Use of *Morinda citrifolia* Aqueous Extract as an Immunostimulant and Prophylactic Agent Against *Edwardsiella tarda* in Tilapia (*Oreochromis niloticus*)

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Abstract
Edwardsiella tarda is an important fish pathogenic bacteria causing massive mortalities and large economic losses in natural environment and fish farming worldwide. Public concerns on the use of antibiotics to control bacterial infectious diseases have been increasing. The objective of this study was to examine the effectiveness of aqueous extract of Morinda citrifolia leaf on nonspecific immunity and disease resistance against E. tarda in the tilapia (Oreochromis niloticus). Parameters used for assessment of the status of innate immunity were phagocytic activity and respiratory burst activity of leucocytes and lysozyme activity in plasma.

Dose-dependent increase of the phagocytic activity was observed at week 3 and week 4 in fish given diets with 0% (Diet 1), 1% (Diet 2), 1.5% (Diet 3) and 2% (Diet 4) of M. citrifolia aqueous extract. Fish receiving diet 2, diet 3 and diet 4 had the increased level of lysozyme activity at week 3 and week 4, compared to the control group, but no significant difference in the activity was found between fish treated with diet 3 and diet 4. Diet 2 did not have any effect on respiratory burst activity, while diet 3 and diet 4 caused the similar degree of increase in the activity at week 3 and week 4. When the fish diets were given to the fish 3 weeks prior to intraperitoneal infection of E. tarda, a dose dependent reduction in mortality of the infected fish was obtained. No mortality was apparent in the fish receiving diet 4. The results of this study suggest that M. citrifolia aqueous extract may be used in fish as an immunostimulant and prophylactic agent against E. tarda.

Keywords: *Morinda citrifolia*, *Oreochromis niloticus*, *Edwardsiella tarda*, Innate immune response.

Introduction

Bacterial infectious diseases are major problems causing heavy loss to fish farmers worldwide. Among bacterial fish pathogens, *Edwardsiella tarda*, a Gram-negative bacillus, is an important pathogen causing edwardsiellosis in various commercial fish species such as chinook salmon (*Oncorhynchus tshawytscha*)[1], eel (*Anguilla japonica*)[19], flounder (*Paralichthys olivaceus*)[21], channel catfish (*Ictalurus punctatus*)[20], carp (*Cyprinus carpio*)[31], turbot (*Scophthalmus maximus*)[6,22] and tilapia (*Oreochromis niloticus*)[8]. It has been responsible for massive mortalities and large economic losses in natural environment and fish farming worldwide[24].

Public concerns on the use of antibiotics to control bacterial infectious diseases are increasing because of their negative effects. Prolonged use of antibiotics could favor the development of antibiotic-resistant bacteria, thereby reducing drug efficacy[38]. Furthermore, accumulation of antibiotics in the environment and in fish can be potentially harmful to consumers and other organisms[4]. To avoid or reduce the use of antibiotics in aquaculture, researchers have been focusing on searching for a safer alternative to control bacterial infectious diseases. The use of natural products as immunostimulants to improve innate (nonspecific) immune response and increase disease resistance in fish has been shown recently to be promising[11,23,25-29,32,37]. Since plants and plant products have been considered to be safe to farmers, consumers and environment, it is of interest to use them as immunostimulants to prevent edwardsiellosis in fish.

Morinda citrifolia is a tree in the family Rubiaceae. It is native to Southeast Asia but has been extensively spread to many parts of the world such as Indian subcontinent, Pacific islands, French Polynesia and the Dominican Republic. It is known by many names including great morinda, Indian mulberry, beach mulberry, cheese fruit and noni. It has been used for both culinary and medicinal purposes[36]. Various parts of the plant such as leaf, flower and fruit have been reported to have a broad range of therapeutic effects including antitumor, analgesic, hypotensive, anti-inflammatory and immune enhancing effects[12-14,35]. The present study focuses on the effectiveness of aqueous extract of *M. citrifolia* leaf on nonspecific immunity and disease resistance against *E. tarda* in the tilapia (*Oreochromis niloticus*)

Material and Methods

Plant material and aqueous extract preparation: *M. citrifolia* leaf were purchased from the herb shop Ban Samunprai in Pranakorn, Bangkok, Thailand. The sample was dried at 70°C for 72 h and sold as dried material. *M. citrifolia* leaf aqueous extract was prepared by finely grinding the dried sample and then thoroughly mixing it with distilled water in a ratio of 1:10 (w/v) for 24 h at room temperature. The mixture was centrifuged at 10,000 xg for

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10 min at room temperature and the supernatant was collected for filtration through a 0.45 µm pore size filter membrane (Schleicher and Schuell, Keene, NH). The filtrate was freeze-dried and kept at -20ºC in a glass vial until use.

