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RESEARCH PAPER

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Effect of *Leucas aspera* Against *Aeromonas hydrophila* in Nile Tilapia (*Oreochromis niloticus*): Immunity and Gene Expression Evaluation

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Abstract

The present study addressed the effects of *Leucas aspera* enriched diet in Nile tilapia. Three hundred Nile tilapia were fed *Leucas aspera* as follows: 0 g kg^{-1} *L. aspera* (C-control), 5 g kg^{-1} *L. aspera* (T1), 10 g kg^{-1} *L. aspera* (T2) and 15 g kg^{-1} *L. aspera* (T3). After 30 days of feeding, significant ($P<0.05$) increase in growth performance was noticed by feeding the fish the T2 diet. Thereafter fish were intraperitoneal injected with *Aeromonas hydrophila* in challenge test. After 21 days of challenge, highest survival rate (70%) was observed in fish fed the T3 diet followed by fish fed T2 diet (65%). Serum immunological parameters such as phagocytosis, alternative complement activity, respiratory burst activity and lysozyme activity were significantly ($P<0.05$) enhanced in fish fed all inclusion levels of *L. aspera* with the maximum activity in fish fed the T2 diet. Hematological parameters were significantly ($P<0.05$) higher in all groups fed *L. aspera* diets *vs*. control fed fish. No histopathological changes in liver were observed in fish fed the T2 diet in the histology study. Gene expression study revealed the upregulation in the expression of COX-2 and GR genes. In conclusion, the current results suggest that dietary administration of *L. aspera* especially the T2 diet, has beneficial effects in improving immunity and can mitigate the adverse effects of *A. hydrophila* infection in Nile tilapia.

Introduction

Aquaculture is one of the fastest growing industries in the world with China, India, Vietnam and Bangladesh being the major producers (FAO, 2017), but one major threat to the industry is the diseases, which causes economic loss and stunted development (Cabello et al., 2020). Previously, disinfectants, chemotherapeutics and antibiotics were used, but the consequences of these treatments were harmful (Ringø et al., 2014; Seyfried et al., 2010). However, during the last decades research has focused on the application of novel ecofriendly and alternative supplements such as pro-, pre- and synbiotics, immunostimulants and medicinal herbs to improve fish growth, immune responses and disease resistance (Carbone and Faggio, 2016; Ringø et al., 2014, 2018; de Santis et al., 2015; Musthafa et al., 2017, 2018; Awad and Awaad 2017; Nath et al., 2017; Van Doan et al., 2019; Elumalai et al., 2020). Of the functional food alternatives, plant derived compounds are promising, and are mainly categorized into tannins, alkaloids, pigments, essential oils, steroids, terpenoids, flavonoids and phenolics (Harikrishnan et al., 2011; Newaj-Fyzul and Austin, 2014; Reverter et al., 2014; Van Hai, 2015). Due to the abundance of secondary metabolites, dietary plant supplementations

are suitable alternative to chemical treatments, as they have potential to prevent fish diseases (Hoseinifar et al., 2020; Rashidian et al., 2020; Van Doan et al., 2019). It is well known, that plant extracts contain immunostimulants, biocidal and anti-stress effects, as well as promoting fish growth (Wang et al., 2009; Harikrishnan et al., 2010; Wu et al., 2010; Yin et al., 2011).

Nile tilapia *Oreochromis niloticus* is a widely preferred fish species, and is one of the commonly cultured fish species in the world, produced in more than 100 countries (Gobi et al., 2018; Gu et al., 2015; van Doan et al., 2018). The global production was estimated to be 6.5 million metric tons in 2018 and is expected to reach 7.3 million metric tons by 2030 (Behera et al., 2018).

Aeromonas hydrophila, a Gram-negative bacterium is an aquatic pathogen that is distributed worldwide, and is an opportunistic pathogen producing symptoms such as tail and fin rot, ulcers etc. (Eddy, 2008; Tawwab and Abbass, 2017). As *A. hydrophila* is a primary bacterial pathogen in many aquatic organisms, it causes great concern to the aquaculture industry (Janda and Abbott, 2010; Mahmoud et al., 2017). It not only results in economic loss, but also has an impact on public health. *A. hydrophila* has the potential to grow at 5°C, that points out its ability to exhibit public health hazards and they are associated with gastroenteritis, intestinal and extra-intestinal infections in humans (Praveen et al., 2016). Several previous investigations revealed the emergence of multidrug-resistant bacterial pathogens from different origins especially fish that increases the need for new natural immunostimulants and antimicrobial alternatives to the commonly used old antimicrobial agents (Algammal et al., 2020; Sayed et al., 2019; Abolghait et al., 2020; Enany et al., 2018).

Leucas aspera (Willd.) Linn. (Family: *Lamiaceae*) commonly known as '*thumbai*' is distributed throughout India, and is traditionally used as an antipyretic and insecticide (Suganya et al., 2014). *L. aspera* contain medicinally active compounds such as triterpenoids, oleanolic acid, ursolic acid and b-sitosterol, nicotine, sterols, glucoside, diterpenes, phenolic compounds (4-(24-hydroxy-1-oxo-5-n-propyltetracosanyl)-phenol) and hence is pharmacologically relevant (Antony et al., 2013). Flowers and leaves are valued as stimulant of menstrual flow, expectorant, aperient, diaphoretic and are considered useful in chronic rheumatism, psoriasis and other chronic skin eruptions (Prajapati et al., 2010). Banu et al. (2012) revealed that, *L. aspera* aqueous extract significantly elevated antioxidant enzymes and acted as a hepatoprotective agent against D-galactosamine induced liver damage in rats. Kripa et al. (2011) emphasized the anti-inflammatory and antioxidant potential in the *L. aspera* extract due to the presence of phytosterols, flavonoids (procyanidin and leucasin) and catechins apart from glycosides, phenolic compounds, and tannins and reported that ethanol extract helped in complete cartilage regeneration in adjuvant induced arthritic rats. A previous study revealed that, *L. aspera* extract encapsulated in *Artemia* nauplii enhanced the growth and survival rate of zebrafish (*Danio rerio*) (Teepica and Srinivasan, 2015). Similarly, Antony et al. (2013) reported that, *L. aspera* engineered silver nanoparticles promote hematological and antioxidant profile in catla (*Catla catla*) challenged with *A. hydrophila*. The current study aims to investigate the effects of *L. aspera* on growth, serum immunity, disease resistance, hematological profile and gene expression in Nile tilapia.

