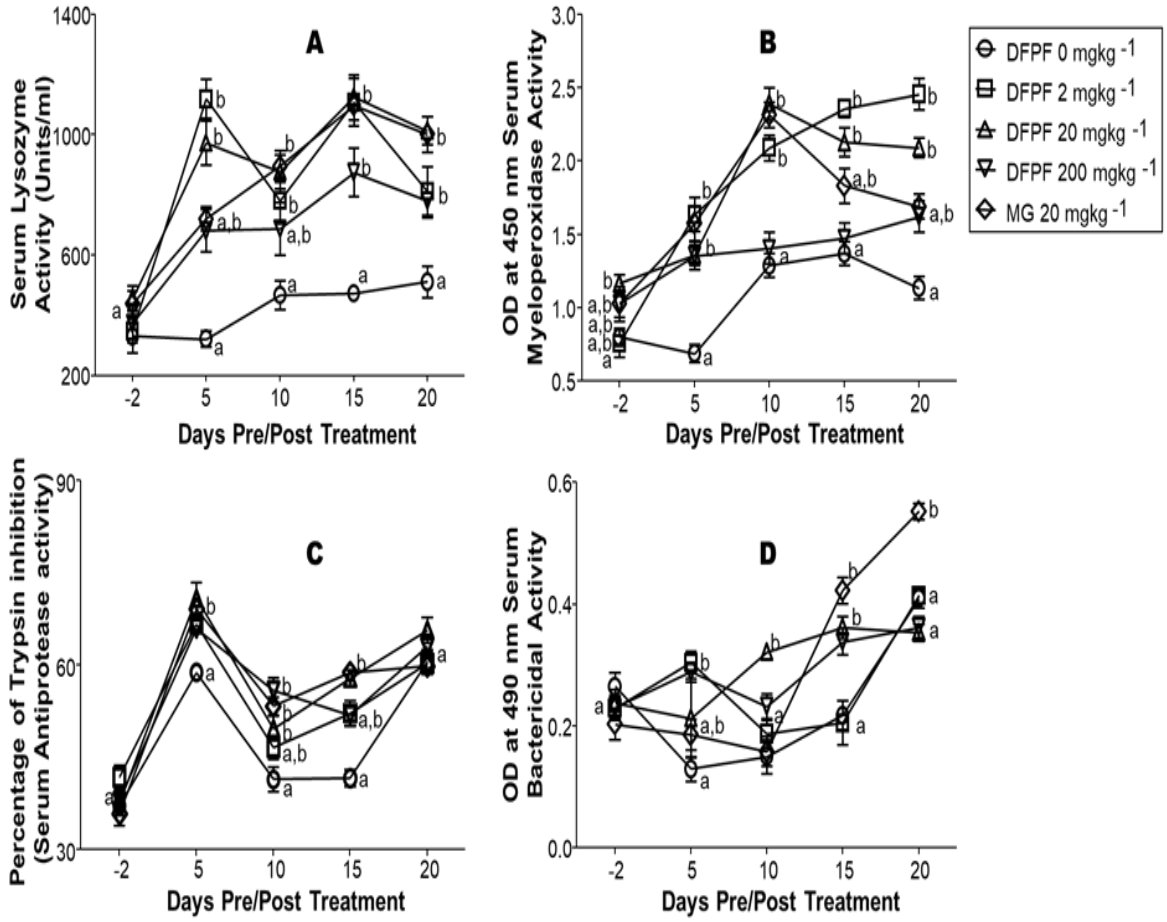

Figure 2: Effect of intraperitoneal administration of DFPF (mgkg^{-1} body weight) on the serum lysozyme activity (A), myeloperoxidase (B), antiprotease (C) and bactericidal (D) activity in Oreochromis niloticus; each point represents mean±S.E. of 12 fish; aposteriori Tukey comparisons of control and treated groups on individual post-treatment days shown with different alphabets represents significant difference (P<0.05)

Table 1: Details of primer sequences used in this study

No	Name of the Gene	Annealing temperature	PCR product size	Reference
1.	β-actin	60°C	100-110bp	J.Qiang *et al.,* (2014)
2.	IL-1β	60°C	205bp	Chen *et al.,* (2016)
3.	Lysozyme	60°C	300bp	Self-designed (http://primer3.ut.ee/)

