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Psidium Guajava Leaf Extracts Fed to Mono-sex Nile Tilapia *Oreochromis Niloticus* Enhance Immune Response Against *Pseudomonas fluorescens*

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Abstract: Plants are being explored as a major source of medicinal compounds. The present study was intended to explore the efficacy of dietary doses of *Psidium guajava* leaf extracts mixed with Maize bran (control) on growth performance, immunological parameters and disease resistance against *Pseudomonas fluorescens* infection in mono-sex Nile tilapia, *Oreochromis niloticus*. Fishes (average weight 16.5±1.5) were fed five different doses of herbal diets for four weeks which prepared with 0%, 2%, 4%, 6% and 8% of *P. guajava* leaf extracts where maize bran used as control (0%). Growth performance and immunological parameters including bacterial activity and phagocyt activity were investigated. Among those doses 8% showed highest significant responses in phagocytic activity, specific growth rate, specific and non-specific immune responses on week 2 and 4 compared to control diet whereas the changes did not manifest on first week. Further the *P. guajava* leaf extracts enriched diet at 8% level resulted in lowest mortality (20%) indicating highest protection (Relative Percent Survival 76.5%) from *P. fluorescens* infection than 2%, 4% and 6% doses diets that resulted 60%, 45% and 25% mortalities respectively. Eight percent (8%) dose also showed highest growth (36g) compare to other doses. The results are suggested that the dietary supplementation of *P. guajava* leaf extracts act as immunostimulants, reduce mortality and disease resistance in *O. niloticus* against *P. fluorescens*.

Keywords: *Psidium Guajava,* Mono-sex Nile Tilapia (*Oreochromis Niloticus*), *Pseudomonas Fluorescens*, Immune Response (Specific and Non-specific)

1. Introduction

Bangladesh is rich in various culture practice of aquaculture resource that are completed by lots of river, ponds, lakes, haors, baors and so on. Aquaculture, probably the faster growing food producing sector of the world and plays an important role in the socioeconomic development of many countries in view of its potential contribution to national income, nutritional security, social objectives and sustainable large export earnings FAO [1]. Tilapia fish was considered as one of the most important fish of all aquaculture in 21st century Fitxzsimmons [2]; Sawhney and Johal [3]. It was first introduced in Bangladesh by ICLARM (International Center for Living Aquatic Resources Management) and BFRI (Bangladesh Fisheries Research Institute) in 1994 from Thailand Hussain *et al.,* [4]. Over last few decades, it has become one of the dominant species of fisheries sector in many Asian countries including China, Thailand, Vietnam, Indonesia, Malaysia, Philippine, Bangladesh and Sri Lanka due to its rapid growth rate, high market demand and increasing consumer acceptance Chowdhury *et al.,* [5]. Tilapias are arguably ideal candidates for aquaculture because of their tolerance of wide pH

