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# The Immunostimulatory Potential and Resistance *Aeromonas hydrophila* of Pineapple Peel Extract in Nile Tilapia (*Oreochromis niloticus*)

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## Abstract

This study described the efficiency of pineapple peel extract (PPE) in increasing the immunity of Nile tilapia (*Oreochromis niloticus*) and in inhibiting *Aeromonas hydrophila* infections. The PPE concentrations used in this study were: 62.5, 125, and 250 mg/mL. 0.1 mL of PPE was administered via intraperitoneal injection into the fish. On day 7, fish was intraperitoneally injected with 0.1 mL *A. hydrophila* (density $10^8$ CFU/mL). Hematology and biochemical serum parameters were observed on day 1 and 7 after injected with PPE and day 7 and 14 post-challenge with *A. hydrophila*. The results indicated that, red blood cells, white blood cells, and hematocrit of PPE-injected fish were significantly higher than those in the control group ($p<0.05$). Alkaline phosphatase, aspartate transaminase, and alanine transaminase in the fish groups receiving PPE were lower than those of the control group. After challenge test, fish injected with 125 mg/mL PPE concentration was able to significantly reduce the mortality rate compare to control (from 80.50% to 47.22%). It can be concluded that PPE was effective in preventing *A. hydrophila* infections, and had a positive impact on the immunity of fish. So that pineapple is an alternative plant that can be used to improve fish health.

## Introduction

In Thailand, Nile tilapia (*Oreochromis niloticus*) is a major fish species for freshwater aquaculture. In 2021, Nile tilapia comprised the highest production volume at 253,489 tons which of 56.30% of the total production from freshwater culture, valued at 11,721.96 million baht (Department of Fisheries, 2022). Due to the concentration and rapid development of the tilapia farming business, intensive aquaculture systems are widely used that involve higher stocking densities for higher yields per unit area. The rapid expansion of aquaculture also has negative consequences, such as poor growth, poor health, increased susceptibility to disease, and death in severe cases. The stress coming from intensive fish farming also contributes and triggers outbreaks. The factor affecting tilapia culture is a bacterial disease especially *Aeromonas hydrophila* causes disease in fish and other aquatic species known as motile *Aeromonas* septicemia (MAS) (Taylor, 2003). *A. hydrophila* causes infections in fish resulting in swollen abdomen, hemorrhage on the exterior surface, around the anal scale sloughing, surface lesions, and septicemia. Clinical indications such as abnormal movement, reddish lesions on the fin bases and anal area, a greyish-white lesion that extended up to the caudal fin in a moribund state, and enlarged, unsmooth and irregular was found in liver (Rashid et al., 2013). The ability of bacteria to invade host cells and disseminate to virtually any organ via the blood and their capacity to produce multiple virulence factors contribute to the pathogenesis of *Aeromonas* infection (Merino et al., 1998). According to Daskalov (2006), *A. hydrophila* pathogenicity and virulence are dependent on its ability to produce components related to gastroenteritis. The pathogenesis of *A. hydrophila* infection is multifactorial and is related to several virulence factors but the mechanisms of disease have not been clearly explained. Bacteria virulence factors are related to the invasion, replication and evasion of the host's immune system, and cause injuries during pathogenesis of the disease (Yu et al., 2004). Several researchers have described different virulence factors in *A. hydrophila*, among them antigen-O, the presence of capsules (Zhang et al., 2002), S-layer (Dooley & Trust 1988), the type III secretion system (T3SS) (Ahangarzadeh et al., 2022), exotoxins such as aerolysin (*aer*A), cytolytic enterotoxin (*act*), and hemolysin (*hly*A) (Yogananth et al., 2009), and exoenzymes such as lipase, amylase, protease, and nuclease seem to play an important role in the pathogenesis (Janda & Abbott, 2010).

Aerolysin is the major contributor to the virulence of pathogenic *Aeromonas* strains and is a pore-forming toxin that binds to receptors on the target cell membrane. After proteolytic activation, this toxin induces pore or channel formation, leading to the destruction of membrane permeability, osmotic lysis, and cell death (Podobnik et al., 2017). Cytotoxic enterotoxin has enterotoxic, hemolytic, and cytotoxic activities (it can induce 100% cell lysis). After initial colonization of the epithelial cells through type IV pili, *A. hydrophila* may cause diarrhea by producing enterotoxins. Cytotoxic enterotoxin is involved in tissue damage and is responsible for fluid secretion in intestinal epithelial cells and causing gastroenteritis (Sha et al., 2002). Cytotoxic enterotoxin also activates proinflammatory cytokine and eicosanoid cascades in macrophages and a rat intestinal epithelial cell line (IEC-6), leading to tissue damage and a fluid secretory response (Chopra et al., 2000). Hemolysin is known to induce hemolysis by destroying red blood cell membranes inducing anemia (Tomás, 2012). Type III secretory system (T3SS) is an effective system for transportation of toxin into the effector proteins of target cells and plays an important role in host-pathogen interactions (Yu et al., 2004). *AscV* and *aopB* genes are important components of T3SS in *A. hydrophila* that encode the T3SS consolidation proteins associated in internal membrane of bacteria and make holes or channels in the host cell (Ahangarzadeh et al., 2022). The presence of virulent factors in clinical isolates of *A. hydrophila* is indicative of the roles of these factors in the mechanisms of pathogenesis. The disease signs are associated with bacterial invasion and the expression of virulence genes such as production of aerolysin, cytotoxic enterotoxin, and hemolysin responsible for tissue damage, hemolysis, hemorrhage, and enteritis (El-Bahar et al., 2019).