Preparation of fish diets: The fish diets supplemented with various concentrations of *M. citrifolia* leaf extract were prepared from the commercial fish diet (C. P. Classic; S. W. T. Co., Ltd., Bangkok, Thailand). Diets 2 to 4 were prepared by mixing dried matter of *M. citrifolia* leaf extract with the commercial fish diet in the ratios (w/w) of 1%, 1.5% and 2% respectively. Distilled water was added to each diet mixture (1 mL/g) and mixed until homogenous. The mixture was passed through a meat mincer producing extruded strings which were dried at 30°C for 24 h and then broken into about 2 mm long pellets. The control diet (Diet 1) was prepared using the same process as the other test diets except no supplement was added.

Fish preparation: Tilapia (*Oreochromis niloticus*) were purchased from Nong Khon Farm, Ubon Ratchathani, Thailand. They were maintained in 500 l plastic tanks at 25°C, under a 12 h light:12 h dark photoperiod and were fed commercial fish diet (C. P. Classic; S. W. T. Co., Ltd., Bangkok, Thailand) at a rate of 3% of fish body weight twice daily for 2 weeks prior to the experiments.

All experiments were conducted in 100 L aquaria. Fish weighing 30 ± 2 g were placed in the aquaria (20 fish per aquarium) 24 h prior to the experiments. Feed was supplied twice daily at a rate of 3% of fish body weight. On the day of stocking, the fish were fed the aforementioned commercial fish diet. Subsequently, each of the diets (Diet 1, 2, 3, or 4) was assigned randomly to two replicate groups for 4 weeks. During the experiment, water temperature 25 ± 2°C, dissolved oxygen concentration 6.0 ± 0.6 mg/L, pH 7.0 ± 0.5, total hardness 34.5 ± 1.8 mg/L as $CaCO_3$, alkalinity 29.5 ± 2 mg/L as $CaCO_3$ and photoperiod 12L:12D were maintained.

Blood, plasma and leucocytes preparation: Blood samples (5 fish/group) were collected from caudal vein at 1, 2, 3 and 4 weeks after treatment. Haparin was used as an anticoagulant. Individual fish was sampled only once to avoid the influence on the assays due to multiple bleeding and handling stress on the fish. Leucocytes were separated from each blood sample by density-gradient centrifugation. One mL of histopaque 1.119 (Sigma-Aldrich, St. Louis, MO, USA) containing 100 µL of bacto hemagglutination buffer, pH 7.3 (Difco, Franklin Lakes, NJ, USA) was dispensed into siliconised tubes. One ml of a mixture of 1.077 density histopaque and hemagglutination buffer and 1 mL of blood were carefully layered on the top. The sample preparations were centrifuged at 700 g for 15 min at 4°C.

After centrifugation, plasma was collected and stored at -20°C until being used. Interface of leucocyte suspension was gently removed with a Pasteur pipette and dispensed into siliconised tubes containing phenol red free Hanks balanced salt solution (HBSS, Sigma-Aldrich). Cells were then washed twice in phenol red free Hank's balanced salt solution (HBSS, Sigma-Aldrich) and adjusted to 2 x 10^6 cfu/mL.

Phagocytic activity assay: Phagocytic activity of leukocytes was determined according to the method described by Butprom et al.[5] This assay involves the measurement of congo red stained yeast cells which have been phagocytozed by leukocytes.

Congo red stained yeast cells (*Saccharomyces cerevisiae*) were prepared as follows: Three mL of a congo red solution and 0.87% in phosphate buffer saline (PBS) pH 7.2 were added to yeast cell suspension (1.5 g). After incubating at room temperature for 15 min, the mixture was thoroughly mixed with 7 mL of distilled water. The resulting mixture was autoclaved for 15 min to kill and to fix the yeast cells. These cells were then washed three time in HBSS and stored at 4°C until use. Prior to use, the cell concentration of was adjusted to 10^8 cells/mL in HBSS.

To perform the assay, 250 µL of the leucocyte suspension was mixed with 500 µL of the congo red stained and autoclaved yeast cell suspension (providing yeast cell/leucocyte ratio of 40/1). The mixtures were incubated at room temperature for 60 min. Following incubation, 1 ml of ice-cold HBSS was added and 1 mL of histopaque (1.077) was injected into the bottom of each sample tube. The samples were centrifuged at 850 g for 5 min to separate leucocytes from yeast cells. Leucocytes were harvested and washed twice in HBSS. The cells were then resuspended in 1 mL trypsin-EDTA solution (5.0 g/L trypsin and 2.0 g/L EDTA, Sigma-Aldrich) and incubated at 37°C overnight. The absorbance of the samples was measured at 510 nm using trypsin-EDTA as a blank.