fluctuations Reite *et al.,* [6]; George [7] extreme temperatures Li Kuang Cong *et al.,* [8]; Denzer [9], high ammonia and nitrite levels Redner and Stickney [10] and low dissolved oxygen levels Maruyama [11]; Lowe-McConnell [12]. Some species can even be cultured at high salinities Chervinski and Hering, [13]; Watanabe *et al.,* [14]. Under the original extensive or semi-intensive culture systems, Tilapias were more resistant to disease than many other fish species Roberts and Sommerville [15]. Approximately 15% of the total of freshwater aquaculture production has been estimated to be lost every year in Bangladesh due to disease infection. Among all other bacteria, *Aeromonas* and *Pseudomonas* are the major bacteria fish pathogens which widely distributed in aquatic organisms in nature Banu and Islam *et al.,* [16]. The condition of aquatic environment undoubtedly important for persistence of bacterial pathogen. Any water which supports long term survival may contribute to an easy outbreaks of disease Chowdhury and Wakabayashi *et al.,* [17]. In Bangladesh, scientific information about bacterial disease in fish are in preliminary stage while it is scare in the field of *Pseudomonas fluorescens* which has been denoted as one of important disease causing agents of both farmed and wild fish *P. fluorescens* were originally described as the causative agent of Bacterial Hemorrhagic Septicemia disease of pond cultured fish Foysal *et al.,* [18]. Certain *Pseudomonas* species may produce additional types of siderophore, such as pyocyanin by *Pseudomonas aeruginosa* Lau, *et al.,* [19] and thioquinolobactin by *Pseudomonas fluorescens* Matthijs *et al.,* [20]. The use of herbal compounds as immunostimulants has been increasing rapidly in aquaculture to avoid the indiscriminate use of hazardous antibiotics. Some herbs are reported to have anti-microbial activity against several pathogenic bacteria and have used as traditional medicines for the treatment of human disease Recently, they have been used to control disease in animals, including fowl Mtambo *et al.,* [21]; calves Kromna *et al.,* [22] and tilapia Abutbul *et al.,* [23] and Yin *et al.,* [24]. Guava (*Psidium guajava*) belonging to family Myrtaceae is a traditionally used plant because of its food and nutrition value. Guava is widely grown in tropical and many areas like Bangladesh, India, Florida, and West Indies. Different parts of the *Psidium guajava* are reported to be used in folk medicine. Various parts of the plant like root, bark, leaves and fruits are found to possess many pharmacological properties as it is used in the treatment of various disorder Begum *et al.,* [25]. Leaf extract of guava has been reported for their antibacterial activity because of the presence of flavonoid glycosides, morin-3-O-alpha-L-lyxopyranoside and morin-3-Oalpha-L-arabopyranoside Arima *et al.,* [26]. Variety of feed ingredients are available in Bangladesh. A major portion of this ingredients are obtained from different plants. Different parts of plant body are used as fish feed. Plant origin feed ingredients are comparatively cheaper than animal origin fish feed ingredients like fish meal, bone meal etc. In the present study effort was made to study to achieve the immune response (specific and non-specific) and the control measure of *P. fluorescens* in mono-sex Nile tilapia against *P. fluorescens* infection by using *P. guajava* leaf extracts.

2. Materials and Methods

2.1. Fish and Husbandry

Apparently healthy three hundred (300) pieces of mono-sex Nile tilapia, *Oreochromis niloticus* (weight 16.5±1.5g, N=300) was obtained from Mayer Dua Hatchery at Chachra, Jessore, Bangladesh and were transported by 2 oxygenated polythene bag to the laboratory of the Dept. of Fisheries and Marine Bioscience (FMB), Jessore University of Science and Technology (JUST), Jessore, on January to June 2016. The health status of the fish was checked upon arrival and the fish were immediately treated with 100 mg L^{-1} formalin for 20 min. Fishes were acclimatized in the indoor aquarium (100 L) with recirculating aerated water for 1 day. Continuous aeration was provided to maintain dissolved oxygen level at 7.5±0.5mgl-1 and two third of the aquarium water was exchanged daily. During the experimental period water temperature, pH and TDS (Total dissolved solid) were 23±0.8°C, 5.94± 0.21 and 434±0.29 mgl^{-1}. At the first day of their arrival no feed was provided. At the end of the acclimation period, the fishes were randomly distributed into 15 aquarium.

2.2. P. Gujava Leaf Extract Preparation and Herbal Diet Preparation

The *Psidium guajava* leaves were collected, cleaned and dried in shade drying at room temperature for 2 weeks. The leaves were grinded in a mechanical grinder. After that the powder sieved by 80 µ diameter pore. Then the powder was stored in sealed plastic container at–20°C until it was used. The herbal powder (100 g) was mixed with 1000 ml of 95% ethanol in a 2000 ml conical flask and stored at room temperature for the next 7 days and during that time it was agitated daily to ensure complete digestion. The extract was filtered through Whatman No. 2 filter paper and the filtrated powder was dried under reduced pressure. The residues obtained after evaporation of ethanol was kept in sterilized screw cap glass container and stored at–20°C until it is use. The experimental diet was prepared by mixing with locally available maize bran diet. At first maize bran was grinded by a grinder and mixed with *P. guajava* leaf extracts. All the ingredients were mixed thoroughly by adding water and pelletized by hand and then sun dried. Five different experimental maize bran diet were prepared which contained different percentages of *Psidium guajava* leaf extracts such as 0%, 2%, 4%, 6% and 8%. The prepared feed was then sun dried under sterile condition for 3-4 days and stored in a plastic air-tight container.