The occurrence of infectious diseases has caused huge economic losses for fish farmers. The excess use of antibiotics and various synthetic chemicals has also resulted in drug residue and resistant pathogens in treated fish (Ferri et al., 2022). Antibiotics that accumulate in the environment and fish potentially threaten consumers and the environment. Moreover, the regular consumption of antibiotic-treated fish can lead to complications in humans such as promote the transfer of antibiotic-resistant bacteria to humans, cause allergies, and induce other severe pathologies, such as cancers, anaphylactic shock, nephropathy, bone marrow toxicity, mutagenic effects, and reproductive disorders (Arsène et al., 2022). The inappropriate administering of antibiotics over time introduces selective pressure, allowing the survival of resistant bacterial strains through adaptive pathways involving transferable nucleotide sequences i.e., plasmids. This is one of the essential mechanisms of antibiotic resistance development in food production systems (Ferri et al., 2022). Moreover, the abuse or misuse of antibiotics coupled with the limited new development of antimicrobials has led to the problem of multidrug-resistant (MDR) bacteria. Multidrug resistance has been increased all over the world that is considered a public health threat. Several recent investigations reported the emergence of multidrug-resistant bacterial pathogens from different origins such as Algammal et al., (2020) reported that the liver of *Oreochromis niloticus* was the most predominant affected organ (54.1%) from *Aeromonas hydrophila* which all isolates were positive for the *gyr*B-conserved gene and harbored *aer*A and *alt* virulence genes, and highly resistant to amoxicillin. Zhu et al. (2020) stated that *A. hydrophil*a isolates from diseased fish were found to be completely resistant to penicillin (100%) and ampicillin (100%) and a high prevalence of resistance to amoxicillin (96.4%), piperacillin (92.9%), cefalexin (78.6%), doxitard (75%), and teicoplanin (67.9%). The biological processes in MDR strain related to carbon metabolism, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, cationic antimicrobial peptide (CAMP) resistance and propanoate metabolism were down-regulated. Algammal et al. (2022a) reported that the isolated *Gallibacterium anatis* strains in layer chickens were highly resistant to sulfamethoxazole-trimethoprim, oxytetracycline, penicillin, ampicillin, kanamycin, neomycin, and erythromycin and carried *bla*ROB and *tet*H resistance genes, MDR *Bacillus cereus* isolates in *Mugil seheli* were to six antimicrobial classes and carried the resistance genes *bla*1, *bla*2, *tet*A, and *erm*A (Algammal et al., 2022b), and *Aeromonas veronii* strains recovered from *Mugil seheli* were MDR to eight classes and possessed blaTEM, blaCTX-M, blaSHV, tetA, aadA1, and sul1 genes (Algammal et al., 2022c). Elbehiry et al. (2022) showed that the antimicrobial resistance levels against *Pseudomonas* isolates in chicken meat were nitrofurantoin (81.16%), ampicillin and ampicillin/sulbactam (71%), cefuroxime and ceftriaxone (65.22%), aztreonam (55%), and ciprofloxacin (49.28%). The emergence of MDR bacterial pathogens from different origins that increase the necessity of the proper use of antibiotics as well as using of safe and potent herbal extracts. An alternative approach to improving the health performance and disease resistance of Nile tilapia are important challenges facing aquarists.

Today, plant extracts are used in aquaculture to treat and control bacterial diseases in place of antibiotics, that induce resistance to specific pathogenic bacteria (Seyfried et al., 2010). The use of plant extracts is another option for disease prevention because it increases fish immunity, which makes it possible to control the spread of the infection. Moreover, plant extracts are easy to find, economically, environmentally friendly, and sustainable methods with minimal side effects are urgently required to improve aquaculture production (Ahilan et al., 2010). To date, a large number of plants have been used for the control of several diseases in fish and numerous studies have explored the potential of agricultural waste in minimizing environmental pollution (Dawood et al., 2022). Pineapple (*Ananas comosus*) is one of these medicinal plants and is a perennial fruit-bearing tropical plant that is well known throughout the world. Most importantly, pineapple is a commercial fruit in tropical regions and some parts of the subtropics. Tropical countries such as Thailand, Malaysia, Indonesia, India, Kenya, the Philippines, and China are among the leading pineapple producers in the world (Lasekan & Hussein, 2018). In 2021, Thailand was reported as the sixth pineapple producer worldwide, with 1.8 million tons (Shahbandeh, 2023). As of April 2023, the sales volume of canned pineapples amounted to approximately 19,000 tons in Thailand. In that same month, Thailand produced more than 21,000 tons of canned pineapples (Office of Industrial Economics Thailand, 2023). Pineapple waste consists of almost 50% of the original fruit which comprises of stem, crown, core, and peel (Sarangi et al., 2022). Based on the physicochemical composition and nutritional values of fruits, pineapple can be considered one of the most useful fruits for manufacturing value-added compounds such as antioxidants, organic acids, bromelain, and phenolic compounds (Mohd Ali et al., 2020). It contains phenolics, citric acid, malic acid, vitamin A, and vitamin B, is high in dietary fiber, demonstrates high antioxidant properties, and contains a proteolytic enzyme, bromelain (Hossain et al., 2015). Pineapple waste also contains high sugars, carbohydrates, protein, and fiber contents (Ketnawa et al., 2012). Pineapple peel waste extraction was carried out using a conventional method in solvents used for the extraction of phenolic compounds and other bioactive compounds (Nurdalilah et al., 2018). It is suggested that the use of pineapple pulp is sources of proteolytic enzymes in fish feed are another option for waste management. Additionally, pineapple has been investigated for several health advantages. It is a plant that many indigenous cultures use for therapeutic purposes. The leather and textile industries, traditional medicine, digestive aids, and nutritional supplements to support health all use pineapples. Bromelain, a crude extract from pineapple, is thought to be responsible for the fruit's therapeutic properties. It contains several closely related proteinases and other compounds, and it has been shown to have various fibrinolytic, anti-edematous, antithrombotic, and anti-inflammatory effects both in vitro and in vivo (Pavan et al., 2012). According to Ketnawa et al. (2012), various pineapple portions have varied amounts of bromelain, which has a wide range of medical and industrial uses. Commercial applications for bromelain have been found in the food sector, some cosmetic products, and nutritional supplements (Walsh, 2002). It is appropriate to consider pineapple as an immunostimulant for aquaculture. Therefore, the objective of this research was to evaluate the efficiency of pineapple peel extract to improve the health and disease resistance of Nile tilapia as an alternative to use of antibiotics in tilapia farming.

