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Immunostimulatory Effect of *Piper betle* Treatment in Nile Tilapia (*Oreochromis niloticus*)

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Abstract

Several herbal plants demonstrated immunostimulatory activity through the enhancement of growth and level of immunity in freshwater fish. The immunostimulatory activity of methanolic extract of *Piper betle* in Nile tilapia (*Oreochromis niloticus*) was evaluated through an experimental feeding with various concentrations of *Piper betle* extracts (0%, 5%, 10% and 15%). The growth performance, blood serum profile and total bacterial load were determined after four weeks of the experimental feeding. Analyses on the growth performance indicated 5% concentration of *Piper betle* treatment enhanced both weight and length gain as well the specific growth rate (SGR) in Nile tilapia. A higher level of total serum protein was demonstrated in Nile tilapia that received *Piper betle* treatment in comparison with the control group. Meanwhile, the glucose level was found greater in the treatment group that received a 10% concentration of *Piper betle*. Treatment with *Piper betle* extract at 5% concentration could reduce the cholesterol level in Nile tilapia. Feeding of Nile tilapia at 5% concentration of *Piper betle* was found able to inhibit the growth of *Vibrio* spp. The incorporation of 5% of methanolic extract of *Piper betle* in the daily diet of Nile tilapia was found appropriate to enhance the growth performance and immunostimulatory activity in Nile tilapia.

Keywords: freshwater fish, feed, immunostimulant, herbal plant

INTRODUCTION

Tilapia (*Oreochromis sp*.) is being cultivated worldwide and it has been considered as one of the important fish species in Malaysia. Intensive culturing activities of this species had exposed them to the risk of infection caused by bacterial or viral pathogens. Several common infectious diseases in farmed tilapia including Streptococcus infections (Zamri-Saad et al., 2014), Columnaris infections caused by *Flavobacterium columnare* (Dong et al., 2015a) and haemorrhagic septicaemia caused by motile Aeromonads (Dong et al., 2015b). Several viral infections such as tilapia larvae encephalitis virus (TELV) and betanodavirus were occasionally reported in tilapia fry (Keawcharoen et al., 2015). Other infections, such as spleen and kidney

necrosis virus (ISKNV), are associated with lethargy, gill pallor, and coelomic cavity distension (Subramaniam et al., 2016). Tilapia Lake Virus (TiLV), an emerging pathogen, has been discovered as the source of massive mortalities in cultured tilapia (Jansen et al., 2019). In Thailand, cultivated red tilapia and Nile tilapia (*Oreochromis niloticus*) were affected due to outbreaks of TiLV infection (Dong et al., 2017a, 2017b; Surachetpong et al., 2017).

The gastrointestinal (GI) tract of fish has been discovered to colonize with a variety of bacterial species obtained from the surrounding aquatic environment and diet. Among the bacterial colonizers include *Acinetobacter, Vibrio*, *Enterobacteriacea*, *Micrococcus*, *Aeromonas*, *Plesiomonas*, *Pseudomonas*, *Clostridium*, *Flavobacterium*, *Fusarium* and *Bacteroides* (Nayak, 2010). Previous studies have reported on the isolation of *Vibrio vulnificus* in Nile tilapia which can cause vibriosis and septiceamia (Mandal et al., 2012; Sumitra et al., 2019). One of the most virulent pathogenic strains of *Vibrio* spp. in the marine environment was identified as *Vibrio cholera*. According to Hounmanou et al. (2019), tilapia has been identified as a reservoir host for the survival and transmission of *V. cholerae* in aquatic habitats. During the second week of the experiment, the El Tor biotypes *V. cholerae* O1 and *V. cholerae* non-O1 colonised tilapia intestines and remained at consistent concentrations.

The mortality rate of cultured tilapia increased as a result of infections caused by pathogenic bacteria and viruses (Jansen et al., 2019). Excessive use of disinfectant and antimicrobial drugs to prevent or control diseases in the aquatic animal may contribute to the development of antibiotic-resistant bacteria. At present, there is no effective treatment or vaccines available to control or prevent the infection in tilapia species. The use of the commercial vaccine is expensive for fish farming practices and very specific for a particular pathogen. Recent studies have been focusing on the investigation of herbal plants as a potential antimicrobial and therapeutic agent to be employed in fish farming. Some plant extracts have been tested for their antibacterial properties and demonstrated the ability to control bacterial and viral diseases (Citarasu, 2010). The herbal extracts can act as immunostimulants which have been proven to promote growth, reducing stress effects and enhance immunity for the prevention against bacterial and viral infections (Kumar et al., 2010).