Lyzozyme activity assay: Plasma lysozyme activity was measured using the method of Butprom et al[5] with some modifications. Briefly, 100 µL of fish plasma was added to 3 mL suspension of *Micrococcus lysodeikticus* ATCC No. 4698 (Sigma-Aldrich). The absorbance of the mixture was examined at the wavelength of 550 nm after incubating at 25°C for 0.5 and 4.5 min. One unit of lysozyme activity was defined as the amount of lysozyme producing a decrease in absorbance of 0.001/min. A suspension of *M. lysodeikticus* was prepared by dissolving 20 mg of the overnight culture of the bacterium grown on nutrient agar plate into 100 ml of 0.067 M sodium phosphate buffer at pH 6.5.

Respiratory burst activity assay: Respiratory burst activity of leucocytes was examined using a modification of the method described by Butprom et al.[5] Briefly, 100 µL of leucocyte suspension and an equal volume of cytochrome *c* (2 mg/L in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at 1 µg/mL were placed in triplicate in microtiter plates. In order to test

specificity, another 100 µL of leucocyte suspensions and solutions of cytochrome *c* containing PMA and superoxide dismutase (SOD, Sigma-Aldrich) at 300 U/mL were prepared in triplicate in microtiter plates. Samples were then mixed and incubated at room temperature for 15 min. Extinctions were measured at 550 nm against a cytochrome *c* blank in a multiscan spectrophotometer. Readings were converted to nmoles O_2^- by subtracting the OD of the PMA/SOD treated supernatant and cnverting OD to nmoles O_2^- by multiplying with 1587. Final results were expressed as nmoles O_2^- produced per 10^5 blood leucocytes.

Bacterial fish pathogen and culture condition: The bacterial fish pathogen used in this study was *E. tarda* PP00124 isolated from naturally infected tilapia in a fish farm in Ubon Ratchathani, Thailand. The identity of the bacterial strain was confirmed by polymerase chain reaction using 2 species specific primers, Eta2-351 (5'TAGGGAGGAAGGTGTGAA3') and EtaEdwsp-780r (5'CTCTAGCTTGCCAGTCTT3') according to the method described by Baird et al.[3] The bacterial strain was cultured on Trypticase soy agar (TSA) and in Trypticase soy broth (TSB) (Difco, Detroit, MI, USA) and incubated at 25ºC. The bacterial stock culture was stored as a frozen culture at -80ºC in TSB containing 20% glycerol (v/v).

Median lethal dose (LD_{50}) determination: Groups of 20 fish with an average weight of 30 g were tested for pathogenicity of *E. tarda*. Each group was kept in 100 l aquarium at 25°C throughout the experiment. From an initial bacterial concentration of 10^8 cfu/ml, 10-fold serial dilutions were prepared. One hundred µl of each dilution was injected intraperitoneally into each fish. The control group was intraperitoneally injected with 100 µl of physiological saline instead of the bacterial suspension. Each dilution trial was conducted in duplicate. Mortalities were recorded daily for 10 days. Dead fish were removed from aquaria daily. Their livers and kidney were aseptically streaked on TSA. After incubation at 25ºC for 24 h, colonies grown on the agar were confirmed to be *E. tarda* by using PCR as described earlier. The LD_{50} value was calculated by the method described by Reed and Muench[30].

Examination of effect of the supplemented fish diets on disease resistance: *In vivo* studies of the effect of *M. citrifolia* extract supplemented fish diets on *E. tarda* infection of tilapia were performed using groups of 20 fish with diets 1 to 4 separately for 3 weeks. Subsequently, fish were infected with *E. tarda* by peritoneal injection of 100 µl of bacterial suspension, at a dose causing 50% mortality (LD_{50}). The infected fish were reared for further 0 days with the assigned test fish diets. The mortality in each group was recorded daily. Dead fish were removed from the aquaria daily and their livers and kidneys were subjected to bacterial isolation on TSA. After incubation at 25°C for 24 h, the bacteria grown on the agar were identified by using PCR as described earlier. The experiments were conducted in duplicate.

Statistical analysis: The data (mean±standard deviation, SD) were analyzed by One way Analysis of Variance (ANOVA) followed by Tukey's test used to compare the means between individual treatments in SPSS at $P < 0.05$ level.

Results

Phagocytic activity: The effect of fish diets supplemented with different concentration of *M. citrifolia* aqueous extract on phagocytic activity of isolated leucocytes in tilapia is shown in figure 1. No change in phagocytic activity was observed in the control (Diet 1) group during the whole experiment. For the groups fed with diets 2, 3 and 4, there was no change in phagocytic activity for 2 weeks. At week 3, a dose dependent increase in phagocytic activity was observed.

