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RESEARCH PAPER

Piper betle Leaf Extract Inhibits Multiple Aquatic Bacterial Pathogens and In Vivo *Streptococcus agalactiae* Infection in Nile Tilapia

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Abstract

An *in vitro* assessment of antimicrobial properties of aqueous and ethanol extracts from solo garlic (*Allium sativum*), garlic chive (*Allium tuberosum*) and betel leaves (*Piper betle*) on six bacterial pathogens in aquaculture, and a challenge of Nile tilapia, *Oreochromis niloticus* with *Streptococcus agalactiae* were performed. Generally, minimum inhibitory concentrations (MIC) ranged from 26.63 to 53.25 mg mL^{-1} for aqueous solo garlic (G) and 14.60 to 29.20 mg mL^{-1} for garlic chive extracts for all pathogens tested. Ethanol extract of betel leaves (P) exhibited the strongest antibacterial activity (0.15 – 0.60 mg mL^{-1}). P and G incorporated in feed at high and low doses as multiples of MIC [High; H (10X for PH and 3X for GH) and Low; L (3X for PL and 1X for GL)] were fed to tilapia followed by *in vivo* challenge against *S. agalactiae* (1 x 10^8 CFU mL^{-1}). Ethanol extract of *P. betle* significantly improved survival ($P<0.05$; PH=100%, PL =77%). White blood cells (WBC), lymphocytes and monocytes differed significantly ($P<0.05$) among treatments and the highest WBC value (1.175 × 10^3) was for PH. Use of ethanol extract of *Piper betle* seems promising for sustainable disease management in aquaculture.

Keywords: Herbal, antimicrobial, risk, haematology, survival.

Introduction

Outbreaks of diseases pose a major threat for sustainable aquaculture development in Asia. Several bacterial species have been reported as current and emerging threats for both fish and shrimp aquaculture industries. These include *Streptococcus agalactiae, Aeromonas veronii, Edwardsiella ictaluri* and *Edwardsiella tarda* affecting farmed tilapia (*Oreochromis* spp.) and striped catfish, *Pangasianodon hypophthalmus* (Dong *et al*., 2015a; Dong*et al*., 2015b); and *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis diseases (AHPND) in white leg shrimp, *Penaeus* (*Litopenaeus*) *vannamei* (Joshi *et al*., 2014; Kongrueng *et al*., 2015; Tran *et al*., 2013). Streptococcus infections have an epidemiological importance in aquaculture because mortalities can be as high as 75% with potential horizontal and vertical (Pradeep *et al*., 2016) transmission. Streptococcus infection in tilapia has caused the industry a colossal loss worth US$250 million in 2008 alone (Klesius, Shoemaker, & Evans, 2008). Current therapeutics against bacterial diseases in Asian aquaculture still relies on antibiotics (Anka, Faruk, Hasan, & Azad, 2013). The use of antibiotics not only increases production cost but also poses a risk to public health due to development of antimicrobial resistance and release of antibiotic residues to the environment. Application of herbal extracts as feed additives to replace the current antibiotic therapy and to combat bacterial diseases provides an alternative approach for sustainable aquaculture.

Dietary herbal additives using the Chinese shrubby sophora (*Sophora flavescens*), allspice (*Pimenta dioica*) and cumin (*Cuminum cyminum*) extracts provided promising results to prevent *S. agalactiae* and *S. iniae* infections in fish (Wu *et al*., 2013; Yılmaz & Ergün, 2014). Bioactive compounds originating from herbs have also been reported to elicit immunoactivation in tilapia by boosting immunological parameters (Abu-Elala, Mohamed, Zaki, & Eissa, 2015). Reports on the use of garlic as immunostimulants in tilapia have shown that it can enhance the immune response of tilapia under cold stress to pathogens such as *Aeromonas hydrophila* and *Pseudomonas fluorescens* (Diab, Aly, John, Abde-Hadi, & Mohammed, 2008; Mesalhy Aly, El Naggar, Mohamed, & Mohamed, 2010). Garlic and betel leaves contain flavonoids (Azzini *et al*., 2014) that possess anti-oxidative properties with benefits for the immune system. A dietary supplementation of

flavonoids and fish oil is known to elicit transcriptomic anti-inflammation response in obese humans (Cialdella-Kam *et al*., 2016). The use of herbal additives therefore, appears to be a practical and cost-effective approach for aquaculture producers considering their antibacterial properties, availability, and reduced concerns about antimicrobial resistance and antibiotic residues in aquaculture products.

In this study, we investigated *in vitro* antimicrobial properties of selected herbal extracts from solo garlic (a variety of *Allium sativum*), garlic chive (*Allium tuberosum*) and piper betel leaves (*Piper betle*) against six pathogenic bacterial strains previously isolated from diseased fish and shrimp. Subsequently, the extracts from solo garlic and betel leaves were formulated in feed for an *in vivo* trial to evaluate the effectiveness of herbal supplemented diets to prevent *S. galalactiae* infection in Nile tilapia, *Oreochromis niloticus* (Chitralada strain: AIT) model.