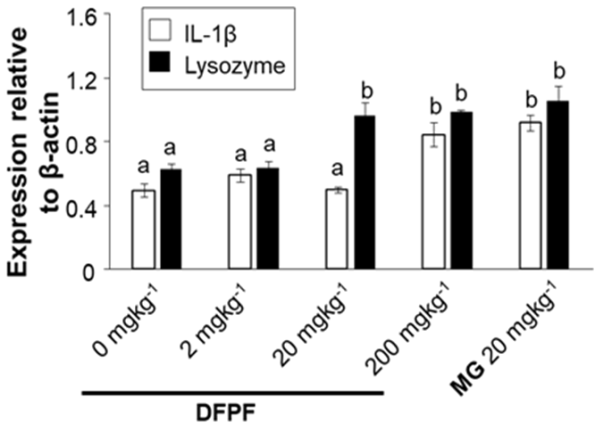

Figure 3: Effect of DFPF on IL-1β and lysozyme genes relative to mRNA expression of the β-actin gene. Each bar represents mean ± SE of 9 fish; a posteriori Tukey comparison of control and treated groups on individual post-treatment periods shown with different alphabets represents a significant difference (P<0.05)

Thus, as shown in figure 2D, while the low dose showed significantly (P<0.05) higher bactericidal activity on day 5, mid dose caused higher bactericidal activity on day 10, and 15 and high dose did it on days 5 and 15. The positive control MacroGard™ enhanced bactericidal activity significantly (P<0.05) on days 15 and 20.

Gene expression studies by semi-quantitative PCR

As shown in figure 3, the highest dose of DFPF up-regulated IL-1β and lysozyme. On the other hand,

only the mid dose upregulated lysozyme gene. MacroGard™ significantly upregulated genes encoding IL-1β and lysozyme.

Disease resistance test against *A. hydrophila*

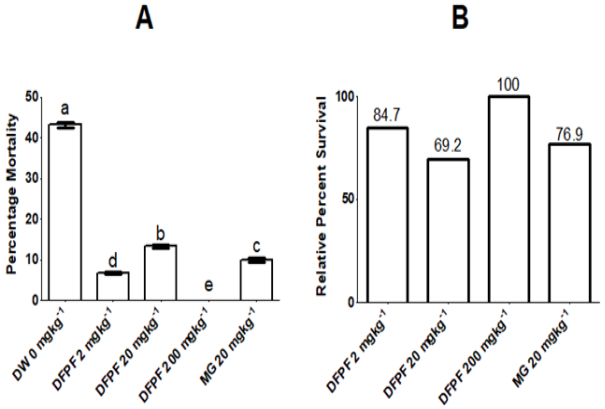

Figure 4: Disease resistance conferred by DFPF in *Oreochromis niloticus* injected 7 days prior to challenge with *A. hydrophila* A. in terms of percent mortality and B. in terms of relative percent survival; each bar represents mean ± SE of 10 fish in triplicates; a posteriori Tukey comparison of control and treated groups on individual post-treatment periods shown with different alphabets represents significant difference (P<0.05)

As depicted in figure 4, when challenged at 7 days after administration of DFPF, all the doses of DFPF tested caused a significant reduction in percent mortality. High dose of DFPF gave the maximum protection of Nile tilapia against live virulent *A. hydrophila* with 0 percent mortality and an RPS of 100. The percent mortality of tilapia treated with a low dose and mid dose of DFPF was 6.6 and 13.3, and the RPS values were 84.7 and 69.2 respectively. MacroGard™ treatment resulted in 10% mortality, and the RPS value was 76.9. (Fig. 3A & 3B).

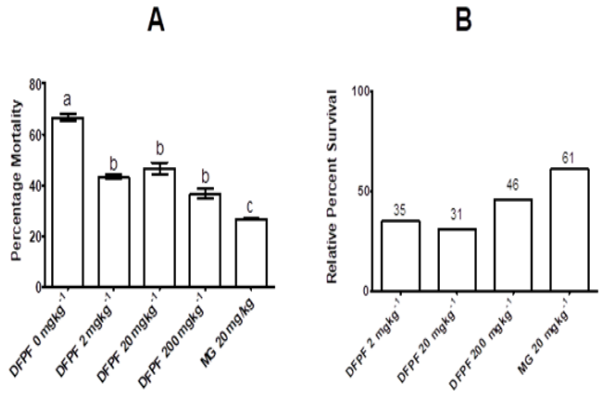

Figure 5: Disease resistance conferred by DFPF in *Oreochromis niloticus* injected 21 days prior to challenge with *A. hydrophila* A. in terms of percent mortality and B. in terms of relative percent survival; each bar represents mean ± SE of 10 fish in triplicates; aposteriori Tukey comparison of control and treated groups on individual post-treatment periods shown with different alphabets represents significant difference (P<0.05)

Even 21 days after treatment with DFPF, there was a significant reduction in percent mortality as shown in fig. 4A with high dose giving RPS values of 46, the mid dose and low dose with RPS values of 31 and 35 respectively. MacroGard™ gave an RPS value of 61 (Fig. 4B).