The activity increased as the concentration of *M. citrifolia* aqueous extract was increased from 0% to 2%. No significant difference in the activity between week 3 and week 4 was observed in all the treatment groups.

Lysozyme activity: The effect of fish diets supplemented with different concentration of *M. citrifolia* aqueous extract on lysozyme activity in plasma of tilapia is shown in figure 2. No change in lysozyme activity was observed in the control (Diet 1) and diet 2 groups throughout the experiment. Furthermore, there was no significant difference in the activity between both groups during 4 week-observation period. For the groups fed with diets 3 and 4, the first increase in lysozyme activity was detected at week 3; after that, there was no further change in the activity in both groups. Throughout the experiment, no significant difference in lysozyme activity was observed between fish fed with diets 3 and 4.

Respiratory burst activity: The effect of fish diets supplemented with different concentration of *M. citrifolia* aqueous extract on respiratory burst activity of isolated leucocytes in tilapia is shown in figure 3. No change in respiratory burst activity was observed in the control (Diet 1) and diet 2 groups throughout the experiment. For the groups fed with diets 3 and 4, the first increase in respiratory burst activity was detected at week 3; after that, there was no further change in the activity in both the groups. However, the levels of superoxide anion (O_2^-) produced by tilapia fed with diets 3 and 4 were found to be significantly different at weeks 3 and 4.

Determination of median lethal dose (LD_{50}): The cumulative mortality of tilapia was observed for 10 days after they were intraperitoneally infected with different doses of *E. tarda* and the results are shown in figure 4. All deaths occurred within 5 d after bacterial infection. The pathogen was found in the livers and kidneys of all dead fish. Based on the mortality, the calculated LD_{50} of *E. tarda* for tilapia was $10^{-3.1}$, this being equivalent to 7.94×10^2 cfu/g of fish.

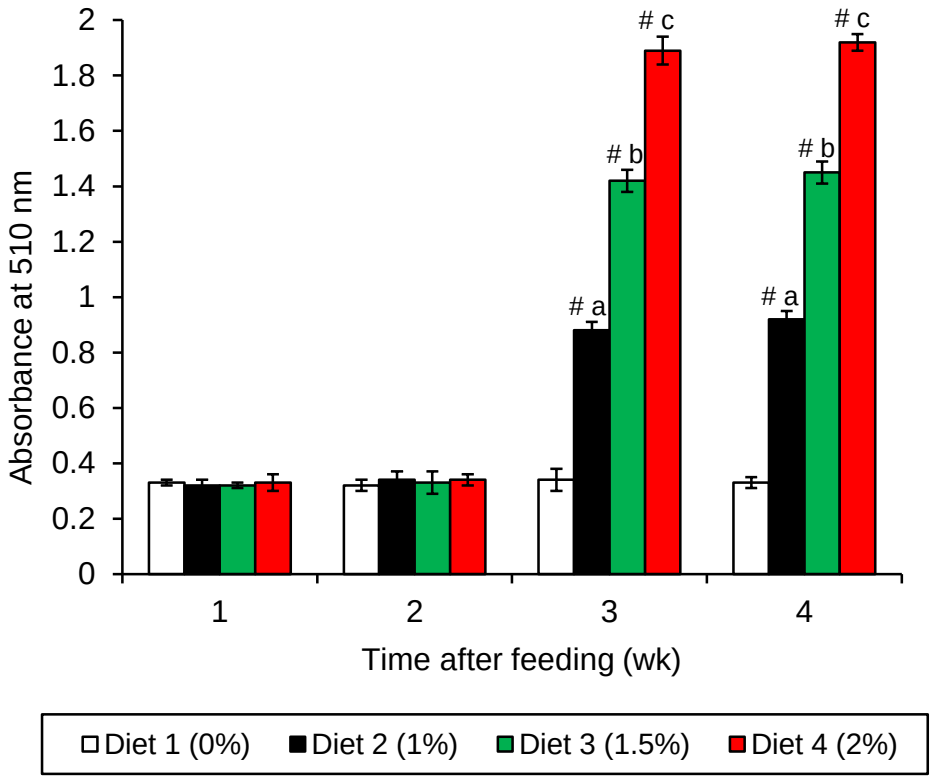

Figure 1: Phagocytic activity of isolated leucocytes in tilapia fed with diets containing different concentration of *M. citrifolia* extract. Values are mean ± SE, n = 5 fish per group. Significant differences ($P < 0.05$) from the control (Diet 1) are indicated by #. Significant differences ($P < 0.05$) among treatment groups are indicated by different letters (a, b and c).