2.3. Pseudomonas Fluorescens Isolation

P. fluorescens strains initially isolated from dropsy and septicemia mono-sex Nile tilapia which were in this study. The disease fish were collected from the pond under the department of Fisheries and Marine Bioscience, JUST during

the winter season. Those strains since their isolation were being maintained in laboratory, by repeated culture in selective agar media (*Pseudomonas* agar media). Stocks were grown in *Pseudomonas* agar media for 24 hrs at 37°C and kept in–20°C until use. The subculture was taken and centrifuged (5000 rpm for 12 min), after centrifugation the supernatant was discarded and the pellet was re-suspended in sterile phosphate buffer saline (PBS). The culture was adjusted at 3.0×10^{-6} colony forming units (CFU) ml^{-1} and incubated at 37°C for 24 hrs.

2.4. Experimental Design

The experiment was performed in 100L rectangular glass aquarium in the laboratory. Fishes were divided into five groups-four treatments or experimental groups (2%, 4%, 6%, 8%) and control group (0%) representing three replicates and maintained 18 aquarium each containing 20 fish and one control group (0%) were placed in the laboratory under the Dept. of Fisheries and Marine Bioscience (FMB), Jessore University of Science and Technology (JUST). At the end of the acclimation period, fishes were fed with experimental diets at a rate of 3% of their body weight twice a day at 10am and 5pm for 4 weeks. The respective diets were continued at the end of the experiment. On the week of 1, 2 and 4 three fishes were selected randomly from each experimental aquarium to collect blood and mucus for specific and non-specific immunological assays. On 28th day of feeding, all groups were injected intraperitoneally (i.p.) with 25µl PBS containing *P. fluorescence* at 3.0×10^{-7} CFU ml-1 for analyzing cumulative mortality.

2.5. Bleeding and Serum Separation (Specific Immune Response Assay)

Three fishes were selected randomly from each group for blood collection. Blood was collected from caudal vein of the fishes from five groups separately with the help of sterilized hypodermal syringes containing EDTA (Ethylene-Diamine-Tetra-Acetic Acid) as an anticoagulant and collected blood was kept in 1ml Eppendorf. For separating the serum from the blood the eppendorfs with blood sample were placed into a centrifuge machine at 5000 rpm for 10 min and the serum collected Azizoglu and Cengizler [27]; Blaxhall and Daisley [28]; Dacie and Lewis [29]. For each group (0%, 2%, 4%, 6%, 8%) three culture plates were prepared. Bacterial stock solution was serial diluted for 10 times and 10^{-3},10^{-4},10^{-5} concentration were selected for further usage. Then 25 μl separated serum of five groups of fishes then separated in different culture plates and finally all plates were placed in a incubator at 37°C for 24 hrs. After 24 hrs all plates were observed.

2.6. Mucus Collection and Bacterial Culture (Non-specific Immune Response Assay)

Mucus was collected by scraping the body surface and gill of fishes with a scalpel from five groups (0%, 2%, 4%, 6%, 8%) and collected mucus was kept in five eppendorf separately. Same like serum and bacteria culture, three culture plates for each group were prepared as followed by disc diffusion method. 25µl mucus from each group was mixed with same volume of three different bacterial solutions (10^{-3},10^{-4},10^{-5}) and finally all plates were placed in a incubator at 37°C for 24 hrs. After 24 hrs all plates were observed.