Betel leaves or *Piper betle* is among of herbal plant that possesses antiseptic properties and suitable to be applied on wound or lesion for healing effects. *Piper betle* also has been reported as a good antibacterial agent against pathogenic microorganisms. *Piper betle* of methanol extract has been shown to exhibit antibacterial action against *Columnare*, *Streptococcus* and *Vibrio* bacteria in previous studies (Hoque et al., 2012). *Piper betle* of ethanol extract showed antibacterial efficacy against a variety of aquaculture bacterial pathogens. In vivo assessment through feeding of Nile tilapia (*Oreochromis niloticus*) with the ethanol extract of *Piper betle* demonstrated enhancement of the number of white blood cells (WBC) and significantly improved the rate of survival in the infected fish following challenge with *Streptococcus agalactiae* (Ataguba et al., 2018).

Hence, this study aimed to look into the possible properties of the methanolic extract of *Piper betle* as an immunostimulant in Nile tilapia (*Oreochromis niloticus*). The methanolic extract of *Piper betle* at various concentrations was incorporated in the commercial pellet and used for feeding tilapia fish. During the experimental feeding, the growth performance of fish was monitored and biochemical markers such as total serum protein, glucose and cholesterol levels as well the total bacterial load were also determined.

METHODOLOGY

Preparation of plant extract

The fresh *Piper betle* leaves were screened for any damage and cleaned with tap water to remove any contaminants. Next, the leaves were dried out at room temperature for a few days and further dried in an oven for 48 hours at 40°C. The dried leaves were ground into powder form by using a Waring blender. Fifty grams of *Piper betel* in powder form were soaked in 250 ml of 80% methanol at a ratio of 1:5 sample to solvent. The mixture of samples was incubated on a rotary shaker for 72 hours. The extract was dried and concentrated under reduced pressure at 60°C in a rotary vacuum evaporator. Then the final extract was stored at 4°C in an airtight bottle.

Preparation of experimental feed

The commercial pellets were ground into a fine powder using an electric grinder. The ground powdered form of the commercial pellet was passed through a 300 um sieve to remove the uneven size of particles. To make the right composition of the formulated feed, all ingredients were measured and mixed according to the respective percentage of crude extract of Piper betle as shown in Table 1. The appropriate volume of water was added into the mixture of formulated feed and mixed thoroughly until the homogenous dough was obtained. The dough was transferred into a container and incubated at room temperature for 30 minutes. A pelletizer machine was used to turn the dough into pelleted feed. The pellets were air-dried and then placed in an oven at 40°C for 24 hours. The feed was then stored in an airtight container and kept in a chiller at 4°C for further use.

Acclimatization of Nile tilapia fish

A total of 80 Nile tilapia fish (average weight is between 15-18 grams per fish) were obtained from a breeding pond located at Bestari Jaya, Selangor and maintained in lab condition. The fish were divided equally into four aquariums whereby each of the aquaria was separated into two compartments using a partition. All aquariums were supplied with continuous aeration and labeled as 0% (control), 5%, 10% and 15% of *Piper betle*. The fish were acclimatized for 1 week by feeding with a commercial pellet.

Table 1 The composition of ingredients according to the respective percentage of *Piper betle* extract.

Ingredients	**Composition of ingredients (in 100 gram)**			
	Diet 1 (0%)	**Diet 2 (5%)**	**Diet 3 (10%)**	**Diet 4 (15%)**
Commercial fish pellet	34.08	34.08	34.08	34.08
Wheat flour	55.42	50.42	45.42	40.42
Baker's yeast	7.5	7.5	7.5	7.5
Piper betle leaves crude extract	0	5	10	15
Corn oil	3	3	3	3

Feeding experiment of Nile tilapia with formulated feed

The feeding experiment was carried out 1 week after the acclimatization period. The Nile tilapia fish were fed with the prepared formulated feed at the rate of 5% of the body weight and feed 2 times daily for 4 weeks. The experimental feed was prepared by incorporating the extract of *Piper betle* at a concentration of 0% (0 g in 100 g), 5% (5 g in 100 g), 10% (10 g in 100 g) and 15% (15 g in 100 g). The water was replaced weekly to maintain the water quality during the experimental period.