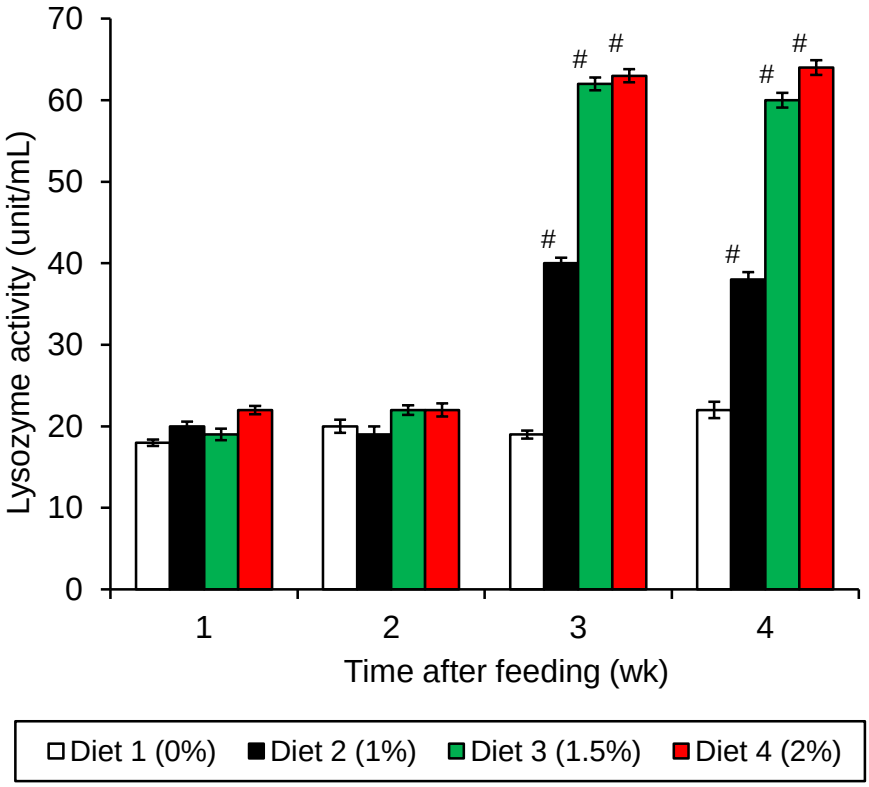

Figure 2: Plasma lysozyme activity in tilapia fed diets containing different concentration of *M. citrifolia* extract. Values are mean ± SE, n = 5 fish per group. Significant differences ($P < 0.05$) from the control (Diet 1) are indicated by #. Significant differences ($P < 0.05$) among treatment groups are indicated by different letters (a and b).

Disease resistance: The effect resulting from the oral administration of the *M. citrifolia* extract supplemented fish diets on the mortality of fish experimentally infected with *E. tarda* is shown in figure 5. The fish diets were given to the fish 3 weeks prior to bacterial infection. After the infection, the diets were given to the infected fish for 10 more days and, during this time, the mortality of fish was observed. In the group receiving the control diet with no *M. citrifolia* extract (Diet 1), the cumulative mortality of the infected fish was 52%. A dose dependent reduction in mortality of the infected fish was obtained when the fish were fed with the diets containing *M. citrifolia* extract. No mortality was apparent in the fish receiving the fish diet supplemented with 2% (w/w) of *M. citrifolia* extract (Diet 4). *E. tarda* was isolated from all of the dead fish, but not from the survivors.

Discussion

The immune system of vertebrates involves both innate (or nonspecific) and acquired (or adaptive) immune responses. The acquire immune response is mediated by antibodies and requires time to develop after exposure to pathogens. On the other hand, the innate immune response is independent of antibodies and constitutes the first line of defense against invading pathogens because it is rapid and requires no prior exposure to pathogens[33]. Because of the intrinsic inefficiency of the acquired immune system due to the evolutionary status, fish primarily rely on the innate immune system in protection against diseases[10,18]. Therefore, it has been widely accepted that improving the innate immunity of fish can help in increasing their ability to resist to diseases.

Many immunostimulants have been successfully used to enhance innate immunity and disease resistance in fish such as aloe vera[17], *Radix astragalin seu Hedysari* and *Radix angelicae sinensis*[15], *Tinospora cordifolia*[32], *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa*[11], *Astragalus radix* and *Ganoderma lucidum*[37]. These positive results have encouraged us to examine the effect of fish diets supplemented with *M. citrifolia* aqueous extract on innate immunity and disease resistance against *E. tarda* in tilapia (*O. niloticus*). Parameters used in this study for the assessment of innate immunity of tilapia were having phagocytic activity and respiratory burst activity of isolated leucocytes and lysozyme activity in plasma.