## Materials and Methods

### Experimental Fish

Nile tilapia with an initial weight of 24.96±0.58 g was obtained from the hatchery of aquatic animal production technology program, Khon Kaen University, Nong Khai Campus, Nong Khai Province. The fish were acclimatized in laboratory conditions for one week before starting the experiment. At the beginning of the experiment, 12 aquaria containers (0.45×0.90×0.45 m, water volume 150 liters) were each stocked with 12 fish with continuous aeration and kept water quality within optimal range such as dissolved oxygen were not less than 3 mg/L, temperature were 25-32°C, and pH were 6.5-8.5.

### Experimental Design

This study was conducted to identify the best concentration of pineapple peel extract for modulating immune of fish against *Aeromonas hydrophila* by intraperitoneal injection (Hardi et al., 2017). The concentrations of pineapple peel extract were taken from the result of the minimum inhibition concentration (MIC) of pineapple extract against *A.hydrophila* in our previous research (Khumsrisuk et al., 2022). After acclimatization, all fish were intraperitoneally injected with 0.1 mL of pineapple peel extract at each treatment concentration as follows; (i) the fish was intraperitoneally injected with distilled water and challenged with *A.hydrophila* (positive control); (ii) the fish was intraperitoneally injected with 62.5 mg/mL of pineapple peel extract and challenged with *A.hydrophila*; (iii) the fish was intraperitoneally injected with 125 mg/mL of pineapple peel extract and challenged with *A.hydrophila* and (iiii) the fish were intraperitoneally injected with 250 mg/mL of pineapple peel extract and challenged with *A.hydrophila*.

**Challenge Test**

On day seven after pineapple peel extract injection, the fish was challenged with *A. hydrophila* via intraperitoneal injection with 0.1 mL of culture suspension of pathogenic *A. hydrophila*. For inoculum preparation, the bacteria were isolated from the liver and spleen of the diseased fish and cross steak on a nutrient agar (Himedia, India) and incubated at 37°C for 24 hours. The predominant bacterial colony was selected and grew on nutrient agar to obtain a pure culture. The species of bacteria was identified by morphology using Gram's stain and biochemical properties using API 20E strip (Biomerieux, USA). Bacteria with identical morphology and biochemical profiles to *A. hydrophila* were selected and cultivated in nutrient broth (Himedia, India) and incubated at 37°C for 24 hours. The final concentration of bacteria was adjusted to equivalent to McFarland turbidity standards No. 0.5 (1.5 x $10^8$ CFU/mL). The fish was then kept for another 14 days. The clinical signs and mortality of fish was recorded every day. The data were calculated for mortality rate percentage and relative percent survival (RPS) followed by Ellis (1988).

**Hematological Test**

Blood sample were collected from 3 fish from each replication of treatments on days 1 and 7 after pineapple peel extract injection and on days 7 and 14 post-challenged with *A.hydrophila*. The Fish blood was drawn from the caudal vein of fish by a sterile syringe containing EDTA (Himedia, India) as an anticoagulant. The collected blood was used for the estimation of hematology parameters as follows; red blood cells (RBCs) and white blood cells (WBCs) counts by Neubauer hemocytometer according to Ross et al. (2000), hematocrit analysis by micro-hematocrit method following Mondal and Lotfollahzadeh (2023) and glucose analysis followed by Galant et al. (2015).

**Serum Biochemistry**

Similarly, blood samples were collected without anticoagulants for serum separation to analysis of blood biochemistry. The blood sample was placed in non-heparinized tubes and left to clot at 4°C for 15 min and centrifuged at 10,000×g during 10 min at 4 °C and the resulting plasma stored at −80 °C until assayed (Guardiola et al., 2019). Serum was analyzed for total protein, albumin, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) activity. These parameters were analyzed by phenyl phosphate colorimetric method and enzymatic colorimetric test according to TLC Udon Lab Center (Udon Thani, Thailand). Globulin was calculated mathematically by subtracting albumin value from the total protein value (Khalil, 2000).

**Statistical analysis**

All values were performed as technical triplicates (n = 3). Data from each treatment were subjected to one-way analyses of variance (ANOVA), using SPSS for windows version 28.0. Means were paired-comparisons to determine significant variations between the experimental groups after analysis of variances by Duncan's New Multiple Range Test (P = 0.05). The level of significance was chosen at P < 0.05, and the results are presented as mean ± standard deviation.

## Results

The present study provided the potential effect of pineapple peel extract (PPE) on hematology, serum biochemistry parameters and resistance to *Aeromonas hydrophila* in Nile tilapia. According to the pineapple has been used as a medicinal plant medical, nutritional supplement to promote health, is attributed to bromelain, and contains cytotoxic, antidiabetic, hyperlipidemia, antioxidant properties (Das et al., 2019). It is appropriate to consider pineapple as a feed additive for aquaculture. This research aimed to evaluate the utilization of pineapple properties to increase the immunological and disease resistance of Nile tilapia. In this study, Nile tilapia was intraperitoneally injected with PPE at 0, 62.5, 125 and 250 mg/mL then after days 7, fish were challenged with *Aeromonas hydrophila* and observed mortality for 14 days. Blood samples were collected on day 1 and 7 after PPE injection and day 7 and 14 after post-challenge for hematological and serum biochemical analysis.