Growth performance analyses of Nile tilapia

The growth performance of treated Nile tilapia was determined to analyze the efficacy of the experimental feed in fish. The body weight and length of the fish were measured weekly from week 1 until week 4. The growth performance of treated Nile tilapia was determined based on the average value of total weight gain, length gain, specific growth rate (SGR) and feed conversion ratio (FCR).

Body weight gain (BWG) = Wt – Wi
Feed conversion ratio (FCR) = total feed intake (g) / total weight gain (g)
Specific growth rate (SGR) = [(lnWt – ln Wi)/T] × 100
(Wt – mean final weight, Wi– mean initial weight, T – feeding trial period in days)

The statistical package SPSS 14.0 was used to conduct the analyses. All of the data were subjected to a one-way analysis of variance (ANOVA) before the Duncan Test was used to compare means. At $p<0.05$, all differences between treatment groups were considered significant.

Biochemical analysis of blood serum

Hematological analysis of Nile tilapia was conducted after 1 month of feeding with the experimental feed. The fish were euthanized using chill water and the blood samples were taken from the tail ablation. The blood was collected using hematocrit tube as the blood flows from the caudal vein. The blood was transferred into an empty tube for the collection of blood serum. The labeled serum samples were sent to Hematology & Clinical Biochemistry Laboratory, University of Putra Malaysia (UPM) for the analysis of total serum protein, glucose and cholesterol *level*.

Total bacterial load

Analyses of the total bacterial load were performed through the isolation of bacteria from the gut samples of Nile tilapia that fed with various concentrations of *Piper betle* extract. After surface sterilization, the gut from control and treated fishes were carefully removed, washed with sterile buffered saline and ground using mortar and pestle. For *Vibrio* spp. isolation, the resulting aliquot was serially diluted and plated on TCBS (Thiosulfate citrate bile salts sucrose agar) agar. After 24 hours of incubation at 37.0°C, colony development was detected.

RESULTS AND DISCUSSION

Growth performance of Nile Tilapia fed with the experimental diets

Growth performance of Nile tilapia was measured based on the growth performance analyses (weight, length and SGR) following feeding with four experimental diets incorporated with different concentrations of methanolic extract of *Piper betle* (0%, 5%, 10% and 15%) as shown in Table 2.

Table 2 Growth performance analyses of Nile tilapia fed with the experimental diets

Parameters	Diet 1 (0%)	Diet 2 (5%)	Diet 3 (10%)	Diet 4 (15%)
Initial weight, g	16.74 [a] ±2.80	22.72 [a] ±6.67	15.77 [a] ±1.29	18.98 [a] ±1.24
Final weight, g	21.94 [a] ±1.73	32.56 [a] ±4.77	23.47 [a] ±0.38	23.59 [a] ±5.51
Weight gain, g	5.20	9.84	7.70	4.61
Initial length, cm	15.20 [a] ± 1.14	15.83 [a] ± 1.55	14.83 [a] ± 0.96	14.70 [a] ± 1.37
Final length, cm	17.03 [a] ± 0.92	19.60 [a] ± 1.13	18.37 [a] ± 0.35	18.40 [a] ± 1.74
Length gain, cm	1.83	3.77	3.54	3.70
Specific growth rate	11.0	15.22	13.61	10.17

Values are expressed as Mean ± SD (Significant at P<0.05%)
Row means with the same superscript are not significantly different (p>0.05) from each other

Data of weight (g) gained performance in treated Nile tilapia fish as Mean SD (Standard Deviation) with a superscript of (a) in the same row, indicates significantly same means at P <0.05. The numbers are not significantly different (P>0.05) since the weight gain (g) mean values share the same superscript. According to the results, the highest weight gain (g) of 9.84 grams was recorded in treated fish fed with diet 2 containing 5% *Piper betle* extract, while the lowest weight gain (g) of 4.61 grams was observed in fish fed with diet 4 containing 15% concentration of *Piper betle* extract.

The length (cm) gained performance of control and experimental Nile tilapia groups are shown as Mean SD (Standard deviation) with a superscript of (a) in the same row, indicating that the means are the same at P<0.05. Analyses of length gain (cm) demonstrated the highest value (3.77 cm) was achieved in the fish that fed with diet 2 containing 5% concentration of *Piper betle* and the lowest value (1.83 cm) was recorded from the control group that received diet 1. Meanwhile, fish that fed with diet 2 demonstrated the highest specific growth rate (SGR) value (15.22) in comparison with other treatments. This finding suggested that adding 5% *Piper betle* extract to the fish diet improved Nile tilapia growth performance.