Material and Methods

Bacterial Isolates and Culture Condition

Six bacterial pathogens that can cause mortality in fish and shrimp were used in this study. Detailed description of these bacterial isolates and their culture conditions are provided in Table 1. Two strains of *Vibrio parahaemolyticus* causing severe hepatopancreatic pathogenesis and mortality in shrimp (Joshi, *et al*., 2014) were obtained from BIOTEC/NSTDA, Thailand. Four pathogenic bacterial isolates (*Edwardsiella ictaluri, Edwardsiella tarda, Aeromonas veronii* and *Streptococcus agalactiae*) causing diseases in freshwater fish were selected based on previous studies (Dong *et al*., 2015a; Dong *et al*., 2016; Dong*et al*., 2015b). Prior to *in vitro* inhibition assay, bacteria were recovered from glycerol stocks by streaking onto indicated agar medium and incubated at 30ºC for 16 – 32 hours (h). Each bacterial pre-culture was prepared by inoculating 5 ml of the same broth medium with a single colony and incubated at 30ºC while shaking for 16 – 32 h. After incubation, bacteria were suspended in 0.85% NaCl solution and the OD_{600} was adjusted to 0.6. Bacterial suspension was then diluted with Mueller Hinton Broth (MHB) at a cell density of 10^6 colony forming units/ml (CFU mL^{-1}).

Herbal Extract Preparation

Solo garlic or single clove garlic (a variety of *Allium sativum*), garlic chive (*Allium tuberosum*) and betel leaves (*Piper betle*) were purchased from a local market in Samut Prakan province, Thailand. Extraction was carried out using two solvents, namely water (solo garlic, garlic chive and piper betle) and ethanol (piper betle). Ethanol extracts of garlic were avoided because the active compounds in their extracts have a short half-life while important cysteine sulphonates and alliin-derived transformation products being removed with time (Lawson, 1993). One kilogram each of solo garlic and garlic chive was homogenized in one litre of distilled water. Homogenates were then filtered through clean UV sterilized cheesecloth followed by Whatman paper. An aliquot of each extract thus obtained was sterilized using 0.22 µm sterile filters and stored at 4°C for further experiments.

The betel leaves were dried in an oven at 50°C for three days, and their extracts were prepared using the boiling method and 40% ethanol extraction method. Before filtering, 375 g of chopped betel leaves was boiled in 750 ml of water for 15 minutes. After cooling down, the extract was filtered through cheesecloth, Whatman paper, and 0.22 µm sterile filters as described above. For alcohol extraction, the same amount of chopped betel leaves was submerged in 750 ml of 40% ethanol in a closed bottle and incubated at 16°C for 60 h. After filtration by cheesecloth and Whatman paper, the ethanol solvent was evaporated at 85°C for 30 min using a rotary-evaporator (Buchi Rotavapor R-200/205). The resulting extract was sterilized using 0.22 µm filters. Refined extracts obtained were at various concentrations (Table 2). Each extract was diluted in

Table 1. Bacterial isolates used for in vitro MIC determination

Species	Culture conditions	Origin	Disease	References
Vibrio parahaemolyticus 5HP	TSB+1.5% NaCl, 30ºC, 16 h	White leg shrimp (*Penaeus vannamei*)	AHPND	Joshi et al. 2014
Vibrio parahaemolyticus 2HP	TSB+1.5% NaCl, 30ºC, 16 h	White leg shrimp (*Penaeus vannamei*)	Collapse epithelium of the hepatopancreas	Joshi et al. 2014
Edwardsiella ictaluri	TSB, 30ºC, 32 h	Striped catfish (*Pangasianodon hypophthalmus*)	Edwardsiellosis	Dong et al. 2015b
Edwardsiella tarda	TSB, 30ºC, 16 h	Nile tilapia (*Oreochromis niloticus*)	Edwardsiellosis	Dong et al. 2016
Aeromonas veronii	TSB, 30ºC, 16 h	Nile tilapia (*Oreochromis niloticus*)	Hemorragic septicsemia	
Streptococcus agalactiae	TSB, 30ºC, 32 h	Nile tilapia (*Oreochromis niloticus*)	Streptococcosis	Dong et al. 2015a

MHB at percentages of 75, 37.5, 18.75, 9.37, 4.68, 2.34, and 1.17% (v:v).

