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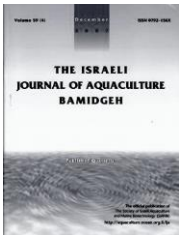

Effect of *Psidium guajava* and *Phyllanthus acidus* Leaf Extract on Immunostimulant Response of Nile Tilapia Against *Streptococcus agalactiae* Infection

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Abstract

A study was conducted to investigate the efficacy of diets supplemented with aqueous extract of plants consisting of leaf-extracts of *Psidium guajava* (PGLE), *Phyllanthus acidus* (PALE), and the mixture of PGLE and PALE at the rates of 5 and 10 g/kg in the feed of Nile tilapia *Oreochromis niloticus.* Following 12 weeks of feeding, the fish were challenged *with Streptococcus agalactiae* for 15 days. Immuno-hematological parameters were measured before and after the challenge. Results showed significantly enhanced innate immune parameters after administration of the plant extracts. Respiratory burst activity, phagocytic assay, and serum lysozyme content were 2 to 3 times higher than that of the control treatment; PGLE-10 group showed the highest values, while fish fed PGLE-10 had the highest post-challenge WBC, RBC, Hb, and Hct values compared to the control. Survival rates were higher in the groups with dietary supplementation of plant extracts compared to the control. The positive immuno-hematological impact of plant extracts show that these can be effectively used as alternative prophylactic and antimicrobial agents in Nile tilapia culture.

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Introduction

Nile tilapia has long been considered an ideal tropical fish species for aquaculture because of its robust nature, quick growth, resilience to sub-optimal water quality parameters, and disease resistance relative to other aquaculture species (Little et al., 2008). However, infectious diseases often pose a significant challenge to tilapia culture causing tremendous losses for the producers (Bondad-Reantaso et al., 2005). The most significant health problems facing tilapia culture is streptococcosis caused by *Streptococcus agalactiae,* a non-motile, oxidase and catalase-negative, gram-positive coccus (Rattanachaikunsopon and Phumkhachorn 2009).

Antibiotics and chemotherapeutics are often used to control disease infections in fish farming. Indiscriminate use of antibiotics in aquaculture is to be discouraged as it can lead to the development and strengthening of drug-resistant pathogens (Rattanachaikunsopon and Phumkhachorn, 2010), and contamination of fish carcass with residues that can pose potential risks to consumers (Abutbul et al., 2004). Methods developed for the protection against bacterial infection have been evolved as a useful prophylactic method (Bei et al., 2015). However, an individual vaccine is usually potent against a single pathogen, and there is no valid immunization against various microorganisms. Hence environmentally safer and cost-effective prophylactic antimicrobial agents to invigorate the immune system of fish against numerous pathogens are needed.

Guava (*Psidium guajava*) is a plant belonging to the family Myrtaceae and is used to isolate flavonoids, and saponins from leaves (Arima and Danno 2002). These leaf extracts are known to have antimicrobial (Metwally et al., 2010) and antioxidant (Chen and Yen 2007) properties. Star gooseberry *Phyllanthus acidus* belongs to the family Phyllanthaceae and is reported to have high phenolic content (Taylor 2003). *P. acidus* leaf extracts have shown potent antimicrobial activity (Jagessar et al., 2008). The objective of the present study was to evaluate the efficacy of aqueous extracts of the plant on immuno-hematological parameters, and disease resistance of Nile tilapia against *S. agalactiae* was investigated.

Materials and Methods

Preparation of plant leaf extracts.

Guava and star gooseberry leaves were collected from the Aquaculture and Aquatic Resources Management (AARM) farm, AIT, Thailand. Leaf-extracts were prepared according to Kamble et al. (2014). The leaves were washed well with distilled water, blotted, and dried in an oven (Memmert, Germany) at 50°C for 96 hours. 10 g of leaf powder and 100 mL of distilled water (BOECO Germany) was homogenized at room temperature using an orbital shaker (Hsiangtai D500) at the rate of 100 rpm for about 20 h. The aliquot was centrifuged (CENTRIKON T-124) at 8000 rpm for 15 min at room temperature; the supernatant was dried in a Rotavapor (BÜCHI R-200/205) at 35°C and further dried at 50°C for 48 h. A mortar and pestle were used to pulverize the dried leaf extracts that were stored at 4°C (Samsung RS265TDWP) until additional use.

Pathogen.