According to Asimi et al. (2015), feeding *L.rohita* fish with cardamom extract improves growth performance, indicating that feeding fish with herbal supplements can improve growth performance. Based on the findings of this study, an optimum concentration of *Piper betle* extract integrated into fish feed can operate as a growth stimulant in Nile tilapia, with the fish fed a diet containing 5% *Piper betle* extract gaining the most weight and length. Meanwhile, fish that treated with diet 1 (0% *Piper betle* extract) and diet 4 (15% *Piper betle* extract) demonstrated among the lowest weight gain and SGR value as compared with fishes that received a diet containing a lower concentration of *Piper betle* extracts (5% and 10%). These findings are most likely related to the herbal extract composition in the fish diet, which influences the ability of the Nile tilapia's digestive system to digest food.

The addition of a methanol extract of *Piper betle* to a Nile tilapia diet may affect growth performance, as the presence of such medicinal plant may affect the fish's capacity to digest and absorb nutrients. Talpur et al. (2013) performed a similar investigation on the effects of ginger (*Zingiber officinale* Roscoe) as a feed additive on Asian sea bass, *Lates calcarifer* culture. The fish that were given a ginger diet demonstrated a significant increase in weight gain, growth, and feed conversion. In the present study, feeding of Nile tilapia with the

experimental diet containing *Piper betle* of methanol extract had shown improvement towards the growth performance. Extract of *Piper betle* at 5% concentration can be considered as the most optimum value to be employed in Nile tilapia daily feeding.

Blood serum analyses

Blood serum analyses were conducted to determine the effect of herbal medicated feed containing *Piper betle* extract at different concentrations towards the level of total serum protein, cholesterol and glucose in Nile tilapia fish. Table 3 shows the level of total serum protein, glucose and cholesterol detected from serum samples of treated Nile tilapia fish after four weeks of feeding treatment. The values are shown as Mean SD (Standard Deviation) in the same row, with a superscript of (a) indicating significantly similar means at $P<0.05$. Based on the present data, there was no significant difference ($p>0.05$) in the total protein, blood glucose and cholesterol level. Nile tilapia fish that fed with *Piper betle* range between 5% to 15% concentration demonstrated better total serum protein level in comparison with the control group (0% of *Piper betle*). In comparison with other treatments, Nile tilapia fed with diet 4 containing 15% of *Piper betle* extract had the greatest total serum protein value. In the meantime, the control group had the lowest total serum protein level.

Table 3 Analyses of blood serum of Nile tilapia fed with the experimental diets

Parameters	Diet 1 (0%)	Diet 2 (5%)	Diet 3 (10%)	Diet 4 (15%)
Total serum protein (g/L)	$21.55^{a} \pm 1.91$	$25.15^{a} \pm 1.83$	$26.45^{a} \pm 2.78$	$27.05^{a} \pm 4.45$
Glucose (mmol/L)	$6.7^{a} \pm 0.57$	$5.1^{a} \pm 0.92$	$8.1^{a} \pm 1.91$	$6.8^{a} \pm 0.91$
Cholesterol (mmol/L)	$3.7^{a} \pm 0.28$	$3.2^{a} \pm 0.28$	$4.6^{a} \pm 0.78$	$4.7^{a} \pm 0.57$

Values are expressed Mean ± SD (Significant at $P < 0.05\%$)
Row means with the same superscript are not significantly different ($p>0.05$) from each other

The glucose level was found to be the highest in fish that fed with diet 3 which contained a 10% concentration of *Piper betle* compared with other treatments. The highest level of cholesterol was demonstrated in fish that fed with diet 4 which contained 15% of *Piper betle* extract in comparison with other treatments. Meanwhile, fish fed with diet 2 incorporated with a 5% concentration of *Piper betle* extract demonstrated the lowest level of glucose and cholesterol in comparison with other treatments. In Asian sea bass, *Lates calcarifer* culture, Talpur et al. (2013) discovered that a ginger diet regulates haematological markers, biochemical indicators, and immunological activities. In comparison with the control group, the treated groups had lower blood glucose, cholesterol, lipid and triglyceride levels.