In vitro Inhibition Assay of Herbal Extracts against Aquatic Bacterial Pathogens

Antimicrobial activity of the herbal extracts was evaluated using a minimal inhibitory concentration (MIC) test. The assay was performed using microbroth dilution method in accordance with guidelines from the Clinical and Laboratory Standards Institute (Cockerill *et al*., 2012). A volume of 50 µL of each bacterial suspension (10^6 CFU mL^{-1}) was loaded to each well of a 96-well plate followed by addition of an aliquot of 100 µL serially diluted herbal extracts giving diluted percentages of 50, 25,12.5, 6.25, 3.125, 1.562 and 0.78% for each initial working percentages mentioned above. Control tests included wells without tested herbal extracts (No H), wells without bacteria (No B), and wells with only medium (Blank). Each test was carried out in triplicate. After incubation for 24 h at 30°C, microtitre plates were read at OD_{600} by a spectrophotometer (SpectraMax). Percentage bacterial growth inhibition was calculated by the formula given below and MIC90 was recorded as the lowest concentration of herb extract at which 90% of the tested bacteria were inhibited.

$$\%\ Growth\ inhibition = 100 - \left[\frac{(OD_{Test} - OD_{No\,H})}{(OD_{No\,B} - OD_{Blank})} \times 100\right]$$

Formulation of Herbal Supplemented Feed for *In Vivo* Test

Aqueous extract from solo garlic (G) and ethanol extracted-betel leaves (P) were chosen for preparation of the test feeds. Doses based on MIC90 of solo garlic extract (26.63 mg mL^{-1}) and betel leaf extract (0.15 mg mL^{-1}) against *S, agalactiae* were considered. Two doses each of solo garlic [1X (Low dose; GL) and 3X (high dose; GH)] and *Piper betle* leaf [3X (Low dose; PL) and 10X (high dose; PH)] extracts were made (where X is the MIC90). Commercial tilapia feed (2 kg each) was homogenized using a blender and each herbal product was added at the following rates: 125 and 375 mL of solo garlic extract/kg feed, and 23.4 and 78 mL of betel leaf extract/kg feed for L and H, respectively to prepare different concentrations (Table 3). The herbal product was mixed well using distilled water (500 ml kg^{-1} feed) and the mixture was re-pelleted and dried in an oven at 50ºC overnight. Proximate composition of the diets were then determined. Control diets were pelleted using distilled water.

In vivo Feeding Trial against Challenge by *S. agalactiae* in Nile Tilapia

Challenge Test with *S. agalactiae*

Tilapia juveniles (24.0 ± 0.2 g) were obtained from the AIT aquaculture facility and maintained in three tanks, each of 300 L capacity using a static water system. Water quality was monitored daily during the acclimatization period of two weeks. Fish were then distributed into eighteen glass aquaria (six groups in triplicate; Table 3), each of 150 L capacity filled with de-chlorinated tap water with ten individuals in each tank.

A pathogenic strain of *S. agalactiae* (Table 1) was used in the bacterial challenge test. A culture of the isolate was prepared as mentioned above. Two control groups (C1 – positive control and C2 – negative control) were fed with feed pellets without herbal additives while four others were fed at a rate of 3% of body weight twice daily with the herbal extract

Table 2. Concentrations of extracts tested for MIC against 6 piscine/crustacean pathogenic bacteria

	Extract Concentration	Serial Dilution Concentration (mg mL^{-1})						
Herbal extract	mg mL^{-1}	0.78%	1.562%	3.125%	6.25%	12.5%	25%	50%
Piper Ethanol	19.3	0.15	0.30	0.60	1.21	2.41	4.83	–
Piper Aqueous	26.7	0.21	0.42	0.83	1.67	3.34	6.68	–
Garlic Chive (Aqueous)	116.8	–	–	3.65	7.30	14.60	29.20	58.40
Solo Garlic (Aqueous)	213.0	–	–	6.66	13.31	26.63	53.25	106.50

Table 3. Details of *in vivo* challenge trial of *S.agalactiae* infection in Nile tilapia

Treatment	Code	Concentration % (Dose mg g^{-1} feed w/w)	*S.agalactiae* (CFU $fish^{-1}$)	% Survivors
Solo garlic; high dose	GH	7.99 (79.88)	1×10^8	56.67
Solo garlic; low dose	GL	2.66 (26.63)	1×10^8	46.67
Piper betel; high dose	PH	0.15 (1.51)	1×10^8	100.00
Piper betel; low dose	PL	0.045 (0.45)	1×10^8	76.67
Positive Control	C1	–	1×10^8	16.67
Negative Control	C2	–	0	100.00

feeds (GL, GH, PL and PH as laid out in Table 3. On day 8, the herbal treated groups and one control group (C1) were injected intraperitoneally with 0.2 ml of bacterial solution at concentration of 1×10^8 CFU mL^{-1} after adjustment to McFarland standards. Prior to injection, fish were anaesthetized using clove oil at a concentration of 10 ml L^{-1} of water. Another control group (C2) was not-injected (Table 3). The same feeding pattern was continued and mortality was observed daily for 15 days post challenge. Infected fish displayed typical signs and symptoms of Streptococcus infection in fish such as upside down swimming, opaque eyes with exophthalmia, and haemorrhage around the base of fins. Abscesses were observed under the inferior jaw in the positive controls but were totally absent in the treated fish. Mean water temperature during challenge was 29.1 ± 0.03 °C as recorded from a digital apparatus (Eutech Cyberscan PC300 multi-parameter meter).