**Challenge Test**

After 7 days of pineapple peel extract (PPE) injection, fish were challenged with *Aeromonas hydrophila,* and mortality was recorded every day for 14 days. The mortality rate of Nile tilapia injected with all concentrations of PPE in response to the challenge test to *A.hydrophila* was lower than that of fish without PPE injection (Figure 1). The lowest mortality rate was found in fish supplemented with 250 mg/mL PPE concentration, with an average of 36.11±4.82%, and showed statistically significant decrease between control (p<0.05). But there was no significant difference between fish injected with PPE 125 mg/mL (p>0.05). Fish supplemented with 250 mg/mL PPE concentration also showed the highest relative percentage of survival (RPS) at 55.15% followed by 44.79 and 24.05% in fish-supplemented PPE concentration at 125 mg/mL and 62.5 mg/mL, respectively (Table 1).

**Hematological Test**

Fish blood was collected on days 1 and 7 after pineapple peel extract (PPE) injection and on days 7 and 14 after *Aeromonas hydrophila* injection. The results of

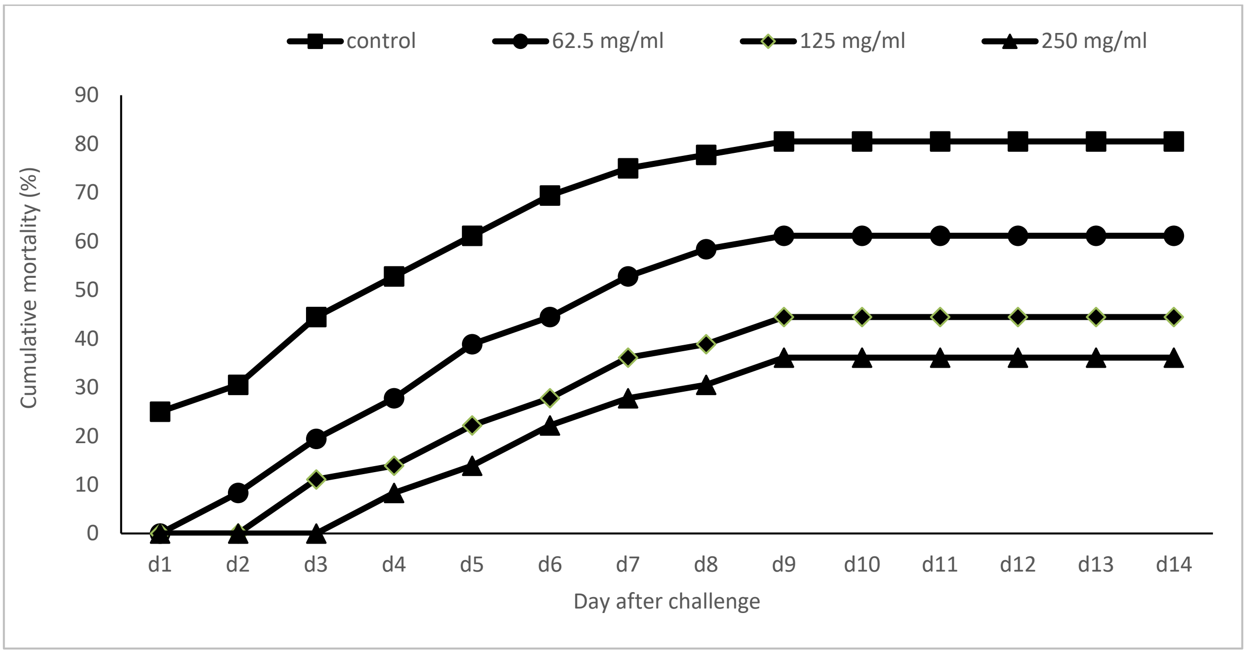


**Figure 1** Cumulative mortality rate of Nile tilapia after challenge with *Aeromonas hydrophila*

red blood cells (RBCs) and white blood cells (WBCs) counts, hematocrit, and glucose values were shown in Table 2. On day seven after PPE injection, the RBCs, WBCs, and hematocrit of fish supplemented with PPE at all concentrations were significantly higher than those in the control group ($p<0.05$). The RBC value of fish treated with each PPE was significantly higher than that of the control (ranging from 4.17-4.24× $10^5$ cell/µL) ($p<0.05$). The hematocrit value of fish supplemented with each PPE was significantly higher than that of the control (ranging from 29.00-32.67%) ($p<0.05$). On days 7 and 14 post-challenge with *A.hydrophila*, the RBCs, WBCs, and hematocrit values of fish groups treated with PPE higher significantly different than the control group ($p<0.05$). The RBCs value of fish treated with each PPE was significantly higher than that of the control (ranging from 4.08-4.28× $10^5$ cell/µL and 4.16-4.48× $10^5$ cell/µL) ($p<0.05$). The WBCs value of fish treated with each PPE was significantly higher than that of the control (ranging from 4.93-4.94× $10^4$ cell/µL and 5.26-7.13× $10^4$ cell/µL) ($p<0.05$). The hematocrit value of fish supplemented with each PPE was significantly higher than that of the control (ranging from 29.00-32.67% and 32.00-35.33%) ($p<0.05$). Glucose values were not significantly different among treatments ($p>0.05$) throughout the experimental period.

### Serum Biochemistry

The results of biochemical serum parameters of Nile tilapia at days 1 and 7 after pineapple peel extract (PPE) injection and days 7 and 14 after *Aeromonas hydrophila* injection are presented in Table 3. On the 1st and 7th days after PPE injection, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) values in all concentrations in the PPE-treated groups were lower than those in the control group. After the challenge test, the values of AST, ALT, and ALP in all PPE groups were slightly increased and tended to be less than the value in the control group but not statistically different from those of the control group ($P>0.05$).

## Discussion

The use of antibiotics as health promoters in the aquaculture industry requires consideration of appropriate use due to the development of cross-resistance between pathogens and the dangers they pose to humans when entering the food cycle. Due to their antibacterial properties, herbal plants and their bioactive substances are becoming relevant as alternative health promoters. It is interesting to consider the immunostimulants used to combat fish diseases. Immunostimulants boost fish defensive systems, both specific and non-specific defense mechanisms, to promote resistance to infectious diseases. Consequently, our study aimed to evaluated the potential role of pineapple peel extract in promoting health and preventing *Aeromonas hydrophila* in Nile tilapia.