Materials and Methods

Leucas aspera Powder Preparation

L. aspera plants were grown at the campus of Kerala University of Fisheries and Ocean Studies in Kochi, India. The whole plant was collected, washed with tap water, shade dried and ground into fine powder described by Kurian et al. (2020).

Fish Diets

The experimental diets were prepared by supplementing different inclusion levels of *L. aspera* powder in the basal diet as follows: 0 (C-control), 5 g kg^{-1} (T1), 10 g kg^{-1} (T2) and 15 g kg^{-1} (T3) (Table 1). The diets were mixed evenly and dried in a vacuum freeze dryer for 15h. Thereafter all diets were ground properly and extruded by a 5 mm mesh sieve. The prepared diets were stored at -20°C for two days until the start of the experiment.

Study Design

Nile tilapia used in the present study was transferred from a fish farm in Alleppey district, Kerala, to the laboratory. The fish were acclimated for two weeks in a 1000 L aerated fiber tank, and fed control diet as described by Kurian et al. (2020). After acclimation, 300 fish were randomly distributed into 15 (150 L) glass tanks (20 fish per tank; average weight=8.11±0.05g), and cultured for 30 days with three replicates per treatment.

(i) Control group - Non- infected fish fed control diet without any *L. aspera* enrichment

(ii) Negative control - Fish fed control diet and challenged by *A. hydrophila* after 30 days of feeding

(iii) 5g kg^{-1} - Control diet + 5 g/kg *L. aspera*

(iv) 10g kg^{-1} - Control diet + 10g/kg *L. aspera*

(v) 15g kg^{-1} - Control diet + 15g/kg *L. aspera*

The fish were fed twice a day at 9.00 am and 5.00 pm *ad libitum.* Throughout the experiment, water quality parameters were monitored daily: water temperature 28±2°C, pH 7.7±0.24, and dissolved oxygen 5.1±0.31 mg/ liter. After 30 days of feeding, all treatment groups except group (i) were intraperitoneally injected with *A. hydrophila.*

Table 1. Ingredients and composition of control and experimental diets

Ingredients (g kg^{-1})	Diet groups %			
	C	T1 (5g Kg^{-1})	T2 (10g Kg^{-1})	T3 (15g Kg^{-1})
Fish meal	270	270	270	270
Corn meal	200	199	203	206
Soybean meal	270	270	270	270
Wheat flour	60	60	60	60
Rice bran	150	150	150	150
L. aspera	0	5	10	15
Cellulose	30	26	17	9
Soybean oil	5	5	5	5
Premix[1]	10	10	10	10
Vitamin C[2]	5	5	5	5
Proximate composition of the experimental diets (g Kg^{-1} dry matter basis)				
Dry matter	88.25	89.07	94.05	93.33
Ash	9.13	9.32	9.70	9.73
Crude Fibre	2.47	2.26	3.06	3.43
Crude lipid	6.36	6.86	6.44	7.24
Crude protein	30.5	31.4	33.8	32.7
GE (Cal g^{-1})[c]	4020	4075	4151	4240

Note: [a] Vitamin premix per kg: Vitamin A=700000 IU; Vitamin D=140000 IU; Vitamin E=500mg; Vitamin B_{12}=1000mcg; Folic acid=100mg; Nicotinamide=1000mg. [b]Mineral premix per kg: Copper=1200mg; Cobalt=150mg; Iron=1500mg; Zinc=3000mg; Iodine=325mg; Selenium=10mg; Magnesium=6000mg; Manganese=1500mg; Potassium=100mg; Calcium=270gm; Phosphorus=130gm; Sulphur=7.2gm; Fluorine=300mg.
[c]GE = Gross energy

Pathogen

A. hydrophila (ATCC 49140) isolated from infected fish was supplied by Aquatic Animal Health Laboratory (AAHL) of C. Abdul Hakeem College located in Melvisharam, *Vellore,* Tamil Nadu, India and was cultured in tryptic soy broth (TSB, Himedia) for 24 h at 37°C with agitation to mid-log growth phase (Musthafa et al., 2018). The broth culture was centrifuged for 10 minutes at 3.000 g. The supernatant was discarded and the pellet was washed twice with 0.15 M phosphate buffered saline (PBS, pH 7.2). The bacterial pellets were re-suspended and divided into aliquots and stored in (TSB) supplemented with 15% (v/v) glycerol and stored at −70°C until further use. Optical density (OD_{456}) of the solution was adjusted to 0.5, which corresponds to 1×10^7 cells ml^{-1}. The bacterial suspensions were serially diluted using standard dilution technique with PBS and used in the challenge experiment.

Challenge Test

After 30 days of feeding, all groups except group (i) were challenged intraperitoneally with 100 µl PBS containing *A. hydrophila* (1×10^7 cells mL^{-1}). Mortality was noted every 12h interval up to 21 days and dead fish were removed immediately (Musthafa et al., 2018). Survival rate (SR) was calculated using the formulae:

SR (%)=(final fish number/initial fish number)x100.

Blood Sampling and Preparation

Six fish were selected randomly from each replication at the end of the challenge test. Fish were anaesthetized with 0.1 ppm MS-222 (Sigma chemicals), and blood was collected from the caudal vein with a 1 ml plastic syringe coated with heparin and stored at 4°C until the next day. Blood samples collected without heparin were allowed to clot for 2h at 4°C, centrifuged at 3.500 g at 4°C for 25 minutes, sera collected and refrigerated. Leukocytes were separated from peripheral blood by the method described by Van Doan et al. (2016).

Hematological Parameters

Hematological parameters were determined according to Nya and Austin (2009).

Phagocytosis Activity

The phagocytic activity assay was performed as described by van Doan et al. (2018). 200 ml leukocyte suspensions $2x10^6$ cells ml^{-1} were spread on coverslips and incubated for 2 h. Non-adherent cells were then removed by washing with RPMI 1640. 200 ml of fluorescence latex beads (Sigma) solution $2x10^7$ of beads mL^{-1} was added on each coverslip and incubated for 1.5 h at room temperature. After incubation, the non-phagocytized beads were washed with RPMI 1640. The coverslips were then fixed with methanol and stained with Diff-Quick staining dye (Sigma) for 10 seconds. The excess stain was removed by washing with PBS (pH 7.4), and the number of phagocytized cells per 300 adhered cells was counted microscopically. The phagocytic index (PI) was determined as follows: PI=Average number of beads per cell divided by the number of phagocytizing cells.