Analysis of Haematological Parameters

Blood samples were collected from the caudal vertebral vein/artery of the fish by venipuncture with sedation. Sedation was achieved using lidocaine hydrochloride at 300 mg L^{-1} of water (Collymore, Tolwani, Lieggi, & Rasmussen, 2014). The lateral approach was employed in collection of blood. A needle was inserted close to the base of the caudal peduncle just below the lateral line and directed towards the ventral side of the vertebra and moved slightly while applying a negative pressure on the plunger. Once blood flow was established, the plunger was pulled up to enable suction of blood. Upon reaching the 1 mL mark, the needle was pulled out and blood was immediately transferred into a K-2 EDTA blood tube and aspirated. A second sample was taken and added to the tube to make up the volume.

The blood cell automatic cell counter HeCo Vet C 9SEAC (Italy) was used to determine erythrocyte count (RBC), Haemoglobin concentration (Hb), Haematocrit value (Hct), Mean corpuscular volume (MCV), White Blood Cell Count (WBC) and Thrombocytes. Haematocrit was determined using the microhaematocrit method followed by automated reading. For haemoglobin, electrolyte lyses preceded the automated method. Mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were determined using mathematical relationships following Sink and Feldman (2004).

Statistical Analysis

Survival analysis followed by multiple comparisons test using the Bonferroni correction factor was performed with GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Other data were analysed using one-way analysis of variance followed by mean separation via Fisher's LSD at 5% level of significance. This was done with the aid of Minitab 16 statistical software (Minitab, 2010). Relative risk of mortality was estimated using epitools package in R (Aragon, 2012) with the Wald method of measurement at a confidence interval of 95% and probabilities being exact fisher's values.

Results

***In vitro* Inhibition of Herbal Extracts against Aquatic Bacterial Pathogens**

Percentage growth inhibition curves for garlic chive (3.65 to 58.40 mg L^{-1}) and solo garlic (6.66 to 106.5 mg L^{-1}) extracts (Figure 1a; Figure 1b) as well as aqueous and ethanol extracts of betel leaves (0.21 to 6.68 mg L^{-1} and 0.15 to 4.83 mg L^{-1}, respectively) (Figure 1c; Figure 1d) indicated that all herbal extracts had an inhibitory effect against the tested fish and shrimp bacterial pathogens. To obtain ≥ 90% growth inhibition (MIC90) for all tested bacteria, at least 29.20 mg mL^{-1} of garlic chive and 53.25 mg mL^{-1} of solo garlic extracts were required (Figure 1a; Figure1b) while the corresponding values were 3.34 mg mL^{-1} and 0.15 mg mL^{-1} for betel leaf extracts from water and alcohol, respectively (Figure 1c; Figure 1d). Based on the concentration of herbal extract used to gain MIC90 (Table 2, Table 4), *E. ictaluri* seemed to be the most vulnerable strain while susceptibility of *E. tarda* and *S.agalactiae* to lower concentrations of the assayed herbal extracts appeared to be relatively lower except for the ethanol extract of *Piper betle*.

Efficacy of Herbal Supplemented Diets against *S. agalactiae* Infection in Nile Tilapia

The survival curve (Figure 2) shows that there was no mortality among fish fed with a high dose of piper betel extract (PH) and the negative control group (C2) during the 15-day experimental assay. This was consistent across the replicates. Groups treated with a low dose of betel leaf extract (PL) yielded a mean survival of ~77% at day 15 compared to 100% with PH but this was not statistically significant ($P = 0.01$; Bonferroni threshold = 0.0036). Although survival rates after feeding of solo garlic extract supplemented feed at low (GL) and high (GH) doses were ~47%and ~57%, respectively, they did not differ significantly (All $P>0.0036$) from the level observed for PL. Survival in the control groups without herbal treatment (C1) was lower (~17%) than all the treatments and was statistically not significant ($P = 0.004$) compared to GL treated fish. In summary, survival of fish treated with various herbal extracts as well as controls was in the order of PH = C2 > PL > GH > GL > C1 (Figure 2).