2.7. Immune Response Assay (Phagocytic Activity)

The phagocytic activity were quantified by following the modified method of Swan *et al.,* [30]. For this assay 24µl blood cell suspension of mono-sex Nile tilapia and 25µl bacterial solution in PBS was previously fixed with glutaraldehyde was placed on a coverslip. After 30 min coverslip was carefully washed with PBS then air dried and fixed with methanol and after that stained with giemsa. The engulfed fish blood cell (phagocytic rate) was determined by using phagocytic microscope (Axiocam ERc 5s with Axio Vision driver Carl Zeiss, Germany)

2.8. Serum Agglutination Titer Assay

At day 14 and 28 of the experiment, blood samples were collected from each group of fish. Serum samples were collected by following centrifugation. Isolated bacterial cell suspension was centrifuged in 5000 rpm for 15min and supernatant was discarded. The resulting pellet was washed twice with PBS solution and then pellet was re-suspended in PBS. Starting with a dilution of 1:10 (10 µl serum and 90 µl PBS) two fold serial serum dilutions were made in 96-well round bottom micro titer plates by adding 25 µl of diluted serum into the remaining wells plate with 25 µl of bacterial cell suspension was added to each well. The plates were covered with plastic film and incubated at 4°C for 2 hrs and 24 hrs incubated at 25°C. Result of agglutination titer was determined by using multi-scanner.

2.9. Challenge Test

For the challenge test virulent *P. fluorescens* strain were prepared from maintaining the serial dilution. Two days after the last bleeding, the fishes from each group were injected intraperitoneally (i.p.) with 1ml of 24 hrs cultured *P. fluorescens* which contained 3.0×10^{-7} CFU ml^{-1} challenge strain. The clinical signs and mortality was recorded upto 28 days of post challenge. The cumulative mortality was calculated by following Amend [31] and Relative Percent Survival (RPS) was calculated as follows

$$\text{RPS}=1-\frac{(\%\ Mortality\ in\ treated\ group)}{(\%\ Mortality\ of\ control\ group)}\times 100$$

2.10. Statistical Analysis

Values for each parameter measured were expressed as the arithmetic mean ± standard error (SE). Effects of *Psidium guajava* leaf extracts diets on growth performance, Immunological parameters were tested using one-way ANOVA and the mean values were compared by using Duncan's multiple range tests at 5% level of significance Zar [32].

3. Results and Discussions

P. guajava tree has a long history of medicinal uses that are still employed today Nwinyi *et al.,* [33]. However lot of pharmacological activity is attributed due to the presence of flavonoids, leutin, zeaxanthine and lycopene. The flavonoids have demonstrated to possess antibacterial activity. The active flavonoid compound quercetine-3-O-alpha-l-arabinopyranoside has been reported for the anti-plague activity Limsong *et al.,* [34].

3.1. Disease Resistance (Challenge Test)

The cumulative mortality was lowest 20% when fed 8% supplemented diet compared with control (85%) and other doses diets, which were 25%, 45% and 60% in case of 6%, 4% and 2% supplemented diets respectively for 28 days against *P. fluorescens*. In this study challenge with *P. guajava* leaf extracts against *P. fluorescens* of mono-sex Nile tilapia have showed 80% survivability and 76.5% RPS with 8% supplemented diets for four weeks which was higher than other treatments (Table 1). In this present study showed decrease in mortality rate with *P. guajava* leaf extracts added diet after injection of *P. fluorescens* was similar in *O. mossambicus* fed with diet containing *Ocimum sanctum* Logambel *et al.,* [35], *L. rohita* fed the diet containing *Achyranthes aspera* Hasan-Uj-Jaman *et al.,* [36], *O. mossambicus* treated with *Eclipta alba* leaf extracts Christybaptin *et al.,* [37] against *A. hydrophila* infection. *A. hydrophila* infected rainbow trout showed a similar result which was reported when fed with *A. sativum*, *L. perennis*, *M. indic*a and *U. dioica* Awad and Austin [38].