Alternative Complement Activity

Alternative complement pathway activity (ACH50) was determined according to Yanno (1992). Rabbit red blood cells (R-RBC) were washed thrice in 0.01 M ethylene glycol tetra-acetic acid-magnesium-gelatin veronal buffer (0.01M-EGTA-Mg-GVB), and the concentration was adjusted to 2 x 10^8 cells ml $^{-1}$ in the same buffer. Exactly 100 ml of the suspension was lysed with 3.4 ml of distilled water. The absorbance of hemolysate was measured at 414 nm against distilled water blank and was brought to be close to 0.740. For the ACH50 test, 100 ml of test serum was diluted with 400 ml of 0.01M-EGTA-Mg-GVB, and serial two-fold dilution was made. Consequently, 100 ml of R-RBC suspension was added to each tube and incubated at 20^0C for 90 min with occasional shaking. After incubation, 3.15 ml of cold saline was added to each tube and centrifuged at 1600 g for 5 min. One hundred ml of the supernatant of each dilution was then transferred to 96-well plate and read at 414 nm. The degree of hemolysis was calculated by dividing the corrected absorbance 414 value by the corrected absorbance 414 of the 100% hemolysis control. The degree of hemolysis and the serum volume were plotted on a log paper. The volume of serum that gave 50% hemolysis was used for calculating the ACH50 using the formula: ACH50 (units/ml)=1/K x r x ½, where K is the amount of serum giving 50% hemolysis, r is the reciprocal of the serum dilution, and 1/2 is the correction factor.

Respiratory Burst Activity

Respiratory burst activity was carried out as described by Secombes (1990) for measuring the production of oxygen radicals from phagocytes. 0.1 mL of heparinized blood was placed into a microtiter plate and equal amount of 0.2% NBT was added and incubated for 30 minutes at room temperature. 0.05 mL of the NBT-blood cell suspension was taken out and added to a glass tube containing 1.0 mL of N, N-dimethylformamide solution. Then the mixture was centrifuged for 5 minutes at 3.000g. The supernatant was taken into a glass cuvette and absorbance was read at 540 nm using a spectrophotometer.

Lysozyme Activity

Lysozyme activity was determined by the protocol of Parry et al. (1965). Briefly, 25 µL of mucus and serum loaded into 96-well plate in triplicate. Then, 175 µL of *Micrococcus lysodeikticus* (Sigma Aldrich - USA) suspension (0.3 mg mL^{-1} in 0.1 M citrate phosphate buffer, pH 5.8) was added to each well. After the rapid mixing, change in turbidity was detected every 30 seconds for 5 min at 540 nm and 25°C using a micro-plate reader. The equivalent unit of activity of the sample (compared with the standard) was determined and expressed as µg mL^{-1} serum. The serum lysozyme activity was measured and expressed as U mL^{-1}.

Histology

For histopathology study of liver, tissues from fish fed 10g kg^{-1} *L. aspera* enriched diet were processed following the methods by Humason (1979) and Pearse (1968). Liver was fixed for 24 hours in 10% formalin solution before embedding in paraffin wax. Sections of liver were stained with hematoxylin and eosin and were analyzed for histopathological alterations.

RNA Extraction, cDNA Synthesis and Real Time-PCR

For gene expression studies, spleen of fish fed 10g kg^{-1} *L. aspera* enriched diet was collected. RNA was extracted using TRIzol (Sigma). Purity and quality of RNA was detected using Nanodrop (Thermo Fisher Scientific) as the 260:280 ratio was 1.8-2.0. Afterward, complementary DNA (cDNA) was synthesized using cDNA synthesis kit (iScript cDNA synthesis kit, Bio-Rad) according to the manufacturer's protocol. Specific primers for gene expression were designed using primer premier 5 software to amplify the selected genes with β-actin as a house keeping gene (Table 4). The q RT-PCR analysis was carried out using CFX96 Touch Real time PCR detection system (Bio- Rad) and Sso Advanced Universal SYBR green supermix (Bio-Rad) following the manufacturer's instructions and as per the method described by Mahfouz (2015).

Growth Performance

Growth performance was calculated at the end of the feeding trial. Weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated using the formulae:

WG = final weight (g) - initial weight (g);

SGR (%) = 100 × (ln final weight - ln initial weight)/ Duration of experiment;

FCR = feed given (dried weight)/weight gain (wet weight);

Statistical Analysis

The data of each parameter were expressed as the mean±standard error of mean (SEM) and the effects of experimental diets were tested using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test using SPSS (version 16 for windows). Differences were considered statistically significant when ($P<0.05$).

Results

Growth Performance

Growth performance was assessed after 30 days of feeding. Weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were significantly (P<0.05) improved in fish fed *L. aspera* diets compared to control. Tilapia fed 10g kg^{-1} diet (T2) displayed the best growth performance among the treatment groups (Table 2).

Hematological Profile

The erythrocyte (RBC) and leukocyte (WBC) levels were significantly (P<0.05) higher in the fish fed *L. aspera* diets *vs*. fish fed the control diet (Table 3). Highest erythrocyte number (6.70±0.0) was observed in fish fed 10g *L. aspera* kg^{-1} (T2) *vs.* the control. Similarly, significant (P<0.05) higher numbers of all other erythrocyte parameters; hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were recorded in all *L. aspera* treatment groups compared to the control, with the highest value in group fed 10g *L. aspera* kg^{-1}. Fish fed dietary *L. aspera* affected the proportions of neutrophil, lymphocyte, monocyte and eosinophil. Highest value of neutrophil was revealed in the group fed 5g *L. aspera* kg^{-1} compared to the other treatment groups. However, a decrease in percentage of lymphocyte and monocyte levels was observed in all treatment groups including the control at all-time points (Table 3).

Phagocytic Activity

All administrations of *L. aspera* revealed significantly (P<0.05) higher phagocytic activity *vs.* control group. However, the maximum activity is revealed in fish fed 10g *L. aspera* kg^{-1} followed by fish fed 15 g kg^{-1} and 5 g kg^{-1} (Figure 1).