Challenge tests are typically used as the standard assay to assess the overall health of the immune system (El-Houseiny et al., 2022) A strong indicator of the effectiveness of immunostimulants is the increase in the resistance of fish to pathogenic microorganisms (Sakai, 1999). The results of the *A. hydrophila* challenge test, the mortality rates in all PPE injections decreased to 36.11% (250 mg/mL PPE extract), 47.22% (125 mg/mL PPE extract) and 61.11% (62.5 mg/mL PPE extract)

**Table 1.** Mortality rate and relative percentage survival of Nile tilapia after *Aeromonas hydrophila* injection for 14 days (Mean±SD)

| Concentration of pineapple peel extract (mg/mL) | Mortality rate (%) | Relative percentage survival (%) |
|---|---|---|
| 0 | 80.50±4.81[a] | - |
| 62.5 | 61.11±4.81[b] | 24.05 |
| 125 | 47.22±4.80[c] | 44.79 |
| 250 | 36.11±4.82[c] | 55.15 |
| P-value | 0.00 | - |

Within a column, means with the different letters are significantly different (P<0.05)

**Table 2.** Blood profile of Nile tilapia was treated with pineapple peel extract for *Aeromonas hydrophila* prevention

| Time | Parameter | Concentration of pineapple peel extract (mg/mL) | | | | P-value |
|---|---|---|---|---|---|---|
| | | 0 | 62.5 | 125 | 250 | |
| D1-Pre | RBC ($\times 10^5.\mu l^{-1}$) | 2.13±1.71 | 3.10±1.69 | 4.04±1.37 | 2.98±0.59 | 0.343 |
| | WBC ($\times 10^4.\mu l^{-1}$) | 3.11±0.06[a] | 2.67±0.05[a] | 4.94±0.12[b] | 4.93±0.31[b] | 0.001 |
| | Hematocrit (%) | 27.67±1.53 | 28.33±0.58 | 28.00±1.00 | 29.33±0.58 | 0.274 |
| | Glucose | 47.33±4.93 | 47.67±4.16 | 50.00±6.08 | 56.67±1.15 | 0.107 |
| D7-Pre | RBC ($\times 10^5.\mu l^{-1}$) | 3.04±0.27[a] | 4.17±0.84[b] | 4.23±0.24[b] | 4.24±0.21[b] | 0.036 |
| | WBC ($\times 10^4.\mu l^{-1}$) | 3.11±0.41[a] | 2.67±0.18[b] | 4.94±0.12[bc] | 4.93±0.26[c] | 0.000 |
| | Hematocrit (%) | 26.33±0.58[a] | 29.00±0.58[b] | 30.00±1.00[b] | 32.67±1.53[c] | 0.001 |
| | Glucose | 56.00±6.08 | 58.33±5.51 | 60.67±6.66 | 62.33±2.52 | 0.540 |
| D7-Post | RBC ($\times 10^5.\mu l^{-1}$) | 3.36±0.14[a] | 4.08±0.50[ab] | 4.27±0.26[b] | 4.28±0.16[b] | 0.075 |
| | WBC ($\times 10^4.\mu l^{-1}$) | 3.37±0.59[a] | 4.88±0.15[b] | 5.05±0.10[b] | 5.43±0.67[c] | 0.001 |
| | Hematocrit (%) | 28.33±0.58[a] | 30.67±1.00[b] | 32.67±0.58[c] | 33.00±1.00[c] | 0.000 |
| | Glucose | 94.33±6.81 | 88.67±9.02 | 90.33±2.52 | 92.00±4.36 | 0.718 |
| D14-Post | RBC ($\times 10^5.\mu l^{-1}$) | 3.26±0.23[a] | 4.16±0.12[b] | 4.38±0.42[b] | 4.48±0.30[b] | 0.003 |
| | WBC ($\times 10^4.\mu l^{-1}$) | 3.49±0.17[a] | 5.26±0.11[b] | 5.67±0.35[c] | 7.13±0.55[d] | 0.000 |
| | Hematocrit (%) | 29.00±1.00[a] | 32.00±1.00[b] | 34.67±2.08[c] | 35.33±1.15[c] | 0.002 |
| | Glucose | 91.67±5.51 | 82.67±3.06 | 85.00±4.36 | 88.67±1.53 | 0.089 |

Values are means ± SD. Within a row, means with the different letters are significantly different (P<0.05).
Note: D1-Pre: day 1 after pineapple peel extract injection, D7-Pre: day 7 after pineapple peel extract injection, D7-Post: day 7 after challenge test, D14-Post: day 14 after challenge test

**Table 3.** Biochemical serum parameters of Nile tilapia were treated with pineapple peel extract for *Aeromonas hydrophila* prevention (means ± SD)