The pathogen *S. agalactiae,* propagated in Brain Heart Infusion (BHI, HI-MEDIA#M211) medium at 35±2°C overnight, was received from Center of Excellence for Shrimp Molecular Biology and Biotechnology (CENTEX Shrimp), Thailand. Glycerol (20%) with 0.85% saline solution was used for the stock culture and stored at -20°C.

Experimental fish.

Chitralada strain of Nile tilapia (8.9±0.2 g, mixed sex) were obtained from the AIT experimental facility. They were acclimatized to experimental conditions in 500 L fiberglass tanks for 15 days and fed with pelleted feed at 4% of body weight, twice a daily. A Eutech Cyberscan PC300 multi-parameter apparatus was used to measure water quality parameters such as temperature, pH, and dissolved oxygen during the experiment. Ammonia-nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N), and nitrate-nitrogen (NO_3-N) were measured following Boyd and Tucker (1992).

Preparation of fish feed.

The experimental feed was prepared by mixing a commercial tilapia feed containing 30% protein (Charoen Pokphand; CP-7710) with the aqueous plant extracts at a rate of 1.5 mL/2g. The wet blend was then passed through a mincer (MITSUYAMA YC80B-4) to produce spaghetti type strings which were broken to form 5-mm long pellets, which were dried at 50°C in a hot air oven for 24 h, and then stored at 4°C until use. The proximate compositions of feeds supplemented with the plant extracts are presented in Table 1**.**

Table 1. Proximate compositions of dietary plant extract treatments (as fed basis)

Treatments	*Moisture (%)*	*Ash (%)*	*Crude Lipid (%)*	*Crude Protein (%)*
C	7.6±0.1	8.2±0.1	6.5±0.1	30.5±0.2
PGLE-5	7.2±0.1	10.2±0.1	5.8±0.1	32.3±0.2
PGLE-10	7.3±0.1	10.2±0.1	5.9±0.1	35.6±0.4
PALE-5	7.5±0.1	10.2±0.1	5.8±0.1	33.9±0.2
PALE-10	7.3±0.1	10.3±0.1	5.5±0.4	35.8±0.4
Mixed-5	7.5±0.1	8.3±0.1	5.7±0.1	33.9±0.2
Mixed-10	7.6±0.1	10.3±0.1	6.4±0.2	35.8±0.4

The given values are the means of three replicates ± SE
C: control; PGLE: *Psidium guajava* leaf extract; PALE: *Phyllanthus acidus* leaf extract; Mixed: mixture of PGLE and PALE

Experimental design.

Glass aquaria of 150 L capacity each, equipped with a water recirculation system and stocked with 50 individual fish were used for the experiment to evaluate hematological and innate immune parameters. The research was laid out in a completely randomized design with three replicates for each of the seven treatments consisting of T_1 (PGLE-5; *P. guajava* leaf extract at 5 g/kg of feed), T_2 (PGLE-10; *P. guajava* leaf extract at 10 g/kg), T_3 (PALE-5; *P. acidus* leaf extract at 5 g/kg), T_4 (PALE-10; *P. acidus* leaf extract at 10 g/kg), T_5 (Mixed-5; a mixture of *P. guajava* and *P. acidus* leaf extract, each at 5 g/kg), T_6 (Mixed-10; the mixed extract, each at a rate 10 g/kg), and the control (no plant extracts). The fish were fed at 4% of body weight, twice daily for 12 weeks. Water quality parameters were also measured during this period.

Challenge test.

After 12 weeks of rearing, the fish were transferred to glass aquaria of 150 L capacity for the challenge test with 30 fish stocked in each tank kept in triplicate for each treatment. The fish were challenged using *S. agalactiae* and mortality was recorded for two weeks. Hematological and innate immune parameters were monitored before and after the challenge test. 100 µL of bacterial suspension (1×10^7 CFU/mL) was injected intraperitoneally (IP) into ten individual fish from each replicate (30 individuals per treatment) and mortality was recorded for 15 days. Clinical symptoms for common *S. agalactiae* infections such as hemorrhage on pectoral fin and body, bilateral exophthalmia, distended abdomen due to ascites, corneal opacity, and pop-eye were observed in the challenged fish. Tissues from dead fish were removed for bacteriological analysis to confirm *S. agalactiae* infection as the cause of mortality. Five fish from each replicate were sampled for serum and blood parameter measurements described in the sections below. Cumulative mortality and relative percent survival (RPS) from the different treatment groups were calculated as follows:

Cumulative mortality (%) = [(total mortality in each treatment after challenge)/ (total number of fish challenged for some treatment)] × 100. Relative percent survival (Amend 1981) = 1-[(Mortality (%) in treated group)/ (mortality (%) in control group)] × 100

*Blood and serum collection***.**

Before blood collection, the experimental fish were starved for 24 h. Five individuals randomly selected from each replicate were anesthetized using Ethyl 3-aminobenzoate methanesulfonate (MS-222, SIGMA-ALDRICH) at a rate of 100 mL/10L. Blood sample was collected from the caudal vein using a heparinized syringe of 1 mL (24 gauge). The blood sample was immediately added to an Ethylenediaminetetraacetic (EDTA) tube (BD Vacutainer®) kept separately for each fish, shaken gently, and refrigerated at 4°C. Additional five fish per replicate were anesthetized for serum separation. The collected

blood sample was kept for clotting in a slanted position at room temperature for 12 h**.** For the separation of serum, samples were centrifuged (Centrisart® A-14) at 8000 rpm for 15 min, and eppendroff tubes with screw caps were used to store the supernatant at -20°C until further use.

*Innate immune parameters**.***

Phagocytic activity was measured using a modified method described by Siwicki and Anderson (1993). For this assay, *Staphylococcus aureus* (1×10^7 CFU) in 0.1 mL of phosphate-buffered saline (PBS), and 0.1 mL of heparinized blood samples were added in each well of a microplate. After mixing thoroughly, the samples were kept for incubation (for 30 min at 25°C). After incubation, the microplate was mixed gently, and 0.05 mL of the contents from each well was transferred to a glass slide for smear preparation. The smear was then fixed using ethanol, kept for air drying, and stained with 7% Giemsa. One hundred phagocytic cells per slide were counted using a microscope; these were used to determine phagocytic activation (PA) according to the following formula: phagocytic activation (PA, %) = [(Number of phagocytic cells with engulfed bacteria)/ (Number of phagocytes)] $\times$ 100

Respiratory burst activity was determined using method of Siwicki and Anderson (1993). An equal portion of 0.2% NBT (Nitroblue tetrazolium) and blood was mixed (1:1), and incubated at 25°C for 30 min. From this 50 µL was put in a glass tube and mixed with 1 mL of dimethylformamide (Sigma, USA) for solubilization of the reduced formazan product. This mixture was centrifuged at 2000 $\times$ g for 5 min. The supernatant was taken, and the amount of NBT reduction measured at an optical density (OD) of 540 nm against the blank Dimethylformamide.

A turbidimetric assay was used to measure serum lysozyme (SL) content (Kumari *et al.* 2006). Microtitre plates (96 well, U bottom) were used to mix 15 µL test serum and 150 µL of *Micrococcus lysodeikticus* (0.3 mg/mL) in 0.02 M sodium acetate buffer, pH 5.5). After immediately adding the substrate, initial OD was taken at 450 nm and final OD after 260 seconds. Serum lysozyme values were expressed in mg/mL.

Hematological parameters.

An automatic cell counter (HeCo Vet C 9SEAC, Italy) was used to determine hematological parameters (red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), and hematocrit (Hct)). Microhematocrit method was applied to measure Hct followed by automated reading. A computerized automated process with a cell counter was used to determine hemoglobin.

Statistical Analysis.

All percentage data were transformed into Arcsine. Statistical package for social sciences (SPSS) software (version 21.0) was used to analyze data. Data were analyzed applying one-way ANOVA followed by Duncan's multiple comparison test at $P < 0.05$. Kaplan-Meier analysis was used to estimate the survival of fish in each challenged treatment group, while the differences among treatment groups were determined using the log-rank (Mantel-Cox) test for Pairwise comparisons.

Results

Water quality parameters.

During the trial, water parameters monitored were: temperature 30.33±0.04ºC, pH 7.34±0.02, dissolved oxygen (DO) 5.00±0.06 mg/L, ammonia nitrogen (NH_3N) 0.16±0.01 mg/L, nitrite nitrogen (NO_2N) 0.12±0.01 mg/L, and nitrate nitrogen (NO_3N) 1.19±0.02 mg/L. During the challenge experiment, average water parameters were: temperature 29.29±0.02ºC, pH 7.31±0.02 units and DO 5.47±0.05 mg/L.