Blood alterations in terms of haematology and biochemistry are significant indicators for monitoring pathological and physiological changes in fish (Satheeskumar et al., 2011). Biochemical markers, according to Ferreira et al. (2007), provide an early indication of potentially hazardous alterations in stressed organisms. Previously, Bello-Olusoji et al. (2006) suggested that alteration in fish haematology in regards to stressing agents are indications of the fish's stressful stage, providing helpful information for preventing any unfavorable conditions that could harm the fish's health.

Saravanan et al. (2011) used haematological, ion regulatory, biochemical and enzymological parameters to assess the toxicity of neem leaf extracts (*Azadirachta indica* A. Juss) in the Indian main carp, *Cirrhinus mrigala*.

Enhancement of innate immune response in fishes is associated with the increase of serum protein, albumin and globulin level (Wiegertjes et al.,1996). According to Das et al. (2004), the concentration of blood plasma protein is a good predictor of a fish's overall health. Meanwhile, Abdali et al. (2011) found that a decrease in plasma protein is a sign of toxin action in the kidney, spleen and liver. The present study demonstrated that the addition of methanol extract of *Piper betle* (between 5% to 15%) in the diet of Nile tilapia able to enhance total serum protein in comparison with fish that fed with basic diet demonstrated in the control group (0% of *Piper betle*). This finding indicated that enhancement of total protein in Nile tilapia fish was associated with the consumption of a diet containing *Piper betle* extract that may influence the condition of fish health. The addition of *Piper betle* extract at an appropriate concentration in the diet of Nile tilapia was considered safe and able to improve the level of immunity in Nile tilapia based on the enhancement of total protein level in blood serum.

Glucose has already been used as a biochemical indication for determining the degree of normality in a person's overall physiological state (Habib et al., 2014). Glucose levels in the blood can quickly fluctuate due to external or internal influences. A previous study by Jimoh et al. (2015) demonstrated the effect of diets containing watermelon (*Citrullus lanatus*) seed meal which had increased blood glucose levels in Nile tilapia. Kumar et al. (2010) found a similar result in fish that fed plant protein-based diets, with higher blood glucose levels. An increase in plasma catecholamine and corticosteroid hormones could cause an increase in blood glucose levels (Pickering, 1981). Meanwhile, the blood glucose level reported in the present study was considered low (range between 5.0 to 7.0) except for fish that received a diet containing 10% of *Piper betle* extract which demonstrated a slightly higher value (8.1).

Cholesterol levels in Nile tilapia fish were shown to be greater in those groups that fed with 10% and 15% *Piper betle* extract, respectively. The increased blood cholesterol levels could be attributed to the mobilization of stored cholesterol from tissue. The increased availability of cholesterol in the serum may be related to a decrease in cholesterol conversion to gonadal steroids (Singh and Singh, 1979). In contrast to the current study, Jimoh et al. (2015) and Kumar et al. (2010) found that fish fed a plant-based diet had lower blood cholesterol levels. Other researchers reported similar results whereby a reduction of blood cholesterol was demonstrated in fish fed with plant protein-based diets (Kaushik et al.,1995; Yamamoto et al., 2007). These occurrences could be linked to the use of anti-nutrients in the production of cortisol as a result of stress caused by eating anti-nutrient-rich diets.

Analysis of total bacterial load in treated Nile tilapia

Table 4 shows an analysis of the overall bacterial count with a focus on *Vibrio* spp. from the guts of treated Nile tilapia. The total bacterial count was expressed as Mean SD (Standard deviation) in the same row, with a superscript of (a) indicating substantially different means at $P<0.05$. Based on the present findings, the gut samples of Nile tilapia treated with the methanol extract of *Piper betle* at 5% concentration demonstrated the lowest bacterial count in comparison with the other treatments.

Nile tilapia that received treatment with a 5% concentration of *Piper betle* extract had a lower bacterial count in comparison with other treatments. This finding revealed that the application of *Piper betle* extract at a low concentration in a fish feed could suppress the growth of pathogenic bacteria, particularly *Vibrio* spp., which could be employed to manage bacterial infection in Nile tilapia fish. According to a previous study by Ataguba et al. (2018), *Piper betle* extract appears to be particularly efficient against *V. parahaemolyticus*, a virus that caused one of the most destructive disease outbreaks in the shrimp industry (Joshi et al., 2014; Tran et

al., 2013). *Piper betle* extract has also been proven to be effective against four pathogenic bacteria that have been known to cause infections in tilapia and striped catfish, including *A. veronii*, a pathogen that has been discovered in farmed tilapia (Dong et al., 2015a; Dong et al., 2016; Dong et al., 2015b).