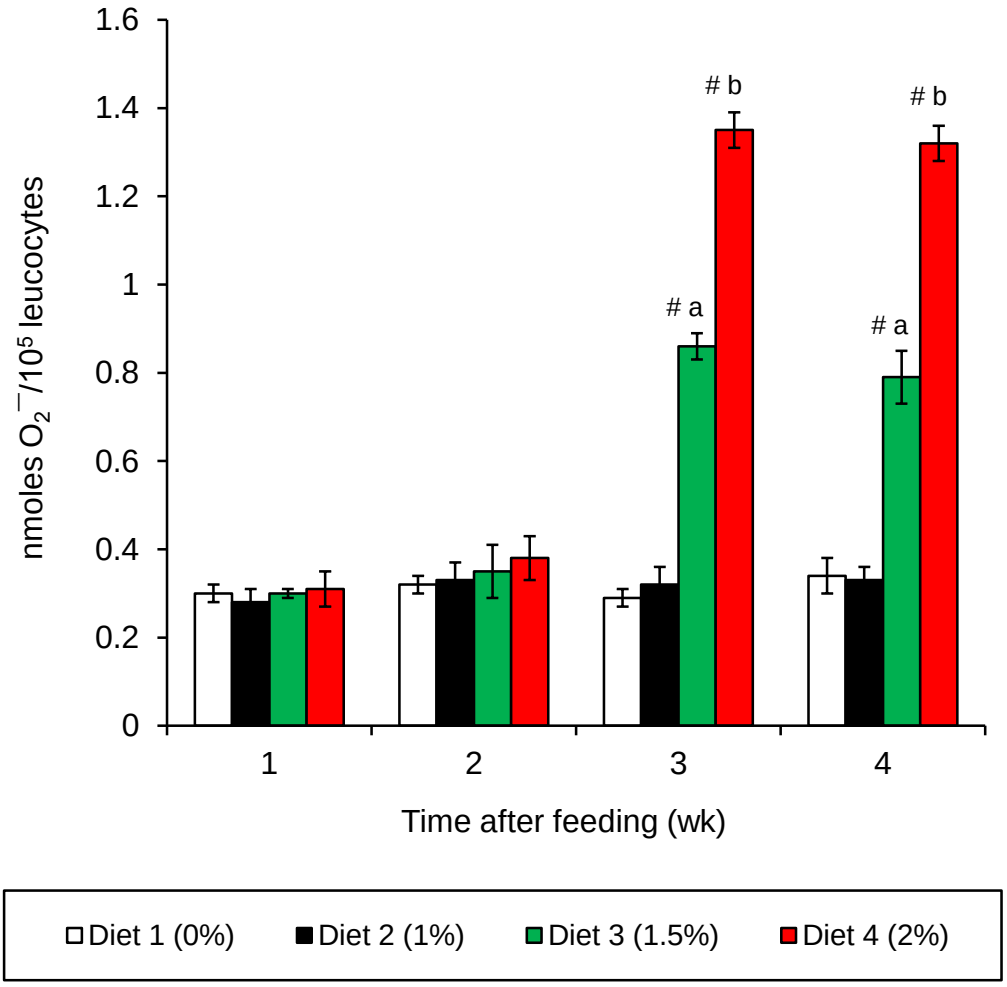

Figure 3: Respiratory burst activity of isolated leucocytes in tilapia fed diets containing different concentration of *M. citrifolia* extract. Values are mean ± SE, n = 5 fish per group. Significant differences ($P < 0.05$) from the control (Diet 1) are indicated by #. Significant differences ($P < 0.05$) among treatment groups are indicated by different letters (a and b).