Innate immune parameters.

Innate immune parameters such as NBT (A_{540}), phagocytic activity (%), and serum lysozyme activity (mg/mL) of Nile tilapia fed dietary phytobiotics are shown in Table 2.

Table 2. Innate immune parameters of Nile tilapia fed plant extract treatments

Treatment	*Respiratory burst activity (NBT (A_{540}))*		*Phagocytic activity (%)*		*Serum Lysozyme Activity*	
	Pre-challenge	*Post-challenge*	*Pre-*	*Post-*	*Pre-challenge*	*Post-*
C	0.53±0.07^{a}	0.33±0.06^{a}	40.89±0.69^{a}	30.56±0.98^{a}	149.8±4.4^{a}	160.1±4.2^{a}
PGLE-5	1.24±0.06^{b}	1.04±0.05^{b}	86.03±1.75^{c}	78.03±2.67^{c}	270.0±11.5^{b}	290.0±10.4^{b}
PGLE-10	1.63±0.12^{d}	1.46±0.12^{d}	93.60±0.44^{d}	86.60±1.06^{d}	355.3±22.8^{d}	376.3±25.1^{d}
PALE-5	1.20±0.12^{b}	1.01±0.12^{b}	81.17±1.93^{b}	72.16±2.39^{b}	267.0±5.7^{b}	289.0±4.8^{b}
PALE-10	1.32±0.09^{bc}	1.13±0.08^{bc}	90.97±1.42^{d}	82.63±1.88^{cd}	316.0±6.5^{c}	341.0±6.4^{c}
Mixed-5	1.27±0.09^{b}	1.07±0.10^{b}	80.86±1.35^{b}	70.87±1.25^{b}	267.0±7.4^{b}	292±10.2^{b}
Mixed-10	1.45±0.10^{c}	1.26±0.09^{c}	90.53±0.53^{d}	83.20±1.69^{cd}	326.7±10.1^{cd}	348.7.±10.2^{cd}

The given values are the means of three replicates ± SE (n = 5 fish/group).
*Duncan Multiple Comparison Test, mean values with same column followed by same superscripts are not significantly different (P>0.05).
C: control; PGLE: *Psidium guajava* leaf extract; PALE: *Phyllanthus acidus* leaf extracts; Mixed: mixture of PGLE and PALE

Innate immune parameters were significantly enhanced after administration of single or mixed plant extracts. However, fish fed diet supplemented with PGLE-10 showed significantly higher pre and post-challenge values compared to the control for all immune parameters (NBT: 1.63±0.12, 1.46±0.12; PA: 93.60±0.44%, 86.60±1.06%, and SL: 355.3±22.8 mg/mL, 376.3±25.1 mg/mL, respectively).

Hematological parameters.

During the pre-challenge period, there were no significant changes (P> 0.05) observed in WBC (Fig. 1A), RBC (Fig. 1B), Hb (Fig. 1C), and Hct (Fig. 1D). However, fish fed the diet supplemented with PGLE-10 showed a higher WBC (9.20±1.3 × 10^2 cells/µL), RBC (2.77±0.35 × 10^6 cells/µL), Hb (12.3±1.4 g/dL), and Hct (35.3±4.2 %) content.

Disease resistance.

Fish were challenged with *S. agalactiae* after 12 weeks of the feeding trial, and cumulative survival was recorded after 15 days (Fig. 2). Data on relative percentage survival (RPS), mortality rate, and survival rate, of plant extract treatments challenged with *S. agalactiae* are given in Table 3. Fish fed diets supplemented with plant extract treatments resulted in significantly higher survival than the control group (P < 0.05 by log-rank test). The highest relative percentage survival was found in PALE-5 and PGLE-10 (81.0%) followed by Mixed-5 (76.0%), PGLE-5 (71.4%), PALE-10 (71.0%) and Mixed-10 (71.0%), respectively.