Complement Activity

Figure 2 shows a significant (P<0.05) increase in complement activity in Nile tilapia by all doses of *L. aspera*, and administration of 10g *L. aspera* kg^{-1} revealed the highest complement activity *vs*. the control group.

Respiratory Burst Activity

Maximum respiratory burst activities of phagocytes were exhibited by fish fed 10g kg^{-1} followed by 15g kg^{-1} feed (Figure 3). All the inclusions levels of *L. aspera* revealed significant (P<0.05) increase in respiratory burst activity compared with the control.

Table 2. Growth performance of *O. niloticus* fed *L. aspera* supplementation diets against *A. hydrophila* after 30 days

Parameters	Control	5g kg^{-1}	10g kg^{-1}	15g kg^{-1}
IW (g)	8.10±0.01	8.10±0.01	8.11±0.01	8.13±0.01
FW (g)	44.46±1.16	51.96±1.0*	58.50±0.51*	50.9±0.55*
WG (g)	36.36±1.17	43.86±1.01*	50.39±0.51*	42.77±0.61*
SGR (g)	3.77±0.06	4.12±0.04*	4.38±0.01*	4.07±0.02*
FCR	1.57±0.01	1.55±0.02*	1.49±0.01*	1.53±0.02*

IW= Initial weight, FW = Final weight, SGR = Specific growth rate, FCR = Feed conversion ratio. The values are expressed as mean ± standard errors of mean.

Table 3. Immuno-haematological parameters of *O. niloticus* fed *Leucas aspera* supplementation diets against *A. hydrophila*

Parameters	Control (0 g kg^{-1})	Negative Control	Percent change	5 g kg^{-1}	Percent Change	10 g kg^{-1}	Percent Change	15 g kg^{-1}	Percent Change
Erythrocyte profile			Con. VS Neg.Con.		Con. VS 5 g kg^{-1}		Con. VS 10 g kg^{-1}		Con. VS 15 g kg^{-1}
RBC(× 10^6 mm^3)	2.30±0.0	1.10±0.0	-52.17↓	2.80±0.3	21.73↑	6.70±0.0*	191.30↑	3.50±0.2	52.17↑
Hb (g dL^{-1})	8.60±0.3	6.30±0.1	-26.74↓	8.70±0.0	1.16↑	14.60±0.1*	69.76↑	10.5±0.0*	22.09↑
PCV (%)	33.5±1.7	12.3±1.0	-63.28↓	39.0±1.7	16.41↑	47.5±3.8*	41.79↑	41.5±2.5*	23.88↑
MCV (fl)	120.2±6.3	78.5±5.3*	-34.69↓	145.2±7.0	20.79↑	162.5±3.7*	35.19↑	149.5±5.3*	24.37↑
MCH (pg)	22.3±1.5	15.5±1.2*	-30.49↓	38.9±1.0	74.43↑	61.2±2.9*	174.43↑	45.2±1.7*	102.69↑
MCHC (g dL^{-1})	28.7±0.1	18.4±0.5*	-35.88↓	34.5±0.5	20.20↑	56.0±2.6*	95.12↑	42.5±2.7*	48.08↑
Leukocyte profile									
WBC(×10^{3}mm^3)	22.50±0.32	17.10±0.1	-24.00↓	27.50±0.3	22.22↑	35.50±0.6*	57.77↑	29.10±0.2*	29.33↑
Neutrophil (%)	26.25±0.17	32.32±0.1*	23.12↑	43.00±0.6*	63.80↑	40.00±0.6	52.38↑	36.40±0.5	38.66↑
Lymphocyte (%)	70.50±1.35	64.62±1.1	-8.34↓	54.15±1.5	-23.19↓	56.65±1.5	-19.64↓	60.15±1.2	-14.68↓
Monocyte (%)	2.25±0.05	1.64±0.0*	-27.11↓	1.60±0.03*	-28.88↓	2.00±0.03	-11.11↓	2.20 ± 0.0	-2.22↓
Eosinophil (%)	1.00±0.02	1.26±0.0	26.00↑	1.25±0.01	25.00↑	1.35±0.01*	35.00↑	1.25±0.0	25.00↑

The superscript symbols in the same line show that there are significant differences among different experimental group (P<0.05).
*Values are means ± SD (n=6 in three replicates).
↑ Symbols indicates the increase percentage of respective parameters.
↓ Symbols indicates the decrease percentage of respective parameters.

Table 4. Primers used for real time PCR

Gene	Sequence	Product size (bp)	Reference
GR	F: CATTACCGAGACGCGGAGTT R: CAGTTGGCTCAGGATCATTTGT	420	Yilmaz et al
β-actin	F: ACAGGATGCAGAAGGAGATCACAG R: GTACTCCTGCTTGCTGATCCACAT	155	Zhi et al
COX-2	F: AGCAGCCAGAAGGAAGGCGG R: GACTGAGTTGCAGTTCTCTTAGTGTGC	130	Chuang and Pan

Lysozyme Activity

The lysozyme activity was significantly (P<0.05) increased in serum, liver and kidney of fish fed the *L. aspera* diets *vs*. with the control. The highest activity was observed in fish fed 10 g kg^{-1} diet (Figure 4).

Disease Resistance

The survival rates after challenging with *A. hydrophila* was 55% (5g kg^{-1} group), 65% (10g kg^{-1} group), 70% (15g kg^{-1}) and 30% in fish fed the control diet (Figure 5). Among the *L. aspera* supplemented groups, survival rate was significantly (P<0.05) highest in fish fed 15g/kg^{-1}.

Histology

Effects of dietary *L. aspera* on liver histology of control and treatment group (10 g kg^{-1} diet) are shown in Figure 6a-b. In the treatment groups, histological analysis of liver revealed normal architectural arrangement with abundant hepatocytes (H) with prominent nucleus and sinusoids (SI).

Gene Expression

The results of immune gene expression are shown in Figure 7a-b. In fish fed the 10 g kg^{-1} diet, expression of cyclooxygenase-2 (COX-2) and glutathione reductase (GR) was upregulated.

Discussion

The present study addressed the potential of *L. aspera* to stimulate growth, immunity, and disease resistance against *A. hydrophila* in Nile tilapia.