Table 4 Total bacterial load isolated from the gut of treated Nile tilapia fish

Treatment	Total bacterial load
0%	$2.87^{a} \pm 5.57 \times 10^{6}$
5%	$1.15^{a} \pm 1.57 \times 10^{2}$
10%	$7.57^{a} \pm 1.37 \times 10^{6}$
15%	$8.62^{a} \pm 1.57 \times 10^{3}$

Values are expressed Mean ± SD (Significant at $P < 0.05\%$)
Row means with the same superscript are not significantly different ($p>0.05$) from each other

Natural plant products have been used in fish as an anti-stress, growth promoter, appetite stimulation, immunostimulant and antibacterial agents due to the existence of active principle components (Citarasu, 2010). Herbal plants possess bioactive compounds that have good antimicrobial activities to prevent bacterial infections and also act as immunostimulants in aquatic species to boost immunity and disease resistance properties. Previous research has shown that herbal supplements such as *Piper betle* were capable to improve fish growth and protect them from sickness. According to Ataguba et al. (2018), *Piper betle* leaves extracted with 40% ethanol had the best in vitro inhibitory activity. *Piper betle* extract supplemented in diets also protected fish from pathogenic *S. agalactiae* infection in vivo, indicating that it could be employed as a feed additive to combat bacterial infections in aquaculture.

CONCLUSION

The effect of a methanolic extract of *Piper betle* on the growth performance and immunity level of Nile tilapia fish was established in this study. The inclusion of *Piper betle* extract at an optimum quantity in the daily diet of cultured Nile tilapia fish resulted in improved growth performance and increased immunity in the Nile tilapia fish. As a result, the methanol extract of *Piper betle* at a concentration of 5% may be considered as the optimum value that can improve the growth performance, enhance immunity level and demonstrated the antibacterial properties against selected bacteria in Nile tilapia fish. Immunostimulants can be used to control diseases in Nile tilapia fish since there are no effective treatments for numerous diseases in aquaculture. The use of *Piper betle* extract as an immunostimulant in aquaculture could help to prevent infections and disease outbreaks.

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REFERENCES

Abdali S., Yousefi Jourdeli A., Kazemi R., & Yazdani, M.A. (2011). Effect of Atrazine (Herbicide) on blood biochemical indices of grass carp (*Ctenopharyngodon Idella*). *Journal of Persian Gulf*, 2, 51-56.

Asimi, O. A., Sahu, N. P., & Pal, A. K. (2015). Immunomodulatory and Growth Response of L.Rohita to Dietary Fortification of Clove and Cardamom Extract. *Global Journal of Fisheries and Aquaculture*, 3(5), 230-237.

Ataguba, G.A., Dong, H.T., Rattanarojpong, T., Senapin, S., & Salin, K.R. (2018). *Piper betle* leaf extract inhibits multiple aquatic bacterial pathogens and in vivo *Streptococcus agalactiae* infection in Nile tilapia. *Turkish Journal of Fisheries and Aquatic Sciences*, 18, 671-680. http://doi.org/10.4194/1303-2712-v18_5_03.

Bello-Olusoji, O.A., Omoare, V.Y., & Nwana L.C. (2006). Comparative Studies on the haematological characteristics of pond cultured and wild tilapia (*Oreochromis niloticus*) Linnaeus, 1857. *Nigerian Journal of Forestry*, 36(2), 134 – 141.

Citarasu, T. (2010). Herbal Biomedicines: A New Opportunity for Aquaculture Industry.

Das, P.C., Ayyapan, S., Jenai, J.K., & Das, M. (2004). Acute toxicity of Ammonia and its sub lethal effect on selected haematological and enzymatic parameters of mrigala, *Cirhinus mrigala*, (Hamilton). *Aquatic Research*, 35, 135-143.

Dong, H. T., Nguyen, V. V., Le, H. D., Sangsuriya, P., Jitrakorn, S., Saksmerprome, V., & Rodkhum, C. (2015a). Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. *Aquaculture*, 448, 427-435. http://dx.doi.org/10.1016/j.aquaculture.2015.06.027

Dong, H. T., Nguyen, V. V., Phiwsaiya, K., Gangnonngiw, W., Withyachumnarnkul, B., Rodkhum, C., & Senapin, S. (2015b). Concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish, Pangasianodon hypophthalmus in Thailand. *Aquaculture*, 448, 142-150.