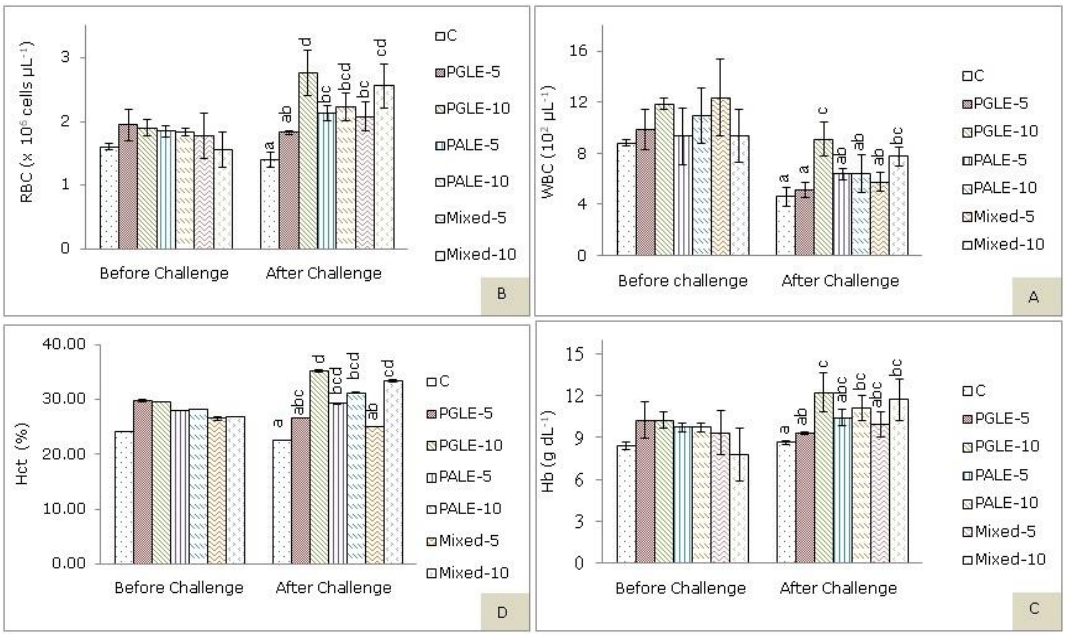

Fig. 1 A) Leucocyte (WBC, × 10^2 cells/µL), B) erythrocyte (RBC, × 10^6 cells/µl), C) hemoglobin (Hb, g/dL) and D) hematocrit (Hct, %) count of Nile tilapia fed plant extracts; Results with the means of three replicates ± SE (n = 5 fish/group).

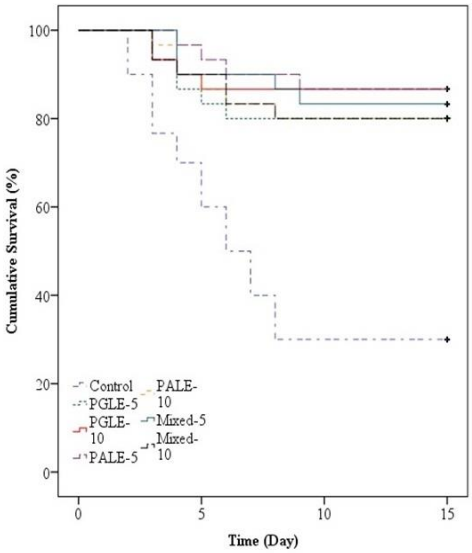

Fig. 2 Kaplan-Meier survivorship curves (cumulative survival (%) over time (Day)) for Nile tilapia after challenge with *Streptococcus agalactiae* (1×10^7 CFU/mL); the fish fed plant extract treatments. A result with the means of three replicates ± SE (n = 30 fish/group).

Table 3. Relative percentage survival (RPS) of challenged Nile tilapia fed plant extracts

Treatments	*Survival rate (%)*	*Mortality rate (%)*	*Relative percentage survival (%)*
C	30.0±0.0[a]	70.0±0.0	-
PGLE-5	80.0±5.8[b]	20.0±5.8	71.4±8.2
PGLE-10	86.7±3.3[b]	13.3±3.3	81.0±4.8
PALE-5	86.7±3.3[b]	13.3±3.3	81.0±4.8
PALE-10	80.0±5.8[b]	20.0±5.8	71.0±8.2
Mixed-5	83.3±3.3[b]	16.7±3.3	76.0±4.8
Mixed-10	80.0±0.0[b]	20.0±0.0	71.0±0.0

The given values are the means of three replicates ± SE (*n* = 30 fish/group).
*Duncan Multiple Comparison Test, mean values with same column followed by same superscripts are not significantly different (*P*>0.05).
C: control; PGLE: *Psidium guajava* leaf extract; PALE: *Phyllanthus acidus* leaf extract; Mixed: mixture of PGLE and PALE

