Efficacy of using Guava leaves (Psidium Guajava) as nonspecific immune stimulant in Nile tilapia (Oreochromis niloticus)

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ABSTRACT

The aim of this study was to evaluate the effects of different doses {0, 0.5, 1 and 1.5 gm} of Guava (Psidium Guajava) leaves powder as immuno-stimulant on immune response of Oreochromis niloticus and investigate antioxidant activity of Guava in the blood of fish on 30 days. In this study, Oreochromis niloticus were obtained from Central Laboratory for Aquaculture Research (CLAR), Abbassa, Abu-Hammad, Sharkia, Egypt. Fish were transported to a laboratory then the health status of the experimental fish was inspected (with average body weight 52 ± 2 g / fish). All immune parameter (lysozyme, NBT and nitric oxide) were higher on 30 days of feeding of 1.0 gm of guava powder.

In this experiment serum total protein and globulin showed significant increase in the 4th week at groups feed 1% guava powder when compared to control group. Significant decrease of serum albumin and A/G ratio was observed in all treated groups and highest decrease were observed at 4th week in 1%, compared to control. All treatment showed significant decrease of creatinine while first and second week show non-significant increase of urea at 0.5 and 1.5% with significant decrease at 1% Guava supplemented diet while fourth week revealed significant increase at 0.5 and 1.5% with non-significant increase at 1% compared with control.

Generally we can say that antioxidant enzymes (GST, SOD & CAT) and MDA showed significant decrease than control group.

All groups were challenged intraperitoneally injection of 0.2 \((1 \times 10^8 \text{ cells / ml / fish})\) of 24 hours broth culture of virulent Aeromonas hydrophila after four weeks of feeding experiment and mortality rate percent were recorded.
INTRODUCTION

Aquaculture fish production has increased drastically over the past few decades to meet the increasing demand for animal protein food. However, the highly demand for Nile tilapia, Oreochromis niloticus (O. niloticus) in our Egyptian market was stimulating the development of intensive tilapia culture due to its desirable characteristics for aquaculture such as rapid growth, tolerance of wide range of water quality parameters, disease resistance, good taste and high market value (Barcellos et al., 1999). Improper fish culture practices and the environmental conditions affect the fish health, in turn resulting in production losses and infectious disease.

In aquaculture, infectious diseases are mainly controlled by chemotherapeutics and antibiotics. However, the use of antibiotics and chemotherapy has recently been criticized because their usage has created problems with drug-resistant bacteria, toxicity and accumulation both in fish and environment. On the contrary, natural products such as plant extracts might have beneficial effects while cause no problems (Citarasu et al., 2002 and Sagdic & Ozcan, 2003). Prevention of disease or immuno stimulation of susceptible fish stock is much desirable. Hence, immunostimulants are being concentrated more upon for disease control measures. The use of natural immunostimulants seems to be the most promising method of preventing fish diseases. Immunostimulants enhances the innate immune systems, thus preventing infectious diseases (Watanuki et al., 2006). Several immunostimulants have been tested in fish as some natural plants were rich sources of compounds including volatile oils, saponins, phenolic compounds, tannins, alkaloids, polypeptides and polysaccharides. These natural plant products were reported to have such various activities as antistress, appetizer, antimicrobials and immunostimulants (Citarasu et al., 2002; Citarasu et al., 2003 and Kumar et al., 2012).