| Time | Parameter | Concentration of pineapple peel extract (mg/mL) | | | | P-value |
|---|---|---|---|---|---|---|
| | | 0 | 62.5 | 125 | 250 | |
| D1-Pre | Total protein | 2.13±0.15 | 2.03±0.47 | 2.03±0.41 | 2.20±0.20 | 0.908 [ns] |
| | Albumin | 1.07±0.15 | 1.15±0.19 | 1.14±0.06 | 1.15±0.06 | 0.835 [ns] |
| | Globulin | 1.07±0.15 | 0.89±0.58 | 0.89±0.45 | 1.05±0.26 | 0.909 [ns] |
| | AST | 18.33±7.51 | 18.80±2.88 | 15.97±2.12 | 14.33±6.66 | 0.716 [ns] |
| | ALT | 18.33±1.53 | 12.00±5.57 | 20.33±5.51 | 14.33±3.06 | 0.149 [ns] |
| | ALP | 35.33±6.66 | 41.67±8.96 | 35.00±3.61 | 29.33±3.79 | 0.192 [ns] |
| D7-Pre | Total protein | 2.33±0.12 | 2.43±0.35 | 2.47±0.25 | 2.37±0.12 | 0.888 [ns] |
| | Albumin | 0.97±0.15 | 1.07±0.21 | 1.00±0.10 | 1.00±0.12 | 0.827 [ns] |
| | Globulin | 1.37±0.06 | 1.37±0.15 | 1.47±0.15 | 1.40±0.20 | 0.827 [ns] |
| | AST | 20.00±3.61 | 19.17±1.90 | 16.33±6.51 | 16.40±3.08 | 0.619 [ns] |
| | ALT | 23.50±5.07 | 16.00±3.61 | 22.33±6.11 | 21.67±7.57 | 0.440 [ns] |
| | ALP | 36.00±6.56 | 42.03±2.25 | 34.67±10.01 | 31.33±3.21 | 0.284 [ns] |
| D7-Post | Total protein | 2.43±0.21 | 2.47±0.06 | 2.47±0.15 | 2.60±0.26 | 0.713 [ns] |
| | Albumin | 0.77±0.25 | 0.87±0.15 | 0.97±0.06 | 0.87±0.06 | 0.501 [ns] |
| | Globulin | 1.67±0.45 | 1.60±0.10 | 1.50±0.10 | 1.73±0.25 | 0.747 [ns] |
| | AST | 23.67±7.77 | 22.67±3.21 | 22.67±10.69 | 21.37±14.19 | 0.996 [ns] |
| | ALT | 24.33±9.50 | 18.00±5.20 | 24.70±3.99 | 24.67±5.86 | 0.541 [ns] |
| | ALP | 45.37±9.09 | 39.83±0.76 | 36.60±8.12 | 33.30±2.07 | 0.183 [ns] |
| D14-Post | Total protein | 2.67±0.15 | 2.60±0.44 | 2.53±0.15 | 2.70±0.26 | 0.884 [ns] |
| | Albumin | 1.10±0.10 | 0.97±0.12 | 0.87±0.25 | 0.97±0.06 | 0.363 [ns] |
| | Globulin | 1.57±0.23 | 1.63±0.38 | 1.67±0.21 | 1.73±0.29 | 0.908 [ns] |
| | AST | 24.73±5.16 | 23.33±2.52 | 26.00±9.64 | 22.00±1.00 | 0.836 [ns] |
| | ALT | 28.33±8.50 | 20.90±6.84 | 24.00±6.08 | 25.10±8.31 | 0.692 [ns] |
| | ALP | 47.50±7.17 | 40.63±7.72 | 39.17±10.56 | 34.53±9.13 | 0.394 [ns] |

Note: D1-Pre: day 1 after pineapple peel extract injection, D7-Pre: day 7 after pineapple peel extract injection, D7-Post: day 7 after challenge test, D14-Post: day 14 after challenge test compared with the control (80.50%). Moreover, all fish groups injected with PPE had better relative percentages of survival (RPS) than the control group. This is one of the most obvious criteria of immunological assessment in the challenge test. The RPS of Nile tilapia injected with PPE (250, 125, and 62.5 mg/L) were 55.15, 44.79 and 24.05% respectively. Based on the observed increase in RPS against *A. hydrophila* in fish injected with PPE compared to the control group in this study, it can be said that PPE injection was effective in preventing *A. hydrophila* infection in fish. PPE could support the Nile tilapia's immune system and better resistance to pathogens. This makes fish more resistant to bacterial invasion. As Pavan et al. (2012) mentioned that the medicinal properties of pineapple crude extract are mainly derived from bromelain and phytochemical factors such as vitamin C, flavonoid, and various combinations of thiolendopeptidases and other compounds such as carbohydrate, cellulase, phosphatase, glucosidase, glycoprotein, peroxidase, and many protease inhibitors. Several fibrinolytic, antiedematous, antithrombotic, and anti-inflammatory properties, as well as a reduction in the body's inflammatory reactions, are among the benefits of bromelain. Although, the primary virulence factors of *A.hydrophila* that influence pathogenicity are extracellular toxins (hemolysin, enterotoxin and protease), structural traits (pilli, S-layer and lipopolysaccharide), adhesion and invasion (Janda & Abbott, 2010). *A.hydrophila* could be colonized on the epithelial cells by using type IV pili, after which produced cytotoxic substances are involved in tissue damage and gastroenteritis. (Sha et al., 2002). Type III secretory consolidation proteins associated in internal membrane of bacteria and make holes or channels in the host cell (Ahangarzadeh et al., 2022). In this study, it was reported that PPE has the ability to inhibit *A.hydrophila*. This finding may be due to the association of bromelain with gastrointestinal secretion signaling pathways, and also prevent bacterial adhesion. Because of this, the bacteria in the gastrointestinal system are unable to attach to several glycoprotein receptors (Praveen et al., 2014). Bromelain plays an important role in bacterial protein degradation. According to Praveen et al. (2014), it is one of the crucial components of bacterial membranes that results in cell damage and death. Bromelain may hydrolyze some of the peptide bonds present in bacterial cell wall. The process that prevents bacterial development is still unclear (Bhattacharyya, 2008).