Dong, H. T., Nguyen, V. V., Mata, W., Kayansamruaj, P., Senapin, S., Nilubol, D., & Rodkhum, C. (2016). Diversity of Non-Flavobacterium columnare Bacteria Associated with Columnaris-like Diseased Fish. *Thai J Vet Med*, 46(2), 251-259.

Dong, H.T., Ataguba, G.A., Khunrae, P., Rattanarojpong, T., & Senapin, S., (2017b). Evidence of TiLV infection in tilapia hatcheries in Thailand from 2012 to 2017 reveals probable global spread of the disease. *Aquaculture*, 479, 579–583.

Dong, H.T., Siriroob, S., Meemetta, W., Santimanawong, W., Gangnonngiw, W., Pirarat, N., Khunrae, P., Rattanarojpong, T., Vani chviriyakit, R., & Senapin, S., (2017a). Emergence of tilapia lake virus in Thailand and an alternative semi-nested RT-PCR for detection. *Aquaculture*, 476, 111–118.

Ferreira, J.G., Hawkins, A.J.S., & Bricker, S.B., (2007). Management of productivity, environmental effectsand profitability of shellfish aquaculture-the Farm Aquaculture Resource Management (FARM) model. *Aquaculture*, 264, 160-174.

Habib, M. A., Sharker, R., & Rahman, M. (2014). Effects of feeding frequency on growth and survival in fry of tilapia (Hamilton) in outdoor rearing system. *International Journal of Fisheries and Aquatic Studies*, 1(4), 97–102.

Hoque, M. M., Rattila, S., Shishir, M. A., Bari, M. L., Inatsu, Y., & Kawamoto, S. (2012). Antibacterial Activity of Ethanol Extract of Betel Leaf (Piper betle L.) Against Some Food Borne Pathogens. *Bangladesh Journal of Microbiology*, 28(2), 58–63.

Hounmanou, Y., Mdegela, R. H., Dougnon, T. V., Madsen, H., Withey, J. H., Olsen, J. E., & Dalsgaard, A. (2019). Tilapia (*Oreochromis niloticus*) as a Putative Reservoir Host for Survival and Transmission of *Vibrio cholerae* O1 Biotype El Tor in the Aquatic Environment. *Frontiers in Microbiology*, 10, 125.

Jansen, M.D., Dong, H.T. & Mohan, C.V. (2019), Tilapia lake virus: a threat to the global tilapia industry? *Rev Aquacult*, 11, 725-739. https://doi.org/10.1111/raq.12254

Jimoh, W.A., Shittu, M.O., Ayeloja, A.A., Ajasin, F.O., Okemakin, F.Y., Abdusalami, S.A. & Adekunle. O.F. (2015). Some haematological and biochemical profile of blood of Nile tilapia (oreochromisniloticus) fed on diets containing watermelon (*Citrullus lanatus*) seedmeal. *Bayero Journal of Pure and Applied Sciences*, 8(1), 109 – 114.

Joshi, J., Srisala, J., Truong, V. H., Chen, I. T., Nuangsaeng, B., Suthienkul, O., & Thitamadee, S. (2014). Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, 428–429, 297-302. http://dx.doi.org/10.1016/j.aquaculture.2014.03.030

Kaushik, S.J., Cravedi, J.P., Lalles, J.P., Sumpter, J., Fauconneau, B., & Laroche, M. (1995). Partial or total replacement of fishmeal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effect, cholestromia and flesh quality in rainbow trout, Onchorhynchus mykiss. *Aquaculture*, 133, 257-274.

Keawcharoen, J., Techangamsuwan, S., Ponpornpisit, A., Lombardini, E.D., Patchimasiri, T., & Pirarat, N. (2015) Genetic characterization of a betanodavirus isolated from a clinical disease outbreak in farm-raised tilapia *Oreochromis niloticus* (L.) in Thailand. *Journal of Fish Diseases*, 38, 49– 54.

Kumar, N., Misra, P., Dube, A., Bhattacharya, S., Dikshit, M., & Ranade, S.A. (2010). Piper betle Linn. a maligned Pan-asiatic plant with an array of pharmacological activities and prospects for drug discovery. *Current Science, 99*, 922-932.