In a previous study, Teepica and Srinivasan (2015) reported higher survival rate (92.45%) and SGR (2.52%) by feeding zebrafish bioencapsulated *Artemia nauplii* with *L. aspera* extract. Moreover, it has also been revealed that ethanol extract of *L. aspera* enhanced the anti-inflammatory and antioxidant potential in adjuvant arthritis female wistar rats and Swiss albino mice of both sexes (Kripa et al., 2011).

Dietary supplementations of medicinal plants enhance growth performance of fish species (Ahmadifar et al., 2019; Gholampour et al., 2020; Rashidian et al., 2019). Recently, two studies (Musthafa et al., 2017, 2018) reported that the inclusion of calabaza (*Cucurbita mixta*) and velvet bean (*Mucuna pruriens*) seed meal at inclusion level of 4g kg^{-1} and 6g kg^{-1} significantly (P<0.05) increased FCR, SGR, feed efficiency (FE), and protein efficiency ratio (PER) in Mossambicus tilapia *Oreochromis mossambicus*. Similarly, Mahmoud et al. (2017) reported effect of dietary curcumin (*Curcuma longa*) in tilapia against *A. hydrophila* and revealed that among the five diets used (0, 50,100,150 and 200 mg kg^{-1}), the 50 mg kg^{-1} diet significantly improved the final weight, daily weight gain and SGR. In the present study, a significant (P<0.05) increase in weight gain, FCR and SGR were observed by feeding tilapia 10g *L. aspera* kg^{-1} compared to the other experimental groups, and revealed that dietary *L. aspera* supplementation has the potential of being a growth-promoting additive.

Hematological profile can be utilized as an indicator to evaluate the physiological status of fish (Burgos Aceves et al., 2019). The increase in RBC count noticed in the present study might be an indicator of the immunostimulant effect of *L. aspera*, and changes in the WBC levels indicate response to infections. Elevation in the level of WBC indicates the activation of defense mechanism in response to microbial attack. Increment in neutrophil levels indicates activation of defense mechanism. Level of hemoglobin gives an index on RBC function and severity of anemia can be determined from the hemoglobin level. Rise in hemoglobin content indicates increased oxygen transportation. In the present study, all erythrocyte parameters were higher in all treatment groups than the control.

Phagocytosis is a prime factor that protects the host from invading microbes (Musthafa et al., 2018). Enhanced phagocytosis has been previously reported in studies where herbal immunostimulants were included in fish feed. In a recent study, Mbokane and Moyo (2018) reported that African worm wood (*Artemisia afra*) leaf powder supplemented at 9% and 12% levels increased phagocytosis activity in Mossambicus tilapia. The result of the current study revealed that *L. aspera* is an enhancer of phagocytosis activity.

Complement system provides the host with vital antimicrobial defense (Van Hai, 2015). Complement pathway has bactericidal activities mainly reported in fish serum and is antibody independent. On the other hand, T cells and T cell receptors, B cells and immunoglobulins, and lymphocytes are the specific immune elements in fish. Cytokines, also act in modulating the immune response and are connected

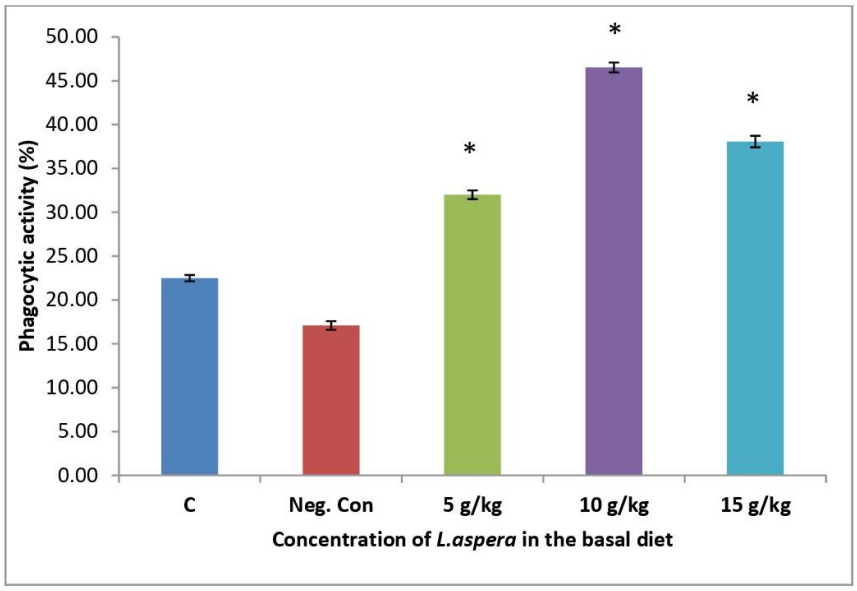

Figure 1. Phagocytic activity (%) of freshwater fish *O. niloticus* (mean±SEM, n=3) fed experiment diets containing *L. aspera* in different levels for 30 days. Significant difference (P<0.05) from the control is indicated by asterisks.

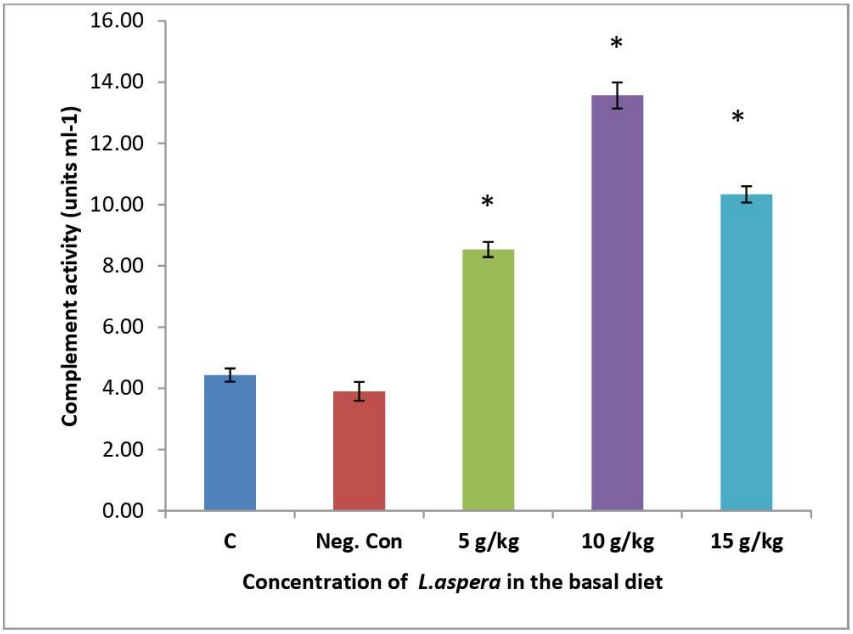

Figure 2. Complement activity (Units/ml) of freshwater fish *O. niloticus* (mean±SEM, n=3) fed experiment diets containing *L. aspera* in different levels for 30 days. Significant difference (P<0.05) from the control is indicated by asterisks.