Table 1. *Treatment challenge of P. gujava leaf extracts against P. fluorescens in mono-sex nile tilapia at 28th day of the experiment.*

Treatment	Challenge dose (cfu)ml^{-1}	Total Fish	No. of infected fish	No. of death fish	Mortality (%)	Survivability (%)	RPS (%)
(0%)	3.0×10^{-7}	20	18	17	85	15	-
2%	3.0×10^{-7}	20	16	12	60	40	29.4
4%	3.0×10^{-7}	20	13	09	45	55	47.1
6%	3.0×10^{-7}	20	09	05	25	75	70.6
8%	3.0×10^{-7}	20	06	04	20	80	76.5

*RPS= Relative Percent Survival

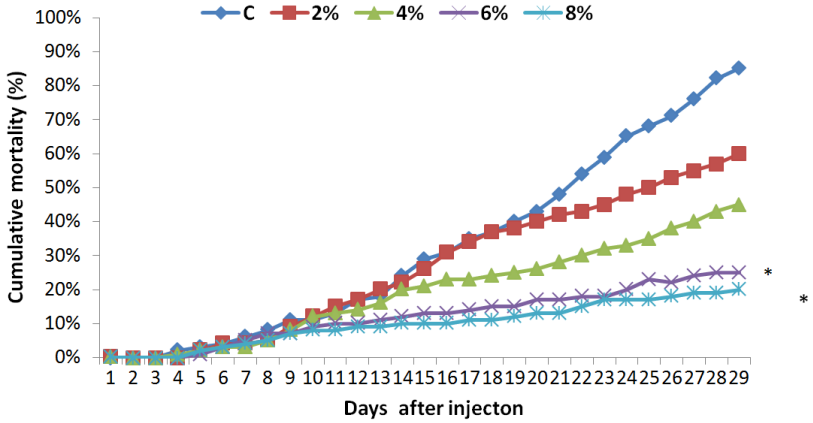

Figure 1. *The cumulative mortalities of mono-sex Nile tilapia fed Maize bran diets containing P. guajava leaf extracts against P. fluorescens. [* indicates relatively significant (P<0.05)].*

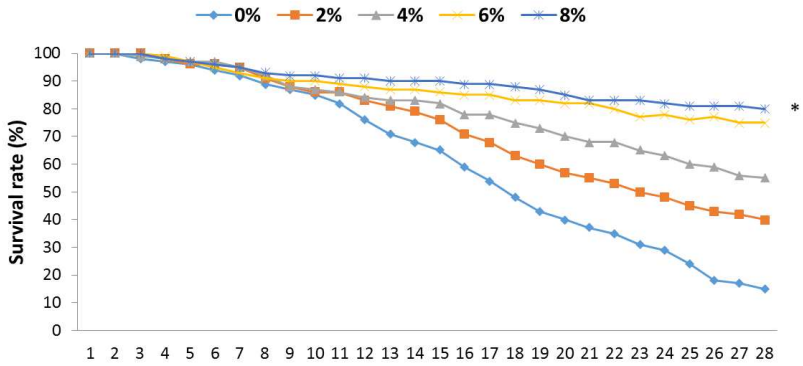

Figure 2. *Survival rate of mono-sex Nile tilapia fed maize bran diet containing different doses of P. guajava leaf extracts against P. fluorescens. [* indicates relatively significant (P<0.05)].*

3.2. Serum Agglutination Titer Assay

Measurement of serum agglutination titer assay fish diets continues 28 days. After 4 weeks of feeding, fish were immunized, spleen and blood were sampled on weekly intervals for three times after immunization. In this period study showed the highest diluted serum (409600) showed positive agglutination (6±1; 3±2) treated by 8% than the control (20±2; 20±1) which was similar to Alam *et al.,* [39]. Similarly, grouper (*E. tauvina*) juveniles fed diets containing highest dose of *Ocimum sanctum* and *Withania somnifera* herb extracts showed a significant increase of their bactericidal activity of serum Sivaram *et al.,* [40].