The trend in the relative risk ratio (Table 5) of exposure to *S. agalactiae* in tilapia that were not

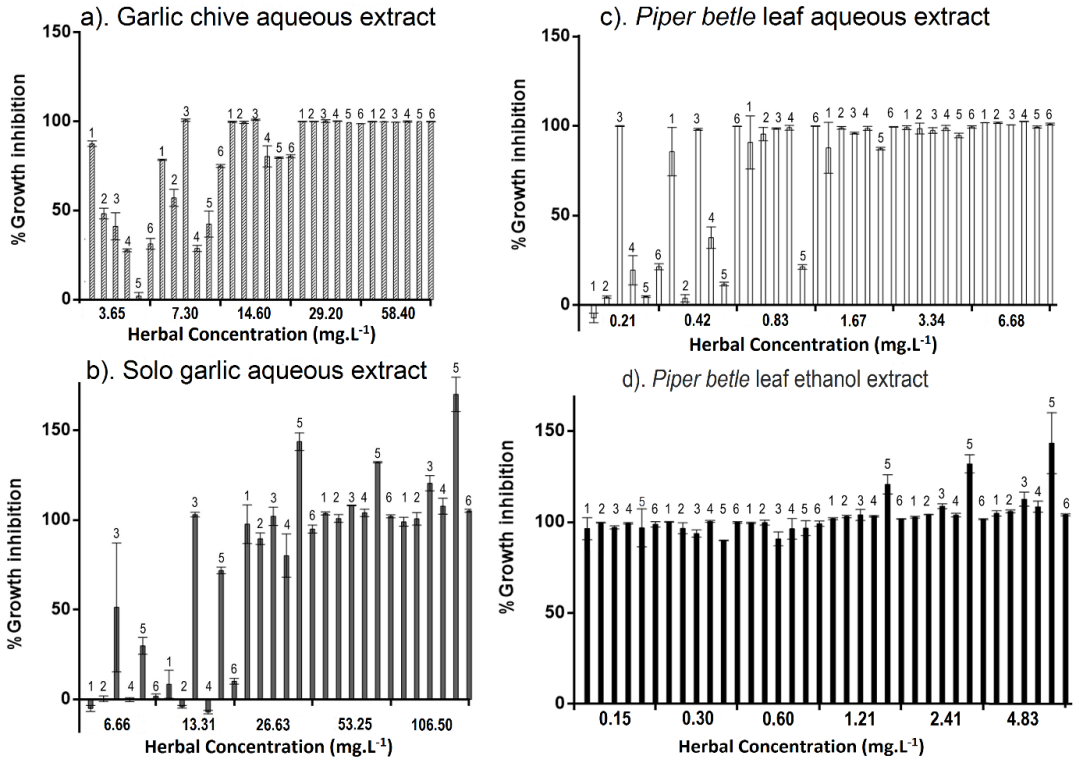

Figure 1. Percent growth inhibition of pathogenic bacteria (1-6) by indicated herbal extract (a-d). Inhibition assay was performed using microbroth dilution methods in which each bacterial suspension was incubated with serially diluted herbal extracts (3.1-50% in a and b while 0.7-25% in c and d). Pathogenic bacteria included 1, *V.parahaemolyticus* 2HP, 2, *V.parahaemolyticus* 5HP, 3, *E.ictaluri*, 4, *E.tarda*, 5, *S.agalactiae*, and *6, A.veronii.*

Table 4. Growth inhibition and MIC_{90} of herbal extract against pathogenic bacteria

Herbal extract	*V. parahaemolyticus* 2HP	*V. parahaemolyticus* 5HP	*E. ictaluri*	*E. tarda*	*S. agalactiae*	*A. veronii*
Garlic chive	99.5±0.6%	99.8±0.3%	100.7±0.6%	100.1±0.1%	99.4±0.03%	99.8±0.1%
Solo garlic	89.6±3.2%	97.6±10.7%	103.1±1.2%	103.9±2.0%	143.7±4.9%	94.9±2.2%
P. betle- aqueous	95.4±3.7%	99.0±1.0%	99.9±0.2%	98.9±1.3%	94.6±1.3%	99.7±0.1%
P. betle-alcohol	96.4±6.0%	99.7±0.2%	97.1±0.8%	99.3±0.4%	96.6±4.1%	98.7±1.6%
Legend	0.78%	1.56%	3.12%	6.25%	12.5%	25%

*Numbers indicate percent growth inhibition of bacteria

*Legend shows the percentage concentration of extract v/v.

given herbs compared to those given the herbs was in the order PH > PL > GH > GL. Risk of mortality was significantly different between positive control and fish treated with herbs. Least risk of mortality considering herbal treatment with GL against no treatment was recorded at 8% and increased to at least 24% in the positive controls than fish treated with GH. The risk increased to at least 83% in positive controls than fish treated with PL and further rose to 224% in positive controls compared to PH treated fish.