Additionally, PPE can be further applied in aquatic feed for bacterial resistance. Previous studies reported the antibacterial activity PPE contained flavonoids, alkaloids, terpenoids, tannins, and saponins. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of PPE against *Aeromonas hydrophila* was 200 µg/mL and 1 × MIC, respectively. Extract of pineapple peel exhibited better antibacterial activity than banana and cassava peels (Mulia et al., 2023). Khumsrisuk et al. (2022) stated that antibacterial activity of PPE against *A.hydrophila* with MIC value was 125 mg/mL. Nahid Hasan et al. (2021) also revealed that the ethanol and methanol PPE showed antibacterial activity and MIC values against *Pseudomonas aeruginosa* and *E. coli*. Additionally, the MIC values of PPE against *S. aureus*, *P. aeruginosa* and *Salmonella typhi* (Okoh et al., 2019), and the MIC of bromelain from pineapple against *Streptococcus mutans*, *Enterococcus fecalis*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis* were reported by Praveen et al. (2014). The antibacterial activity of the PPE may be due to the presence of polyphenols, saponins, flavonoids, and other secondary metabolites in the extract. The pineapple fruits also displayed the chemical profile consisting of eight phenolic compounds, i.e gallic acid, gentisic acid, syringic acid, vanillin, ferulic acid, sinapic acid, isoferulic acid, and o-coumaric acid. (Yapo et al., 2011). Flavonoids play a significant role in antimicrobial activity and have antimicrobial potential by binding to adhesions or complexes to the cell wall and inhibiting enzyme activity (Akinpelu et al., 2008). Phenols can denature proteins, and their lipophilic property allows them to attract lipid molecules present in cell membranes and destroy bacterial cell membranes (Maurer, 2001). Phenol also alters membrane permeability, which may lead to metabolite loss by membrane degradation, oxidative phosphorylation uncoupling, and suppression of active transport (Lubaina et al., 2019). The ability to act as a non-ionic surface agent for phenolic compounds is the primary mechanism of antimicrobial activity. It can damage proteins, interfere with lipid-protein interactions, or prevent pathogen enzyme function.

Improving the immune systems has been a target goal for feed additive supplementation in aquaculture. As Van Doan et al. (2021) mentioned that the inclusion of 10 g/kg pineapple peel powder significantly enhanced the lipopolysaccharide binding protein (LBP) gene expression of Nile tilapia. LBP is a soluble acute-phase protein, which plays a vital role in lipopolysaccharide signaling and non-specific immune response (Kono & Sakai, 2003). The LBP gene-encoded protein is linked to the acute-phase immunologic response to gram-negative pathogenic bacteria. *A. hydrophila* is a Gram-negative pathogen, which possesses a membrane component known as lipopolysaccharide (LPS). LBP is a specific protein that binds with LPS and is responsible for eliciting an immune response against LPS in vertebrates. The LBP gene itself plays a role in the resistance to *A. hydrophila*, or the LBP gene is linked to a gene or several genes, which play a role in the resistance to *A. hydrophila*, that reported in Nile tilapia (Fu et al., 2014) and Crucian carp (Raetz & Whitfield, 2002). Meanwhile, the mechanism by which pineapple peel stimulates the immune response in Nile tilapia has not yet been elucidated. It may be due to the bioactive substances was found in the pineapple peel. Pineapple contains several compounds with pharmacological properties, such as bromelain and ferulic acid, which could stimulate immune response of fish (Wiszniewski et al., 2019).

Our findings agree with those of Khumsrisuk et al. (2022), who reported that Nile tilapia fed 1% PPE for 8 weeks showed the lowest mortality and the highest relative survival percentage after fish challenged with *A. hydrophila*. Van Doan et al. (2021) who stated that Nile tilapia fed the 10 g/kg pineapple peel powder supplemented diet achieved the highest ($P<0.05$) survival rate against *S. agalactiae*. Pineapple waste also has a protective effect against pathogenic bacteria like *E. coli* and *Listeria monocytogenes* (Russo et al., 2014; Rahman & Yang, 2018). Indeed, several studies have demonstrated that the pineapple leaf or its ingredients have a wide range of antibacterial, antiprotozoal, and anthelmintic actions (Ali et al., 2015). After *A. hydrophila* injection, the mortality of fish fed PPE decreased that was comparable with previous studies of other herbs for example, Nile tilapia fed a diet that included *Morus alba* (Mapanao et al., 2019), *Moringa oleifera* (Mapanao et al., 2021), *Ananas comosus* (Khumsrisuk et al., 2022), and *Allium sativum* (Nithikulworawong, 2023). Our results suggested that pineapple peel extract can be used as an important supplementary for fish to enhance immunity against disease in fish.

The profitability and sustainability of aquaculture can benefit from reliable and reproducible fish health monitoring. Aquaculture studies are increasingly using hematological and blood biochemical characteristics as useful tools. Hematological and blood biochemistry parameters are reliable and efficient methods for keeping track of fish health. Fish health and growth can be monitored considerably more reliably and precisely using blood performance. The present study's findings demonstrated that various health indicators in Nile tilapia could be successfully improved through pineapple peel extract (PPE) injection. On day seven after pineapple peel extract injection, values of red blood cells (RBCs), white blood cells (WBCs), and hematocrit were higher than those found in tilapia by Martins et al. (2008) and Sayed and Moneeb (2015) in normal conditions. An increase in hematological indices shows immune system stimulation and function of organs related to blood cell formation such as thymus, spleen, and bone marrow (Iheanacho et al., 2007). The best results were found in the group of fish injected with the 250 mg/mL, as indicated by a significant improvement in RBCs, WBCs, and hematocrit values compared to fish in the control group ($p<0.05$). These observations are consistent with those of Gopalraaj et al. (2022) who reported that dietary inclusion of PPE significantly enhanced the hematocrit and red blood cell count ($p < 0.05$) in *O. niloticus* with an increase in PPE, Rahman and Yang (2018) reported that red blood count, hemoglobin, and hematocrit were improved in the 3% pineapple leaf powder-supplemented broiler performance group, and Fahturohman et al. (2022) stated that the addition of pineapple core extraction in commercial feed carp (*Cyprinus carpio*) was a significant difference ($p<0.05$) in the average value of carp hematocrit.