Herbs and herbal products are incorporated in livestock feeds instead of chemical products and antibiotics in order to stimulate the effectiveness of feed nutrients which result in more rapid gain, higher production and better feed efficiency. Herbs and the logically active substances content stimulate body metabolism, improve digestion, have bactericidal, immuno stimulant action and improved productivity of poultry (Sabra and Mehta, 1990). Practically, reports have shown that supplementing fish diets with various herbs have favorable effects on the performance and health of reared birds (El–Gendi, 1996).
P. guajava or guava is a plant in the family Myrtaceae along with clove, allspice and eucalyptus. Native to tropical America, it is now cultivated in many tropical and subtropical countries for its edible fruit (Perez et al., 2008). It has been used as an ingredient in many food recipes and desserts. The leaves of guava are rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Much of guava's therapeutic activity is attributed to these flavonoids in particular quercetin, saponins, tannins, alkaloids anthraquinones, phlobatannins and cardiac glycosides. Moreover, these flavonoids had antibacterial activity. High amounts of phenolic compounds in the leaves of Psidium guajava showed antioxidant activity (Haida et al., 2011). Psidium guajava is very rich in antioxidants and vitamins as guava fruit is higher in vitamin C than citrus (80 mg of vitamin C in 100 g of fruit) and contains appreciable amounts of vitamin A and also high in lutein, zeaxanthine and lycopene (Hobert and Tietze, 1998).

The objective of the present study was to investigate the effect of dried guava leaves supplementation on diet of Oreochromis niloticus immune response, antioxidant activity and biochemical parameters.

**Material and Methods**

**Plant material and sample preparation:**

Fresh leaves were purchased from herb shop at local market and the identification was done according to (Arabshahi-Delouee & Urooj, 2007). Leaves were cleaned and dried at 70°C for 3 days and then ground well. Finally, dried herbal powders were stored at 4°C until use.

**Feed formulation and diets:**

The experimental diets were prepared by incorporating dried guava leaves to the feeds, containing 0.5, 1 and 1.5 gm. powder of the guava. Control diet was prepared using the same composition of ingredients, except the powder. To prepare the diets, dry ingredients were first mixed thoroughly and 1% binder was added. Sufficient water along with the powder ingredients was then added to make a paste of each diet. The paste was cold extruded and pelletized using a hand pelletizer. Finally, the diets were dried and were sealed in plastic bags and stored at 4°C (Eloff, 1998 and Citarasu et al., 2006) until fed.

**Fish and experimental design:**

Oreochromis niloticus were obtained from Central Laboratory for Aquaculture Research (CLAR), Abbassa, Abu-Hammad, Sharkia, Egypt. Fish were transported to a laboratory then the health status of the
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Experimental fish was inspected and both the fish and tank water were disinfected. Before the initiation of the feeding trial, fish were acclimated to experimental conditions in 600-L containers with recirculated water for 2 weeks by feeding the basal experimental diet at 3% body weight twice daily without supplementation of the plant extracts. After the acclimation period, the fish (mean initial weight of (52 ± 2gm) were randomly distributed in 12 experimental aquaria (120 L) each stocked with 15 fish. The aquaria were daily cleaned by siphoning out the fish faces and uneaten food debris. Four treatments (A = 0, B = 0.5, C = 1 and D = 1.5gm powder kg _1 feed) were applied to each aquaria to evaluate the effects of dried guava powder on the Oreochromis niloticus. Each treatment was given in triplicate aquaria. The fish were fed twice a day at 9:30 and 16:30 hours at 3% of the body weight until the end of the experiment. During the experimental period, the water quality parameters were monitored every day and maintained at optimal levels by regular water exchange (temperature, 24.5°C; dissolved oxygen, 7.2mg L _1; salinity, 0.32 ppt; pH, 6.44 units; ammonia-nitrogen <0.21 mg L _1).

Immune analysis:

Lysozyme was estimated based on the turbidity measurements according to Schaperclaus et.al., (1992). However, 10 µl of serum were added in the cuvettes to 200 µl of Micrococcus suspension (35 mg of Micrococcus dry powder/95 ml of 1/15 M phosphate buffer + 5.0 ml of NaCl solution). The change in the extinction was measured at 546 nm, by measuring the extinction immediately after adding the solution which contained the lysozyme (start of reaction) and after a 20 minute incubation of the preparation under investigation at 40°C (end of reaction). The lysozyme content is determined based on the calibration curve and the extinction measured Respiratory burst (Rb) was assayed according to the method of Siwicki (1989). Nitric oxide was assayed spectrophotometrically (5010, Photometer, BM Co. Germany) according to the method of Montgomery and Dymock (1961).