Serum agglutination titer assay (Table 2) was done on 14th day and 28th day of the experimental period. 2.0% *P. guajava* added diet with maize bran fed fishes and highest diluted serum (409600) showed positive agglutination (6±1; 3+2) response (Fig 3)

Table 2. *Different immune parameters of mono-sex Nile tilapia at 14th and 28th days of the experiment.*

Immune parameter	Control		2%		4%		6%		8%	
Serum agglutination	14 days 20±2	28 days 20±1	14 days 18±1	28 days 15±3	14 days 15±2	28 days 12±1	14 days 10±1	28 days 9±3	14 days 6±1	28 Days 3±2

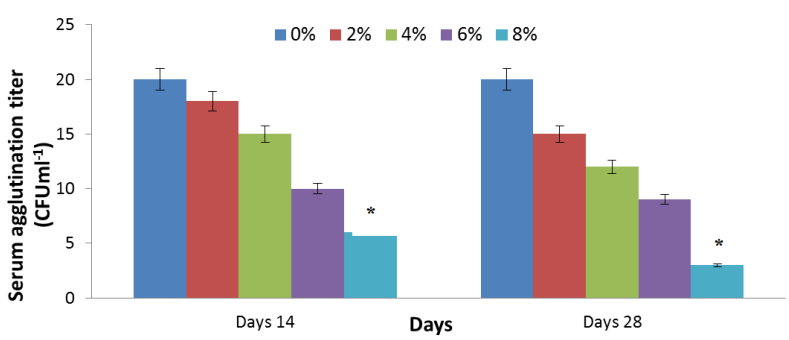

Figure 3. *Serum agglutination titer assay of mono-sex nile tilapia fed with different doses of P. guajava leaf extracts supplemented diets against P. fluorescens. [* indicates relatively significant (P<0.05)].*

3.3. Phagocytic Activity

The phagocytic activity was significantly (P<0.05) higher in test group of fish than the control group on weeks two and four. Phagocytic activity did not significantly enhance with 2%, 4%, 6% the activity significantly increased on week 2 and 4 (Fig 4) but not with 2% and 4% doses of supplemented diet, as compared with the control group. The present findings are in line with the report in grouper *E. bruneus* fed with the diet containing *L. indica* Harikrishnan *et al.,* [41], *O. niloticus* fed with Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) containing diet Yin *et al.,* [42], marine ornamental fish *Amphiprion sebae* with diet containing *Excoecaria agallocha* Dhayanithi *et al.,* [43], *Cyprinus carpio* fed with *Eucalyptus sp* and *Plelargonium roseum* herbs containing diet Mohamadi *et al.,* [44], *O. niloticus* fed with *Nigella sativa* and *Bacillus subtilis* herbs containing diet Elkamel *et al.,* [45]. An increase in phagocytic activity indicated the significant role of *P. guajava* leaf extracts enhancing the immune response. Similar findings has been reported in *O. mossambicus* fed with *Solanum trilobactum* leaf extracts Divyagnaneswari *et al.,* [46] and *Cyprinus carpio* fed with *Nigella sativa* seed extract against *Pseudomonas fluorescens* Khondoker *et al.,* [47].