As Moreno et al. (2000) mentioned that the hematocrit value indicates the volume of RBCs in plasma. It is generally agreed upon that a higher hematocrit, showing higher viscosity, is advantageous to health. Higher hematocrit values are closely related to higher production. An increasing hematocrit within a normal range, but not an endless increase, can represent a positive indicator of optimized oxygen transport and health (Reynolds, 1953). Moreover, various types of stress in fish result in a decrease in hematocrit value. The increased plasma level (decreased hematocrit) enhances electrolyte and protein movements through the blood flow to compensate for the oxygen demands of tissues. However, from the results of this experiment, it was found that after injecting PPE into Nile tilapia, there was no significant decrease in the hematocrit value. That would indicate that injecting PPE does not cause stress for the fish. As Esmaeili (2021) mentioned that RBCs are the most common type of blood cells in vertebrates and are responsible for delivering oxygen ($O_2$). RBCs absorb oxygen in the gills and release it into the tissues. A higher RBCs can indicate oxygen delivery to the tissues have improved. The increased RBCs are also a positive response to immune system improvement. The fish's enhanced erythropoiesis and hemosynthesis activities were demonstrated by improvements in RBCs and HCT (Ashrafizadeh et al., 2020), as we found in the results of this study. These findings also reflected that the fish were in good health and there are no problems such as anemia and malnutrition (Enis Yonar et al., 2012). From our results, serum biochemical parameters such as alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) were also improved in the PPE-treated group (Table 3). So, we also confirmed the non-toxicity of PPE based on our hematological and serum biochemical parameters. These did not change after the PPE injection. Additionally, other studies also showed that the PLP treatment improved the red blood count, hemoglobin, and hematocrit values within the normal ranges (Rahman & Yang, 2018). Thus, the present results indicate that PPE has nutritional benefits for Nile tilapia.

In this study, post-challenge with *A.hydrophila*, red blood cells (RBCs), white blood cells (WBCs), and hematocrit values of fish groups treated with PPE injection increased as the amount of PPE increased (Table 2). This indicated that the health of Nile tilapia improved with the addition of pineapple peel extraction. Similar results have been reported in previous studies of other herbs. For example, Nithikulworawong (2023) reported that Nile tilapia-fed dietary garlic had significantly higher red blood cell and white blood cell counts than the control group ($p<0.05$) at post-challenged with *A. hydrophila*. Pakravan et al. (2012)

stated that common carp fingerlings fed with *Epilobium hirsutum*-containing feed increased leucocyte count before and after *A. hydrophila* infection. RBC count increased with the PPE injection, which might indicate an immunostimulant effect. The high RBCs count in PPE-injected fish groups are presumed to be due to pineapple's antioxidant properties. PPE also contains bromelain that can act as antioxidants in blood cells such as lipid peroxidation inhibition and free radical scavenging (Nyi et al., 2019). Free radicals can damage red blood cell membranes and other cells. The antioxidant can act as a reservoir of free radicals to protect red blood cell membranes. Vitamin C is the body's primary water-soluble antioxidant, defending all aqueous areas of the body against free radicals that attack and damage normal cells (Joy, 2010). WBCs are circulatory cells that help with both innate and adaptive immune responses. It is debatable whether the higher number of WBCs caused by the supplements or result of optimal health and growth regardless of additives. The increase in WBCs indicates an increase in the immune status and the suppression of infection in these groups. Lymphocytes are the largest number of cells consist of the leucocytes, which is responsible for producing antibody, and the chemical act to defense against infection (Ayoola, 2011). Likewise, African catfish fed with 10% to 50% moringa leaves as fish meal substitution in diets showed an increase in WBCs count (Ozovehe, 2013) and Nile tilapia fed moringa leaves supplements had increased RBCs and WBCs counts as the amount of moringa leaves in the diet increased (Mapanao et al., 2021).

Total serum protein is one of the most used and useful blood parameters to measurement. Serum proteins serve a variety of functions, comprised of maintaining osmotic pressure and pH, transporting various metabolites, and interacting closely with the immune system. The total protein content in the blood is considered an index of the nutritional status of the body indirectly (Zheng et al., 2017). Total protein plays an important role in fish humoral immunity and the innate immune response (Jha et al., 2007). Total protein in fish, and the values ranged from 0.74 to 7.5 g/dL with an average of 3.6 g/dL (Esmaeili, 2021). In this study, fish PPE-injected had higher levels of total serum proteins and globulin than the control group. This finding suggests that PPE can increase protective proteins, which can stimulate the immune system. High levels of protein in the blood, especially globulin, is a good predictor of liver function and innate immune response (Asadi et al., 2012). Similar results were observed in Nile tilapia-fed diets supplemented with *Annona squamosa* leaf extract (Almarri et al., 2023) and *Alchemilla vulgaris* (Mansour et al., 2022). Moreover, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) are released into the blood during organ damage. Thus, detecting high levels of ALP, ALT, and AST in the blood provides information on organ damage, particularly liver cells. The current study confirmed that fish injected with PPE were normal and displayed no effects in terms of serum biochemical parameters like the control group, indicating normal organ function. The present study suggested the idea that the fish that received PPE led to a reduction of liver functional enzymes (ALT, AST, and ALP) and kidney function indicators.

## Conclusion

The immunostimulatory potential of pineapple peel extract (PPE) was demonstrated. The PPE at 125 mg/mL improved hematology, serum biochemical parameters, and reduced mortality post challenged with *Aeromonas hydrophila*. Based on Nile tilapia performance, pineapple peel extract can be used as a natural component in fish to promote fish health.

## Ethical Statement

This study was carried out in strict accordance with the recommendations in the guidelines and regulations for Ethics of Animal Experimentation of the National Research Council of Thailand. The protocol was approved by Institutional Animal Care and Use Committee of Khon Kaen University (Reference No. 660201.2.11/497 (84).

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## Author Contribution

Nudtha Nithikulworawong: Conceptualization, Methodology, Investigation, Writing—original draft, Supervision. Wirat Jiwyam: Conceptualization, Supervision. All authors have read and agreed to the published version of the manuscript.

## Conflict of Interest

No competing interests are reported.

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