Fourier Transformed - Infrared Spectrum (FT-IR) of DFPF

The FT-IR spectra of DFPF showed a total peak of 58 (Fig. 5). The typical absorption peaks of polysaccharides with wavenumbers 3427, 2918, 1622, 1406, 1109-1024 were noticed in the spectra.

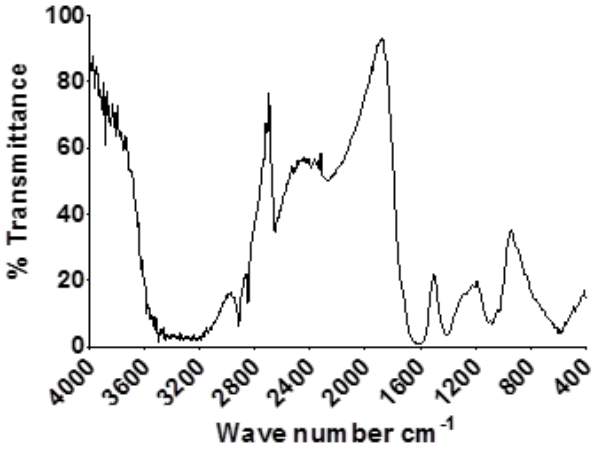

Figure 6: FT-IR spectrum of DFPF showing typical Polysaccharide peaks

DISCUSSION

Among the vertebrates, fishes depend more on innate/nonspecific immunity for disease protection than mammals (Magnadottir 2006). Lysozyme is a bactericidal enzyme and also acts as an opsonin to activate the complement system (Roy, Kumar *et al.,* 2017). In the present study, all the doses of DFPF tested significantly enhanced the lysozyme activity on most of the post-treatment days at varying degrees. Our results are in agreement with Zahran, Risha *et al.,* (2014) which reports the increment of serum lysozyme activity in *Oreochromis niloticus* after 21 days of dietary administration of *Astragalus sp.* polysaccharides. Similarly, there was an increase in serum lysozyme activity when fed for 3 weeks with PF of *Padina gymnospora* in *C. Carpio* (Rajendran, Subramani *et al.,* 2016). The increase of serum lysozyme activity implies an elevation of antimicrobial defence that protects the host during infection (Jawahar, Nafar *et al.,* 2016).

Myeloperoxidase enzyme is abundant in fish neutrophils released from the azurophilic granules by the process of degranulation involved in the killing

of invading pathogens (Strzepa, Pritchard *et al.,* 2017). The toxicity of hydrogen peroxide is greatly enhanced by the haem enzyme, MPO, which uses hydrogen peroxide to convert chloride into hypochlorous acid (Alvarez-Pellitero 2008). In this study, serum MPO activity has been significantly enhanced by DFPF on most of the days tested. A similar significant increase in MPO activity was also reported earlier in rainbow trout, *Oncorhynchus mykiss* fed with glucan supplemented feed for 1 week (Siwicki, Anderson *et al.,* 1994). In our earlier studies, we have observed the MPO activity of *O. mossambicus* increased by different plant extracts (Alexander, Kirubakaran *et al.,* 2010; Kirubakaran, Subramani *et al.,* 2016).

Antiproteases, namely α1-protease inhibitor, α2-macroglobulin, α2-antiplasmin play a major role in restricting the entry of pathogens by acting against the proteases of the invading pathogens (Rao and Chakrabarti 2004) and inhibiting the multiplication of bacterial pathogens selectively (McKerrow, Engel JC Fau - Caffrey *et al.,*). In the present study, enhanced antiprotease activity was noticed in all the groups treated with different doses of DFPF. Expression of protease inhibitors having different biological activities was greatly upregulated upon challenge with bacteria, fungi or dsRNA in *Channa striata* (Kumaresan, Harikrishnan *et al.,* 2015) and *Oplegnathus fasciatus* (Bathige, Umasuthan *et al.,* 2015).

In teleosts, complements present in the serum are responsible for its bactericidal activity (Ellis 2001) and are shown to be stimulated by immunostimulants (Matsuyama, Mangindaan *et al.,* 1992; Jeney and Anderson 1993). In the present study, increases in bactericidal activity of DFPF- treated groups were noticed. Our results are in agreement with that of Misra, Das *et al.,* (2006) wherein bactericidal activity increased in *Labeo rohita* after a series of β-glucan injections. Similarly, studies in Nile tilapia and grass carp, the dietary administration of polysaccharides *Astragalus sp.* and *Ficus carica* resulted in increased serum bactericidal activity (Zahran, Risha *et al.,* 2014; Yang, Guo *et al.,* 2015). Polysaccharides from *Radix isatidis* and *Schisandra chinensis* (Wang, Chen *et al.,* 2016) and Polysaccharide fraction of *Padina gymnospora* (Rajendran, Subramani *et al.,* 2016) was also shown to increase serum bactericidal activity.