with innate and adaptive immunity (Magnadottir, 2006). Thus, with the aid of the immune elements, fish immune system protects the host from the attack of pathogens (Zapata et al., 2006; Bruce and Brown, 2017). The results of the present study revealed that inclusion of *L. aspera* activate the complement system that is crucial in recruiting phagocytes and elimination of pathogens.

Production of oxidative radicals, peroxidase and activation of neutrophils account to the non-specific defense mechanism in fish as oxidative burst or respiratory burst, different cells release reactive oxygen species that are noxious for bacterial pathogens (Ainsworth et al., 1991; Semple et al., 2018). In this context, it is worth to notice that Nile tilapia fed the *L. aspera* enriched diets significantly (P<0.05) increased respiratory burst activity of phagocytes and that *L. aspera* enriched diets stimulated phagocytic cell membrane, and production of reactive oxygen species that are toxic to pathogens.

Lysozyme, a non-specific immune element acts on the peptidoglycan layer of bacteria resulting in lysis (Yousefi et al., 2019). In fish, lysozyme is mainly produced by the liver, and its presence can be detected in mucus, plasma and other fluids (Van Doan et al., 2019). In the present study, significant (P<0.05) increase in the lysozyme activity was observed in the serum, liver

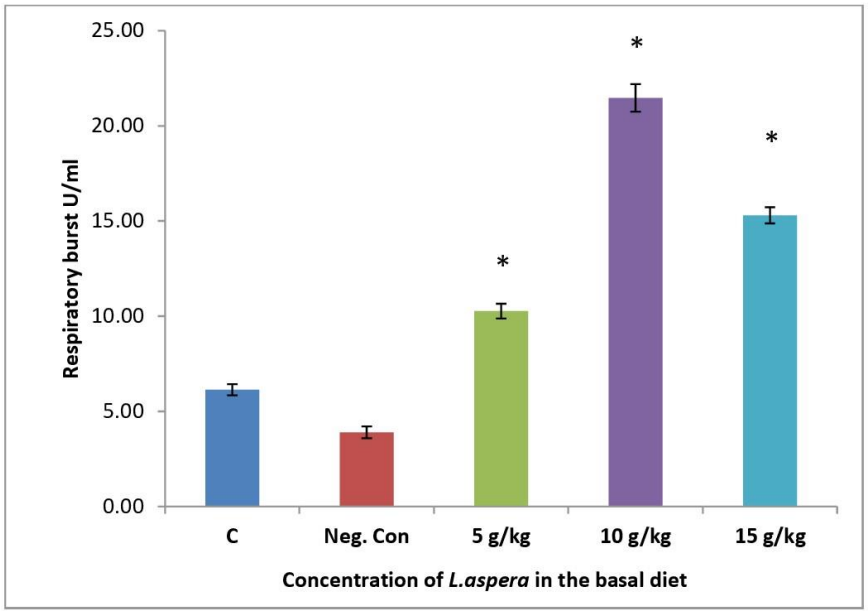

Figure 3. Respiratory burst activity (U/ml) of freshwater fish *O. niloticus* (mean±SEM, n=3) fed experiment diets containing *L. aspera* in different levels for 30 days. Significant difference (P<0.05) from the control is indicated by asterisks.

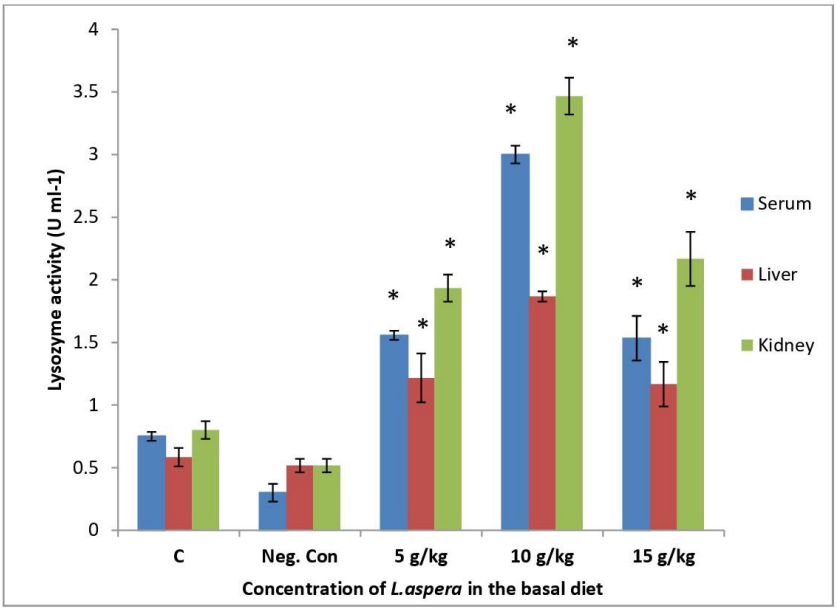

Figure 4. Lysozyme activity (U ml^{-1}) of freshwater fish *O. niloticus* (mean±SEM, n=3) fed experiment diets containing *L. aspera* in different levels for 30 days. Significant difference (P<0.05) from the control is indicated by asterisks.

and kidney samples fish fed different inclusion levels of *L. aspera* in the diets *vs*. control group, and indicate that *L. aspera* administration improved involvement of lysozyme in defense mechanisms in Nile tilapia.

Polyphenols of *L. aspera* and its potent antioxidant activity is believed to be mainly due to their redox properties, which play a pivotal role in neutralizing the inflammation in fish challenged with a pathogen bacterium. The enhanced survival rate of Nile tilapia challenged with *A. hydrophila* may be due to the activation of innate immune defense, due to the presence of different bioactive compounds and essential oils present in *L. aspera* that can break the lipid bilayer of the cell membrane, and its hydrophobic action resulting in losing the permeability and leakage of cell contents.