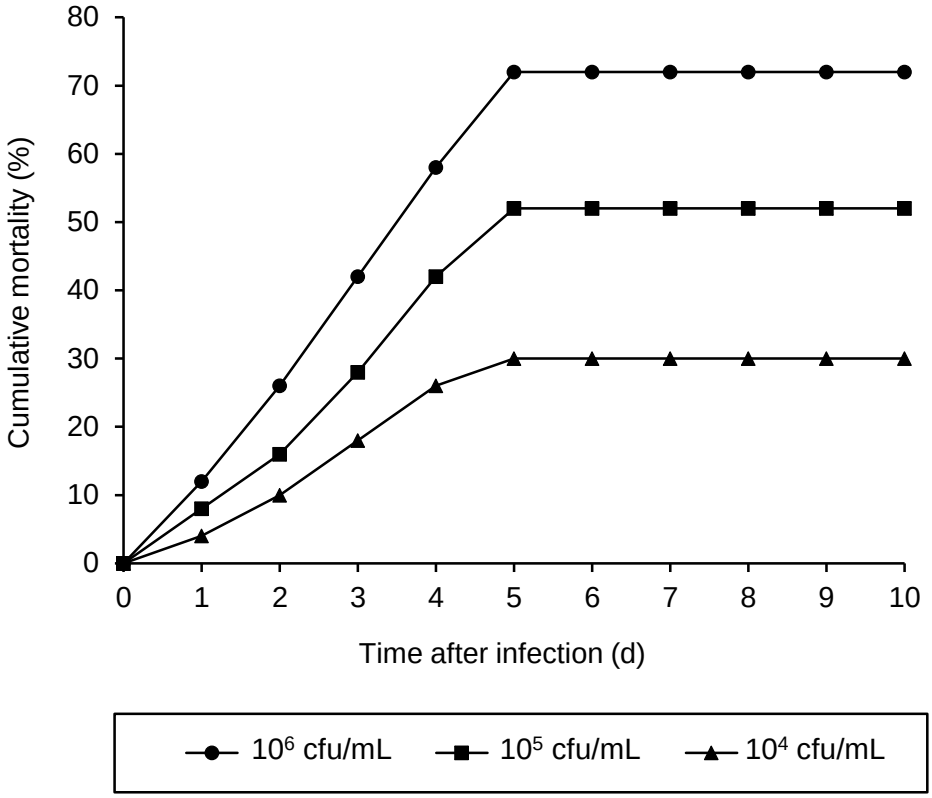

Figure 4: Cumulative mortality after experimental infection with different concentration of *E. tarda* in tilapia fed with the commercial fish diet without any supplement.

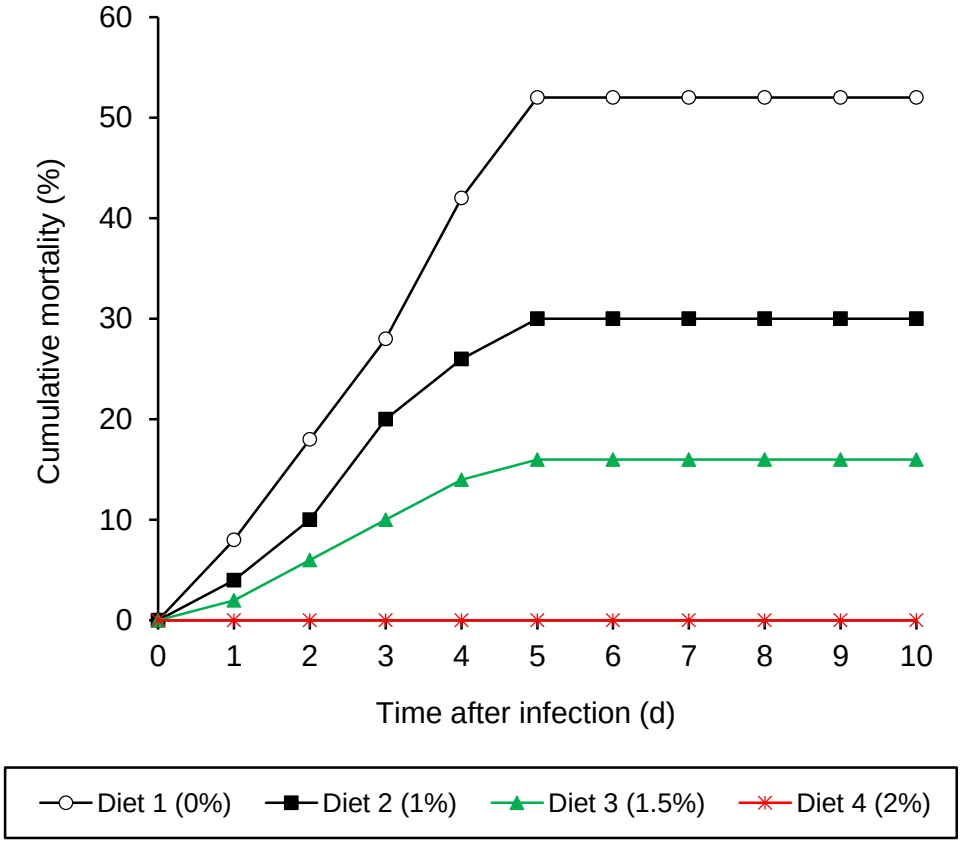

Figure 5: Cumulative mortality after experimental infection with *E. tarda* in tilapia fed with diets containing different concentration of *M. citrifolia* extract.

Time required for immunostimulants to increase innate immunity in fish has been found to be dependent on how they are given to fish. The administration through injection needs less time than the oral administration for immunostimulants to enhance the innate immunity, generally, 2 to 7 days for the injection method[2,7] and 20 to 30 days for the feeding method[9,15,16]. However, the oral administration, as used in this study, is more practical than the administration through injection. Furthermore, it is considered to be noninvasive and produces no stress on fish.