IL-1β is a pro-inflammatory cytokine involved in innate immunity (Pooley, Tacchi *et al.,* 2013). In fish, IL-1β plays a major role in microbial invasion (Bo, Song *et al.,* 2015). IL-1β triggers the expression of another pro-inflammatory cytokine namely TNF-α, which is involved in cell differentiation, proliferation and induction of other cytokines (Wei, Sun *et al.,* 2009). Monocytes, macrophages and neutrophils are the first responders of immunostimulatory polysaccharides and secrete IL-1β (Ferreira, Passos *et al.,* 2015). In the present study, the high dose DFPF administration resulted in the up-regulation of the cytokine IL-1β and lysozyme genes. This result is in line with our previous findings wherein the polysaccharide fraction of *Padina gymnospora* administered as a feed supplement in *Cyprinus carpio* upregulated IL-1β and lysozyme gene expression (Rajendran, Subramani *et al.,* 2016). Similarly, when grass carp fed with *Ficus carica* polysaccharides for 3 weeks, it resulted in strong upregulation of IL-1β and TNF-α (Yang, Guo *et al.,* 2015). Arabinogalactan containing polysaccharides from *Juniperus scopolorum* increased the production of IL-1β and TNF-α in human and murine macrophages (Schepetkin, Faulkner *et al.,* 2005).

Disease resistance against pathogens is the ultimate test of the efficacy of an immunostimulant (Boshra, Li *et al.,* 2006). DFPF reduced the percent mortalities of fish experimentally challenged with LD_{50} of *A. hydrophila*. This finding is in line with the study in juvenile *Cyprinus carpio,* fed with 0.5% dietary microbial polysaccharide; levan resulted in RPS of 100 (Rairakhwada, Pal *et al.,* 2007). Polysaccharide fraction of *Padina gymnospora,* when administered as a feed supplement to *Cyprinus carpio,* it resulted in enhanced protection against *A. hydrophila* (Rajendran, Subramani *et al.,* 2016). Earlier studies with an injection of polysaccharides like barley glycan, krestin, scleroglucan, and zymosan into *O. aureus* resulted in significantly lower mortality of fish challenged with *Edwardsiella tarda* (Wang and Wang 1997). Wang and Wang (1997) also pointed out that these polysaccharides significantly increased macrophage activation compared to other polysaccharides. In the present study, we showed DFPF upregulating IL-1β which has been shown to activate macrophages.

The FT–IR analysis was carried out to define the various functional groups in the sample. The results of FT–IR spectra obtained in our study was similar to the results reported by (Haleem, Arshad *et al.,* 2014; Mao, Shao *et al.,* 2014; Li, Yuan *et al.,* 2017) which show typical polysaccharide peaks in their spectra.

Plant polysaccharides have been known to activate macrophages (Schepetkin and Quinn 2006) through receptors known as pattern recognition receptors (PRR) (Takeuchi and Akira 2010). It has been found that macrophages might bind immunostimulatory plant polysaccharides via Toll-like receptor (TLR) (Stafford, Ellestad *et al.,* 2003). Upon activation, TLR stimulates intracellular signalling cascades, resulting in the increased production of downstream pro-inflammatory cytokines

(Ferreira, Passos *et al.,* 2015; Zhang, Qi *et al.,* 2016). There is a great deal of evidence about these molecules are present in fish (Meng, Zhang *et al.,* 2012; Pietretti, Vera-Jimenez *et al.,* 2013; Rauta, Samanta *et al.,* 2014; Zhang, Kong *et al.,* 2014; Zhao, Liu *et al.,* 2015; Kiron, Kulkarni *et al.,* 2016). DFPF effects in *O. niloticus* reported herein might have been orchestrated by the in vivo activation of TLR.

CONCLUSION

To conclude with, the polysaccharide fraction of *Dendrophthoe falcata* has significantly enhanced the non-specific immune mechanisms, immune-related genes' expression and protection against *A. hydrophila* in Nile tilapia. Appropriate field trials using DFPF- supplemented feed is to be preceded before the use of DFPF as an immunostimulant to prevent diseases in finfish aquaculture systems.

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