Discussion

Antibiotics are routinely used in aquaculture to control bacterial diseases, but they have adverse environmental impacts, are hazardous to consumers, and promote the increase in development of antibiotic-resistant bacteria (Abutbul et al., 2004). The use of environment-friendly prophylactic and therapeutic agents can reduce such risks. Various herbal bioactive extracts have been used as an alternative approach to control diseases in aquaculture (Gültepe et al., 2014; Gabriel et al., 2015; Laith et al., 2017), and as immunostimulants (Ataguba et al., 2014; Kamble et al., 2015). The present work evaluates the efficacy of aqueous extracts of plants on immuno-hemato parameter and disease resistance of Nile tilapia against *S. agalactiae* and other fish pathogens (Abutbul et al., 2005).

A rapid improvement in phagocyte function can be seen in response to inclusion of plant extracts in diets (Dügenci et al., 2003). Plant extract treatment-groups significantly enhanced phagocytic, nitroblue tetrazolium assay, and lysozyme activities of tilapia fed plant extracts during the pre- and post-challenge periods. Non-specific immune response was more pronounced with dietary inclusion rates higher than 5 g/kg, and fish fed PGLE-10 demonstrated the highest activity. Alkaloids, flavonoids, and polysaccharide types of active components are available in plants or herbs and they serve as immunostimulants for the enhancement of innate immunity of fish against pathogenic diseases (Ardó et al., 2008). Enhanced innate immunity had also been reported in tilapia fed mangrove species (Laith et al., 2017), basil (Panprommin et al., 2016), and ginger (Brum et al., 2017).

RBC, Hb, and Hct are hematological indices which are useful in detecting changes in the health of fish during the evaluation of immunostimulants. Pre-challenge RBC, WBC, Hb, and Hct counts of tilapia were not significantly different among plant extract treatments groups, and the control group. It was observed that supplementation of plant extracts of more than 5 g/kg significantly increased these indices during the post-challenge period; PGLE-10 treatment showed the highest increase during both the pre- and post-challenge periods indicating that guava leaf extract acts as an immunostimulant. The inclusion of plant extract at a rate of 10 g/kg induced erythropoiesis and lymphopoiesis that resulted in increases in RBC, WBC, Hb, and Hct thereby strengthening the oxygen carrying capacity and resistance mechanisms across the physiological stress of common carp *Cyprinus carpio* (Pratheepa et al., 2010). The presence of antioxidants in plant extracts triggered erythropoiesis by reducing the rate of oxidant-induced hemolysis (Sheeja et al., 2006). Polyphenols, alkaloids, glycosides, and sugar reduction could also be responsible for increased WBC (Antai et al., 2009). The results of the present study agreed with some previous reports that highlighted significant increase in hematological indices in Nile tilapia fed garlic (Supa-aksorn et al., 2017), aloe (Gabriel et al., 2015), and rocket (Khalil et al., 2015). The results of hematological parameters reveal that supplementation of plant extracts acts as an immunostimulant.

The relative percentage survival and survival rate of plant extract treatments were substantially higher than in the control group. Fish fed diets supplemented with PGLE-10 & PALE-5 resulted in significantly higher survival rates and relative percentage survival of Nile tilapia than in the control. After a bacterial challenge, the cumulative survival (Kaplan-Meier survivorship curves) of Nile tilapia was recorded for 15 days. The post-challenge enhancements in innate immune and hematological parameters were positively correlated with improved survival, thereby protecting Nile tilapia against *S. agalactiae.*

The present experiment and some of the earlier reports have demonstrated that the inclusion of plant extract as a dietary supplement for fish has a prophylactic effect in managing disease outbreaks. Previous investigations revealed that diets supplemented with leaf extracts of buta-buta (Laith et al., 2017), basil (Panprommin et al., 2016), betel (Ataguba et al., 2018), African basil and ginger (Brum et al., 2017), enhanced disease resistance of Nile tilapia against *S. agalactiae*.

In conclusion, diets enriched with plant extracts had a relatively higher percent survival of Nile tilapia against the *S. agalactiae* infection. Addition of 10 g/kg of guava leaves (PGLE-10) improved the hematological and immunological parameters of Nile tilapia. Since the present study showed positive effects of plant extracts, especially guava extract, on the resistance against pathogenic *S. agalactiae*, it can be used efficiently as an alternative prophylactic and antimicrobial agent in Nile tilapia culture.

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