Additionally, proximate analysis of the diets revealed that diet GH had the lowest protein (27.7 %) while the control diet had the highest protein (34.1 %). Nevertheless, the protein content of the diets were > 30% as recommended for tilapia except for diet GH. Lipid content of diets ranged from 5.3% to 6.7% while fibre was below 5% in all diets. Ash content in all diets was above the recommended 6% with the control diet having the highest (10.1 %) content while diet GH had the least (7.1 %).

Haematological Parameters

Dietary herbal feed supplementation at high and low levels with solo garlic and betel leaf extracts significantly increased the mean corpuscular haemoglobin concentration (MCHC) ($P<0.05$) compared to the pre-feeding level (day 0) (Table 6a). On the other hand, thrombocyte counts declined generally from the initial levels in all treatments except for C1. Red blood cells (RBC) and associated indices: mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), haematocrit (Hct) and haemoglobin (Hb) were not significantly different from initial values and also among treatments (Table

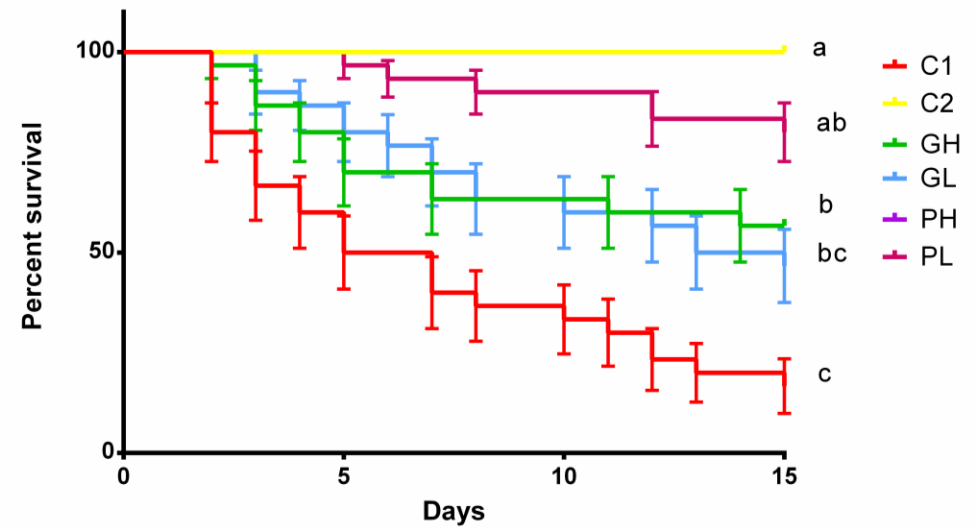

Figure 2. Kaplan-Meier survival curve for Nile tilapia fed with herb additive feed for 7 days and then challenged with *S. agalactiae*. (Curves bearing the same letters are not significantly different; Bonferroni corrected P>0.0036).

Table 5. Relative Risk Ratios (RRR) of mortality in treated fish against positive control

Treatment	RRR	Confidence Interval (CI)		p-value	Least % increase in risk
		Lower	Upper		
GH	1.92*	1. 24	2.98	0.003	24
GL	1.56*	1.08	2.26	0.025	8
PH	50.83*	3.24	798.07	0.000	224
PL	3.57*	1.83	6.97	0.000	83

GH= Garlic High dose; GL = Garlic Low dose; PH = Piper High dose; PL = Piper Low dose

* = Significant (P<0.05)

*Least % risk increase is calculated using: $1 - Lower\ CI \times 100$

6a). White blood cell (WBC) count decreased generally among treatments except for PH where it rose far above other treatments (Table 6b). Among the white blood cell differential counts, only neutrophils exhibited significant differences (P<0.05) among treatments and in deviation from the initial value. Other white blood cell differential cells were not significantly different among treatments. Basophils were barely detected while eosinophils were not detected at all.

Discussion

In view of the emerging threats from new diseases and the consequent antibiotic abuse in aquaculture, it is important to investigate products of plant origin to be used as feed supplements to prevent bacterial diseases and cultivate antibiotic-free products of fish and shrimp for human consumption. Many of the previous studies have searched for herbs that could kill or inhibit the growth of bacteria and stimulate native immune response in fish through oral administration. Solo garlic, garlic chive and piper betel leaves are common herbs in the Southeast Asian region, which have been used for domestic food preparation (solo garlic, garlic chive) or for chewing together with Betel nut and lime (piper betel leaves) by several groups of ethnic communities throughout Southeast Asia and India (Fazal *et al*., 2014). Antimicrobial properties of these plants were previously reported, and various natural bioactive compounds have been identified such as alliin, isoallin, methiin, cis and trans isomers of g-glutamyl-S-1-propenylcysteine, g-glutamyl-S-allylcysteine (Charron, Milner, & Novotny, 2016), and phenols (Fratianni *et al*., 2016) among others from garlic; flavonoids from garlic chive (Knuthsen & Justesen, 1999); and polyphenols and alkaloids from *Piper betle* leaf (Parmar *et al*., 1998). These plants have been proven safe for consumption through a long history of use by humans. Therefore, application of their extracts for farmed fish and shrimp is not likely to pose any concern for product safety especially for export and import. Additionally, availability and affordability of the selected herbs are also critical advantages considering their use in aquaculture farms.