Based on the absence of histopathological changes in the treatment groups it is suggested that diet supplemented with *L. aspera* can exert a protective effect to the liver, the main organ for detoxification. Expression of inflammatory related gene cyclooxygenase-2 (COX-2) and antioxidant related gene glutathione reductase (GR) was evident after challenge test. COX-2 is involved in inflammatory response, and its

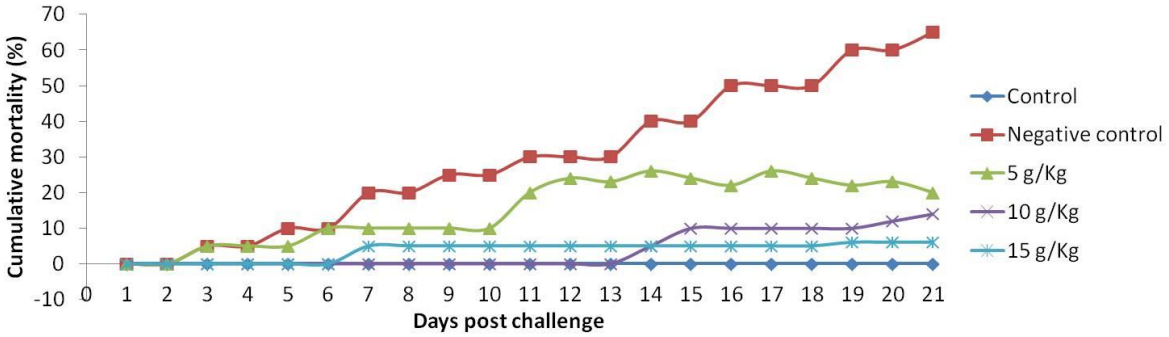

Figure 5. Survival rate (%) of *O. niloticus* (mean±SEM, n=20) fed different concentrations of *L. aspera* during 21 days post challenge with *A. hydrophila.* Significant difference (P<0.05) from the control is indicated by asterisk.

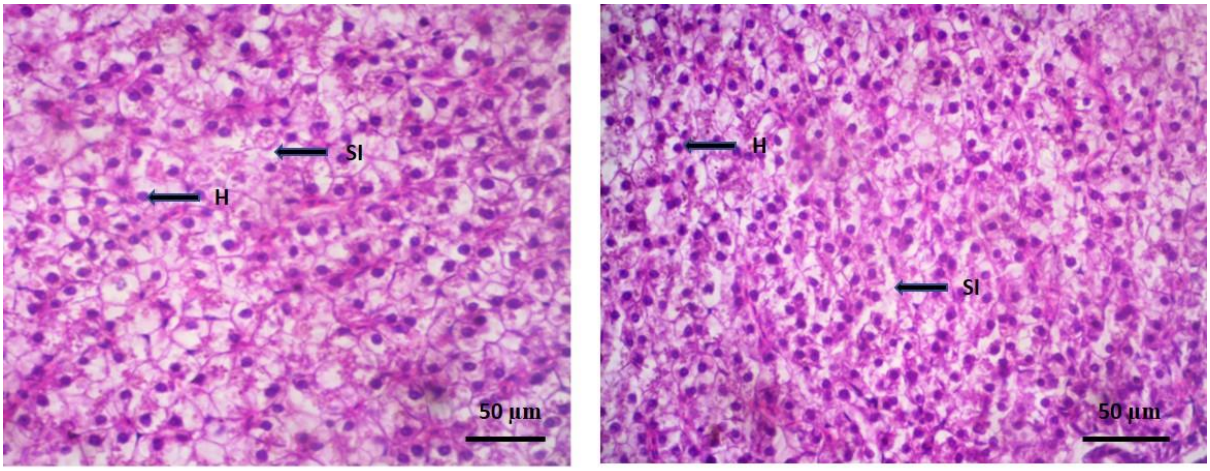

Figure 6a-b. Histology of liver section of *O. niloticus* fed control diet b: Histology of liver section of *O. niloticus* fed *L. aspera* 10g kg^{-1} diet.

role in aquatic animals is that it acts in the arachidonic acid cascade which can eventually synthesize the inflammatory mediators called eicosanoids (Oxley et al., 2010). GR expression suggest that *L. aspera* enriched feed can augment the antioxidant capacity of fish and may neutralize the free radicals.

Inclusion of *L. aspera* at inclusion level of 10g kg^{-1} enhanced the immunological parameters in Nile tilapia by exhibiting enhanced phagocytosis, complement activity, respiratory burst and lysozyme activity, and significantly enhanced growth performance compared with the control and other treatment groups. Results of the present study are in agreement with previous studies where Nile tilapia were fed different herbal supplements. Recent studies such as use of purslane (*Portulaca oleracea*) leaf powder enriched diet enhanced immune response, antioxidant activity and protection against *A. hydrophila* in Nile tilapia (Abdel-Razak et al., 2019), and guava (*Psidium guajava*) extract improved growth, nutrient utilization, immunity and resistance against *A. hydrophila* of Nile tilapia (Omitoyin et al., 2019). These results and the present study proved the efficacy of herbal feed additives in health improvement of Nile tilapia. A possible explanation of the beneficial effect revealed in the present study might be due to the presence of bioactive phyto constituents in *L. aspera*. However, this hypothesis merits investigations, but it is worth to mention that the presence of secondary metabolites in *L. aspera* is responsible for the immune-modulation, for instance sterols can activate phagocytosis and lysozymal enzyme activity (Ghosal et al., 1989). According to previous studies, *L. aspera* leaves contain phenolic compounds like gallic acid and egallic acid; flavonoid compounds such as procyanidin, leucasin, acacetin and apigenin (Meghashri et al., 2010); phytosterols (beta-sitosterol); tannins (Kripa et al., 2011). The results of the present study are in accordance to those reported in velvet bean (*M. pruriens*) (Musthafa et al., 2018). The presence of tannins in *L. aspera* has been accredited to the plant's ability to hasten the healing of lesions and inflamed mucous membranes of aquatic organisms (Prajapati et al., 2010). Flavonoids are free radical scavengers that prevent oxidative cell damage, phytosterols are known to up-regulate the immune response (Meghashri, 2010). Thus, the presence of antioxidant components and other bioactive compounds might be responsible for the activity of *L. aspera* in ameliorating infections caused by pathogens (Kurian et al., 2020). Bindhu et al. (2014) noticed that immunological parameters including