The effect of fish diets supplemented with different concentrations (0%, 1%, 1.5% and 2%) of *M. citrifolia* aqueous extract on phagocytic activity of isolated leucocytes in tilapia was found to be dose dependent. At week 3 and week 4 after feeding, the phagocytic activity increased as the concentration of *M. citrifolia* aqueous extract increased from 0% to 2%. However, this is not the case for the effect of *M. citrifolia* aqueous extract on plasma lysozyme activity and respiratory burst activity of isolated leucocytes observed in this study. Although the increase of concentration of *M. citrifolia* aqueous extract supplemented in the fish diet from 0% (Diet 1) to 1% (Diet 2) and from 1% (Diet 2) to 1.5% (Diet 3) enhanced the lysozyme activity at week 3 and week 4, no significant difference in the lysozyme activity between fish fed with diet 3 (1.5%) and diet 4 (2%) was observed during the same observation period.

For the respiratory burst activity, when the concentration of *M. citrifolia* aqueous extract was increased from 1% (Diet 2) to 1.5% (Diet 3) and from 1.5% (Diet 3) to 2% (Diet 4), significant increase in the number of superoxide anion (O_2^-) in tilapia was observed at week 3 and week 4. However, during the same observation period, no significant increase in the number of superoxide anion was observed between fish fed with the diets supplemented with 0% (Diet 1) and 1% (Diet 2) of *M. citrifolia* aqueous extract. No positive correlation between the effect of immunostimulants on innate immunity and dosage was also found in several previous studies. In some cases the innate immunity of experimental fish did not improve with the increase of the dosage of immunestimuants[9,11,15,16]. Jian and Wu[15] reported that diets supplemented with 1 % and 1.5% of traditional Chinese medicine significantly increased lysozyme activity and complement activity of large yellow croaker (*Pseudosciaena crocea*) after feeding for 20, 25 and 30 days, but no significant differences in both the activities were observed between the two groups. Futhermore, in other cases, high dosage of immunostimulants might inhibit the immune response[17]. Kim et al[17] reported that the chemiluminescent response of head kidney leucocytes from rockfish (*Sebastes schlegeli*), given the diet supplemented with 0.1% of aloe was significantly higher than that of the fish given the diet containing 0.5% of aloe.

The use of immunostimulants for disease resistance in fish as prophylactic agents is more preferable than their use as therapeutic agents because high numbers of mortality might be avoided if they are applied before outbreaks of diseases. The present study investigated the possibility of using aqueous extract of *M. citrifolia* as an immunoprophylactic agent to control *E. tarda* infection in tilapia. In this work, the minimal time required for the phagocytic, lysozyme and respiratory burst activities in tilapia fed with the diets supplemented with aqueous extract of *M. citrifolia* to increase to the maximal level was 3 weeks, the fish were therefore fed on the diets for 3 weeks prior to disease resistance experiment. The dose dependent reduction of cumulative mortality of tilapia experimentally challenged with *E. tarda* was observed.

The cumulative mortalities of bacterial infected tilapia fed with fish diets containing 0.5% (Diet 2), 1% (Diet 3) and 1.5% (Diet 4) of aqueous extract of *M. citrifolia* were 30%, 16% and 0% respectively, compared to 52% in the control group. However, there are several studied showing a negative correlation between concentration of immunostimulant and mortality.

Kim et al[17] reported that rockfish (*S. schlegeli*) fed with a diet supplemented with 0.5 % of aloe showed lower moratlity than the fish fed with a diet supplemeted with 1% of aloe. Wahli et al[34] also found that high dose of vitamic C resulted in lower mortality of rainbow trout (*Oncorhynchus mykiss*) infected with viral hemorrhage septicemia virus. These results suggest that aqueous extract of *M. citrifolia* has prophylactic potential against *E. tarda* infection.

Conclusion

M. citrifolia leaf aqueous extract was examined in this study for its effect on innate immune response and disease resistance in tilapia. Four fish diets containing different amounts (0, 1%, 1.5% and 2%) of the extract were given to tilapia for 4 weeks. The effect of the diets on innate immune response of the fish was examined every week after feeding the diets by determining phagocytic activity, lysozyme activity and respiratory burst activity.

The groups received the fish diets containing 1.5% and 2% of the extract and showed the significant increase in innate immune response 3 weeks after feeding. Furthermore, the fish diet containing 2% of the extract resulted in no mortality rate of *E. tarda* infected tilapia. These results suggest that the aqueous extract of *M. citrifolia* has a potential to be used as an immunostimulant to prevent fish diseases.

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