Four herbal extracts examined in this study exhibited *in vitro* antimicrobial activity against all tested bacteria. The ethanol extract from betel leaves appeared as the most promising extract because it inhibited all bacterial strains at the lowest concentration tested. Antibacterial effectiveness was

Table 6. Effect of two dietary levels of solo garlic and betel leaf extracts on haematological parameters of *Oreochromis niloticus* (Chitralada IV) after 7 days feeding compared to that of day 0. A) Effects on RBC and associated indices

	RBC (10^6/µL)	Hb (g/dl)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (%)	Thrombocytes (10^4/µL)
D0	2.17±0.62	10.60±1.89	31.33±6.33	151.10±13.60	52.33±8.43	34.23±2.50^{b}	6.45±0.75^{ab}
GH	1.86±0.05	11.27±0.47	29.33±1.33	157.83±3.18	60.63±1.00	38.43±0.17^{ab}	1.40±0.20^{c}
GL	1.47±0.11	9.83±0.33	25.00±1.00	171.00±6.35	67.33±2.87	39.37±0.23^{a}	1.30.00±0.20^{c}
PH	1.64±0.18	10.60±0.67	27.33±2.03	168.57±8.16	65.53±3.79	38.87±0.43^{ab}	3.20±0.40^{bc}
PL	1.74±0.11	10.73±0.39	27.67±1.20	159.43±4.17	61.90±1.95	38.80±0.27^{ab}	1.80±0.30^{c}
C1	1.71±0.25	11.07±1.23	28.67±3.71	168.30±4.75	65.33±2.66	38.77±0.64^{ab}	7.23±0.80^{a}
C2	1.72±0.17	10.63±0.57	27.33±1.67	160.13±6.29	62.40±2.95	38.93±0.33^{ab}	3.80±0.78^{bc}
SEM	0.39	1.35	4.31	10.43	5.71	1.43	0.90
P-value	0.715	0.961	0.855	0.495	0.247	0.039	<0.001

*Means in each column with different superscripts differ significantly (P<0.05)
*D0= day 0; RBC= red blood cells; Hb, haemoglobin; Hct= Haematocrit; MCV= mean corpuscular volume; MCH= mean corpuscular haemoglobin; MCHC= mean corpuscular haemoglobin concentration; GH= Garlic High dose; GL = Garlic Low dose; PH = Piper High dose; PL = Piper Low dose; SEM = Standard Error of Mean

B) Effects on WBC and differential counts (x 10^3/µL)

	WBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
D0	87.50±2.50^{ab}	1.33±0.48	83.95±0.65^{a}	2.23±1.37^{b}	0.00	0.00±0.00
GH	42.50±2.50^{b}	7.55±2.35	34.10±0.10^{b}	0.65±0.25^{bc}	0.00	0.20±0.20
GL	35.00±10.40^{b}	3.75±2.85	30.55±7.4^{b}	0.42±0.07^{c}	0.00	0.28±0.16
PH	117.50±12.50^{a}	24.9±11.90	88.00±25.00^{a}	4.56±0.68^{a}	0.00	0.00±0.00
PL	41.00±1.00^{b}	9.89±2.35	30.43±1.97^{b}	0.40±0.23^{c}	0.00	0.28±0.14
C1	52.50±2.50^{b}	9.50±1.50	42.20±0.70^{b}	0.80±0.30^{bc}	0.00	0.00±0.00
C2	70.00±25.00^{ab}	8.30±0.70	60.80±25.70^{ab}	0.93±0.03^{bc}	0.00	0.00±0.00
SEM	15.04	6.17	17.14	0.73	–	0.19
P-value	0.003	0.079	0.026	0.003	–	0.382

*Means in each column with different superscripts differ significantly (P<0.05)
*WBC= white blood cells; GH= Garlic High dose; GL = Garlic Low dose; PH = Piper High dose; PL = Piper Low dose; SEM = Standard Error of Mean

different (indicated by percent growth inhibition, Figure 2 and Table 2) for ethanol and aqueous extracts of betel leaves when constrained at the same dilution rate. This suggested that the composition of bioactive compounds are solvent-dependent and ethanol would be a better choice as solvent in terms of their antibacterial properties. Similarly, Albert and Ransangan (2013) also reported that *Piper betle* ethanol-extract showed very strong *in vitro* inhibition activity against various bacterial strains obtained from American Type Culture Collection (ATCC) including Vibrio species (*V. parahaemolyticus* ATCC 17802, *V. harveyi* ATCC 35084, *V. alginolyticus* ATCC 17749 and *V. anguilarium* ATCC 19264), *Aeromonas* species (*A. hydrophila* ATCC 7965, *A. caviae* ATCC 15468 and *A. salmonicida* ATCC 33658, *Pseudomonas fluorescence* ATCC 13252, *E. tarda* ATCC 15947 and *Yersinia ruckeri* ATCC 29473.