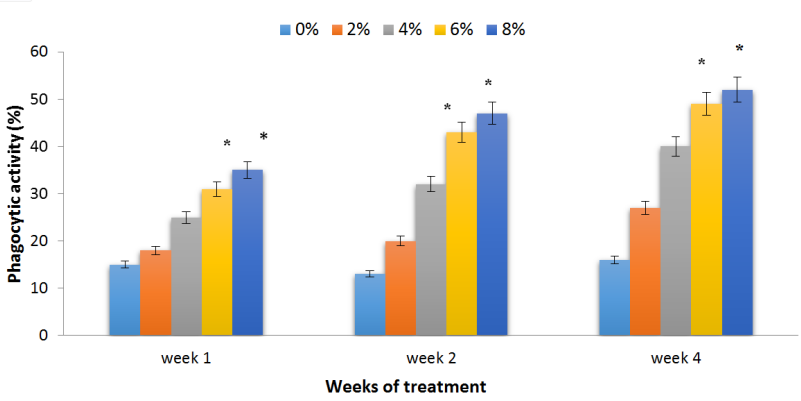

Figure 4. *Phagocytic activity of O. niloticus fed with different doses of P. guajava leaf extracts with maize bran against P. fluorescens. [* indicates relatively significants (P<0.05)].*

3.4. Specific Immune Response Assay (Serum, Bacteria Culture)

Fishes feeding with different doses of *P. guajava* (2%, 4%, 6% and 8%) did not significantly change immune response on first week. Immune response level significantly increase with 6% and 2% supplemented diets on week 2 and 4 (Fig 5). However immune response level did not significantly change in control group. The efficiency of antigen clearance was also enhanced in *Catla catla* treated with *Achyranthes aspera* Chakrabarti *et al.,* [48]. Hemagglutination antibody titers were significantly higher in the test group of fishes compared with the control group Rao *et al.,* [49]. Bactericidal activity of serum was significantly increased in 6% and 8% groups on week 2 and 4 compared to control group.

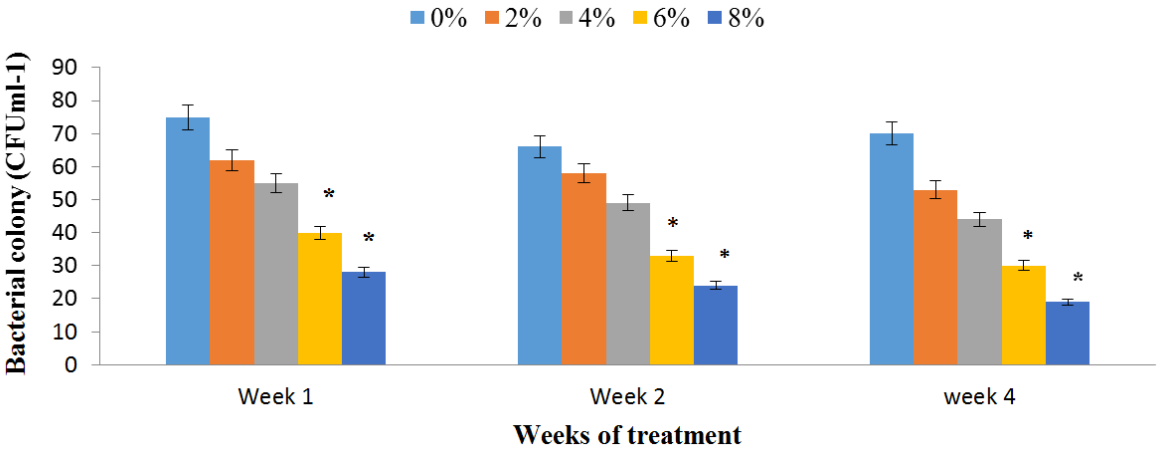

Figure 5. *Bacterial activity of serum of mono-sex Nile tilapia fed diets supplemented with 0%, 2%, 4%, 6% and 8% of P. guajava leaf extracts against P. fluorescens [* indicates relatively significant (P<0.05)].*

3.5. Non-specific Immune Response (Mucus, Bacteria Culture)

Fish feeding with 2%, 4%, 6% and 8% *P. guajava* leaf extracts enriched diet did not significantly enhance the immune response at 7th day in mono-sex Nile tilapia against *P. flurescens* compared to control diet (0%). However, fish fed with 6% and 8% *P. guajava* enriched diets, the non-specific immune response significantly enhanced (Fig 6) from week 1 to 4 compared to the control group. Same like as bactericidal activity of serum the bactericidal activity of mucus also showed efficient result. Fishes fed with 6% and 8% *P. guajava* leaf extracts added diet dramatically enhanced non-specific immune response Hobert *et al.,* [50].