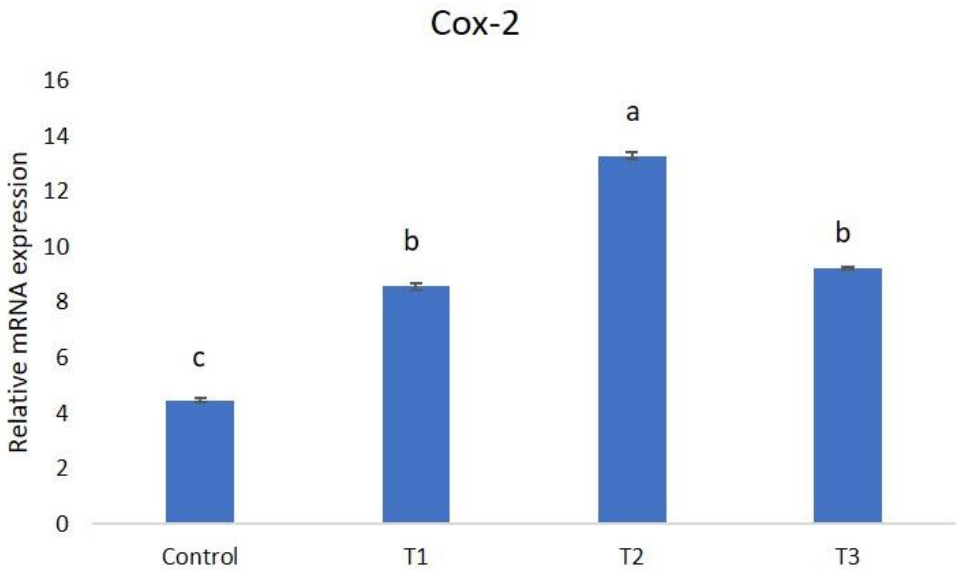

Figure 7a. Immune gene expression of COX-2 in *O. niloticus*

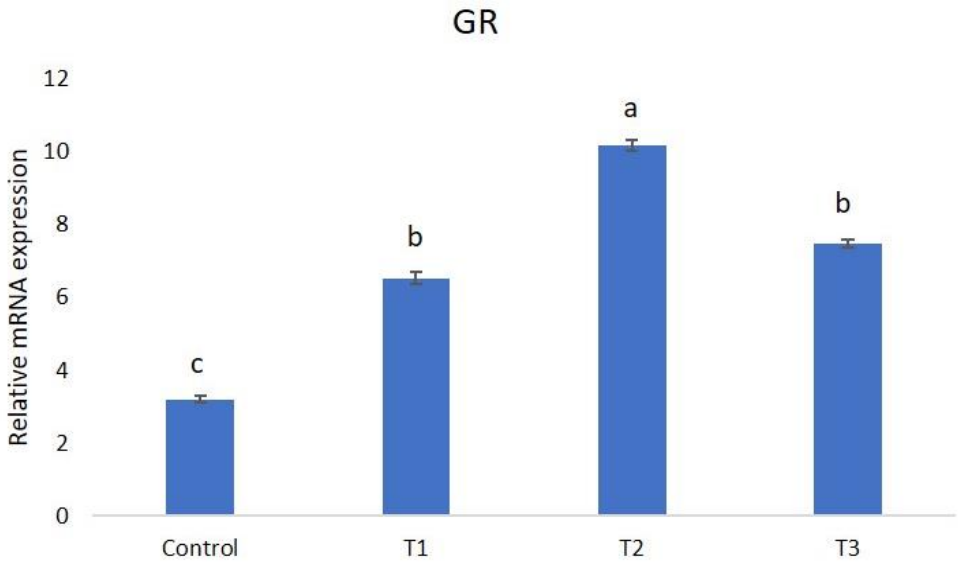

Figure 7b. Immune gene expression of GR in *O. niloticus* fed 10g kg^{-1} *L. aspera* enriched diet.

prophenol oxidase (proPO) activity, intracellular superoxide anion production and intra-agar lysozyme activities were significantly (P<0.01) improved in the combined herbal extract of humming bird tree (*Agathi grandiflora)* with *L. aspera* at 300 and 400 mg kg^{-1} fed to Indian white shrimp (*Fenneropenaeus indicus)* after white spot syndrome virus challenge. Results from the current study indicated that *L. aspera* possess immunostimulatory properties, and this might be due to the strong occurrence of polyphenolic compounds such as alkaloids, flavonoids, tannins and steroids that serve as free radical inhibitors or scavenger. However, to fully conclude, further studies are needed.

In conclusion, *L. aspera* exhibits beneficial effects such as antioxidant and anti-inflammatory activities due to the presence of flavonoids, phytosterols, tannins, catechins, glycosides and phenolic compounds. Protection against bacterial infection makes this medicinal plant a better immune strengthening agent. This is mainly believed to be the action of polyphenols which are free radical scavengers. Compounds of *L. aspera* might be used as lead compounds for designing potent immune-modulatory herbal drug, which can be used as treatment of microbial diseases in various aquatic organisms. Hence, we conclude that *L. aspera* can be included in the diet of Nile tilapia for strengthening the immunity and promoting growth and disease resistance. Further scientific exploration on molecular aspect as well as immunological studies is needed to clarify the role of *L. aspera* in strengthening the health status of tilapia and to establish the therapeutic efficacy of this plant.

Ethical Statement

All experiments were performed in compliance with the guidelines for animal welfare and the use of animals as prescribed by the Institutional Animal Ethics Committee (No. SOST/ PhD001/2019) of Kerala University of Fisheries and Ocean Studies, Kochi, Kerala, India.

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The authors do not declare any funding information.

Author Contribution

First author: experimental design of the study, acquisition and interpretation of experimental data, and drafting of the manuscript; Second author: interpretation of the data, and reviewing of the manuscript; Third author: revising the article for important intellectual content and critical reviewing of the manuscript; Fourth author: Editing and finalization of the manuscript.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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