The data presented in this study showed that *in vivo* protection using different herbal supplemental diets were consistent with *in vitro* inhibition results. Piper betel extract had proved its effectiveness in both *in vitro* and *in vivo* assays. The fish which had received solo garlic supplemented feed exhibited less protection, as evidenced by the poorer inhibition results found in *in vitro* assay. This suggests that piper betel extract has a greater potential as feed additive for disease prevention in aquatic animals. This study is the first to indicate that *Piper betle* extract appears to be very effective against *V. parahaemolyticus*, a pathogen that has assumed great significance recently by causing one of the most devastating disease outbreaks in the shrimp industry (Joshi *et al*., 2014; Tran *et al*., 2013). *Piper betle* extract has also been found to be effective against the four pathogenic bacteria that have been reported to cause diseases in tilapia and striped catfish including *A. veronii*, a newly reported pathogen in farmed tilapia (Dong *et al*., 2015a; Dong *et al*., 2016; Dong *et al*., 2015b). Most of the previous studies lacked any *in vivo* evidence of the efficacy of *Piper betle* extract while presenting promising results of *in vitro* trials. More importantly, *in vivo* trials would provide confirmatory evidence of the relative efficacy of herbal applications for their potential use as feed additives to prevent bacterial diseases in fish and shrimp. Such trials are necessary before considering their field trials and commercial farm applications. The present study has revealed clear evidence of acquired protection for fish against *S. agalactiae* by the piper betel extract supplemented feed used. However, further investigations on several other pathogens that infect

the economically critical fish and shrimp host species are warranted to explore broader commercial applications for this treatment.

(Do Huu, Sang, & Thanh Thuy, 2016) reported that dietary intake of β-glucan induces an inflammatory response that confers resistance to infection through increased levels of leukocytes. Similarly, plant, herb and algal extracts have been reported to boost immune response (Vallejos-Vidal, Reyes-López, Teles, & MacKenzie, 2016) against bacterial infection (Devi, Dhayanithi, Kumar, Balasundaram, & Harikrishnan, 2016). Herbal diets supplemented in the present study showed upregulation of neutrophils among all granulocytes investigated except for GL. High doses of the two herbal extracts upregulated neutrophil levels in tilapia with PL being an exception. A high level of organic acid in the diet of tilapia elicited similar response in neutrophils (Reda, Mahmoud, Selim, & El-Araby, 2016). Incorporation of stinging nettle (*Urtica dioica*) in diets of *Labeo victorianus* also elicited the same effect on neutrophils (Ngugi *et al*., 2015). It might suggest that *Piper betle* supplemented feeds act through the upregulation of lymphocytes and monocytes to induce immune preparedness in fish. The increased level of WBC in blood of fish fed PH, as well as the increased lymphocyte and monocyte levels, might be key factors in the modulation of their immunity against *S. agalactiae*. Increased WBC levels through herbal oil supplementation have been induced in tilapia at higher doses (Baba, Acar, Yılmaz, Öntaş, & Kesbiç, 2017) showing the effectiveness of herbal treatments at high dose to positively affect WBC in tilapia. Fish that were not fed any herbal supplement, yet challenged with *S. agalactiae*, as well as fish fed GH and GL, could not withstand the infection. The risk of mortality as a result of infection is actually linked to the dosage and route of infection, with injection being more lethal (Xia, Wang, Liu, Jiang, & Wang, 2015). Hence the hazard posed by *S. agalactiae* to *O. niloticus* challenged by injection was effectively mitigated by treatment using ethanol extract of *Piper betle* leaf in contrast to the relative risk of mortality going far above 100% without treatment with the herb. Our results also show that economic losses will increase by more than 200% without treatment using PH. There is an obvious trade-off between WBC and neutrophil level that played out in the response of fish fed *Piper betle* leaf at both high and low levels leading to the high survival rate.

The present study revealed that extracts from *Piper betle* leaves, solo garlic, and garlic chive exhibit bacterial inhibition properties against various pathogens affecting economically important aquaculture species. *Piper betle* leaves extracted by 40% ethanol exhibited the best *in vitro* inhibition activity. *In vivo* trials also proved that *Piper betle* extract supplemented in diets could protect the fish in experimental infection with pathogenic *S. agalactiae* and has the potential to be used as a feed additive to prevent bacterial disease in aquaculture.

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