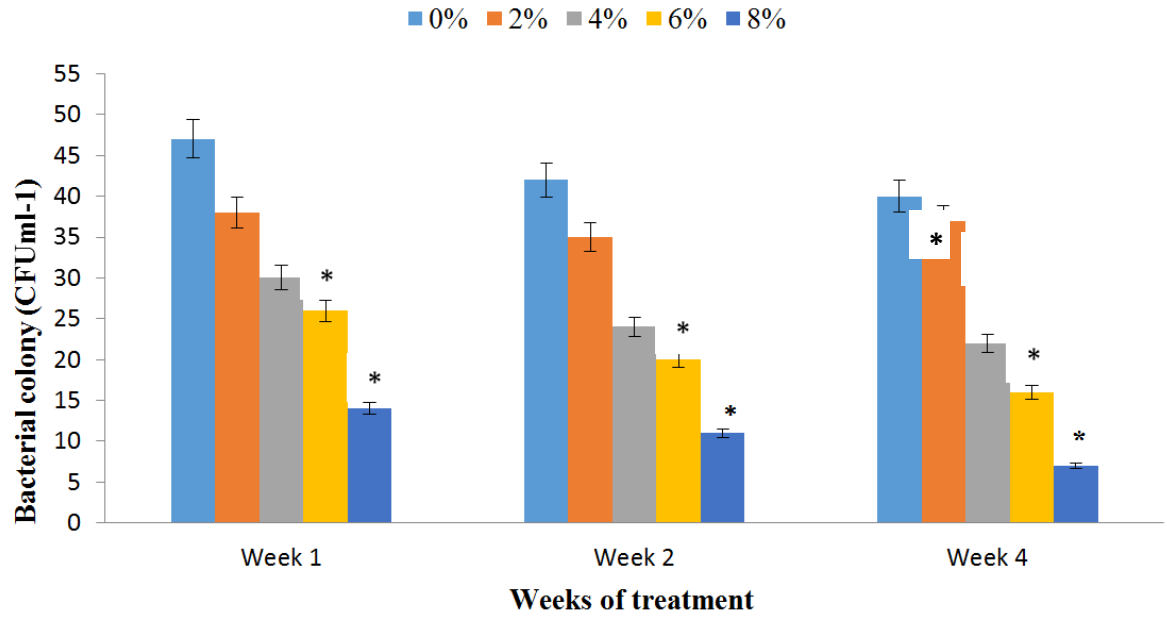

Figure 6. *Bactericidal activity of mucus of mono-sex nile tilapia fed with 0%, 2%, 4%, 6% and 8% P. guajava leaf extracts supplemented diets against P. fluorescens. [* indicates relatively significant (P<0.05)].*

4. Conclusion

The present study has helped in demonstrating the potential bioactive compound of natural plant extracts that are eco-friendly, economical and available in bulk to the farmers with easy preparation protocols. Results of this experiment suggest the protective ability of *Psidium guajava* leaf extracts through specific and non-specific immune responses, as evidence the enhanced hematological and immunological parameters such as, total serum protein, phagocytic activity, bactericidal activity, growth and survival rate against *Pseudomonas fluorescens* and no toxic effects were absorbed. The *Psidium guajava* leaf has been shown to contain major antimicrobial compounds which may act as potential immunostimulant. In this whole experiment eight percent (8%) *Psidium guajava* leaf extracts enriched diet showed highest positive response against *Pseudomonas fluorescens* and act as immunostimulants, reduce mortality, growth promoter and disease resistance (survival rate 80%) in *O. niloticus* against *P. fluorescens* infection. The results presented here indicate the potential of using the *Psidium guajava* leaf as an environmental friendly antibiotic for controlling *Pseudomonas fluorescens* infection in mono-sex Nile tilapia and might act directly even in low concentration.

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