Kumar, V., Makkar, H.P.S., Amselburger, W., & Becker, K. (2010). Physiological, haematological and histopathological response in common carp (Cyprinus carpio L.) fingerlings fed with differently detoxified Jatropha curcas kernel meal. *Food and Chemical Toxicology*, 48, 2063-2072.

Mandal, S.C., Hasan, M., Rahman, M.S., Manik, M.H., Mahmud, Z.H. & Islam, M.S. (2012). Prevalence and characterization of *Vibrio vulnificus* in Nile tilapia, *Oreochromis niloticus* (Linnaeus). *Int. J. Ecotoxicol. Agric. Technol*. 2(1), 101-112.

Nayak, S.K. (2010). Role of Gastrointestinal Microbiota in Fish. *Aquaculture Research*, 41, 1553-1573.

Pickering, A.D. (1981). Stress and compensation in teleostean fishes. Response to social and physical factors, in: Pickering, A.D. (Edn.), Stress and Fish, Academic press., New York/London, 295-322.

Saravanan, M., Ramesh, M., Malarvizhi A. & R. Petkam (2011). Toxicity of Neem Leaf Extracts (Azadirachta indica A. Juss) on Some Haematological, Ionoregulatory, Biochemical and Enzymological Parameters of Indian Major Carp, Cirrhinus Mrigala. *Journal of Tropical Forestry and Environment*, 1(1), 14-26.

Satheeshkumar, P., Ananthan, G., Kumar, D.S. & Jagadeesan, L. (2012). Haematology and biochemical parameters of different feeding behaviour of teleost fishes from Vellar estuary, India. *Comp Clin Pathol*. 21, 1187–1191. https://doi.org/10.1007/s00580-011-1259-7.

Singh, A.K. & T.P. Singh (1979). Seasonal fluctuation in lipid and cholesterol content of ovary, liver and blood serum in relation to annual sexual cycle in Heteropneustes fossilis (Bloch). *Endocrinologie*, 73, 47-54.

Subramaniam, K., Gotesman, M., Smith, C.E., Steckler, N.K., Kelley, K.L., Groff, J.M. *et al*. (2016). Megalocytivirus infection in cultured Nile tilapia (*Oreochromis niloticus*). *Diseases of Aquatic Organisms*, 119, 253– 258.

Surachetpong, W, Janetanakit, T., Nonthabenjawan, N., Tattiyapong, P., Sirikanchana, K., & Amonsin, A. (2017). Outbreaks of tilapia lake virus infection, Thailand, 2015–2016. *Emerging Infectious Diseases*, 23, 1031– 1033.

Sumithra, T.G., Reshma, K.J., Anusree,V.N., Sayooj, P., Sharma, S.R.K., Suja, G.P.V., *et al.* (2019). Pathological investigations of *Vibrio vulnificus* infection in genetically improved farmed tilapia (*Oreochromis niloticus* L.) cultured at floating cage farm of India. *Aquaculture*, Volume 511, 734217, ISSN 0044-8486.

Talpur, A.D., Ikhwanuddin, M., & Abol-Munafi, A.B. (2013). Nutritional effects of ginger (Zingiber officinale Roscoe) on immune response of Asian sea bass, Lates calcarifer (Bloch) and disease resistance against *Vibrio harveyi*, Aquaculture, 400-401, 46-52. https://doi.org/10.1016/j.aquaculture.2013.02.043.

Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K., & Lightner, D. V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms*, 105(1), 45-55.

Wiegertjes, G. F., Stet, R. J. M., Parmeatier, H. K., & Van Muiswinkel, W. B. (1996). Immunogenetics of disease resistance in fish: A comparable approach developmental and comparative immunology. *Journal of Experimental Biology*, 20, 81-365.

Yamamoto, T., Suzuki, H., Furuita, H., Sugita, T., Tanaka, N. & Goto, T. (2007). Supplemental effect of bile salts to soybean meal based diets on growth and feed utilization of rainbow trout, Onchorhynchus mykiss. *Fisheries Science*, 73, 123-131.

Zamri-Saad, M., Amal, M.N.A., Siti-Zahrah, A. & Zulkafli, A.R. (2014). Control and prevention of Streptococcosis in cultured tilapia in Malaysia: A Review. *Pertanika J. Trop. Agri. Sci.* 37, 389–410.