Physiological analysis:

Weekly during feeding trial, three fish from each aquarium were taken for physiological investigation. Fish were anesthetized using buffer tricaine methane sulfonate (20mg/L), and blood was collected from the caudal vein with a sterile syringe and divided equally among three clean and dry tubes. The first part was centrifuged at 3,000 g for 15 min and the serum was stored at −20°C for further assays. Total protein content was determined color metrically according to Henry, (1964),  albumin was measured according to Doumas et.al.,
globulin and A/G ratio determined according to Henry (1964). Urea was determined according to Patton and Crouch (1977). Creatinine was determined calorimetrically according to Henry (1974).

**Antioxidant activity:**

Liver samples were collected from the euthanized fish for antioxidant enzymes assay. The liver tissue was homogenized in 9 volumes of 20 mM phosphate buffer (pH 7.4) containing ethylene diamine tetra acetic acid (EDTA) and 0.1% Triton X-100. The homogenates were centrifuged at 600 ×g for 10 minutes and the supernatants were collected in clean Epindroff tube for antioxidant enzymes assay. Superoxide dismutase activity (SOD) was measured according to (Kakkar et al., 1984), MDA, Catalase activity (CAT) was measured according to (Luck, 1974) and Glutathione-peroxides activity (Habig et al., 1974).

**Experimental challenge:**

On day 30 after feeding, 30 fish from each treatment were injected intraperitoneally (Schaperclaus et al., 1992) with 100 IL of live A. hydrophila (1 × 10^8 cells / ml / fish) (Harikrishnan et al., 2003). Mortality of the challenged fish was monitored up to 10 days.

**Statistical analysis:**

All experimental data were analysed using one way analysis of variance (ANOVA) using the soft ware SPSS version 17 (SPSS Inc., Chicago, IL, USA). Differences between means were determined and compared using Duncan’s test at a significance level of 5%.

**RESULTS AND DISCUSSION**

**Effect of Guava on immunity of Nile tilapia:**

All immune parameter {lysozyme, NBT(nitroblue tetrazolium) and nitric oxide} results revealed that Guava leaves powder enhance the immune response of fish from 1st week till 4th week compared to normal control group. It was noticed that immune response of fish increase at 4th week in groups feed 1% guava powder (Fig.1, 2 & 3). Lysozyme activity is an important index of innate immune response of fish. It is well documented that fish lysozyme possess lytic activity against bacteria. It is also known to be opsonic in nature and activates the complement system and phagocytes. Lysozyme is an important defence molecule of the non-specific immunity and plays a significant role in mediating protection against bacterial invasion (Saurabh & Sahoo 2008) also it was found that significant increase in serum lysozyme activity in all groups fed leaves extracts of Achyranthes aspera compared with the control group. This may be due to the ability of the leaves extracts to modulate lysozyme
activity of the fish also. It was reported that it increase plasma lysozyme activity level in Nile tilapia fed Chinese medicinal herbs (Astragalus membranaceus and Lonicera japonica) alone or in combination after first 7 days of feeding (Ardo et al., 2008). Also it was noticed that increase of lysozyme in shrimp serum and hepatopancrease fed with Guava leaf than control (Yin et al., 2014). It was reported that dietary administration of guava increase NBT and nitric oxide at group feed 1% guava so NBT & NO assay were mostly used to measure the oxidative radical production by leukocytes in the defense against pathogens (Cook et al., 2003; Sahoo et al., 2005). Verlhac and Gabaudan (1994) showed that respiratory burst activity of salmonid stimulated were significantly enhanced by vitamin C treatment. (Lin and Shiau, 2005). A significantly higher respiratory burst activity was observed in the group fed leaf (P. guajava and M. indica) extracts, which suggest that the extracts enhanced the formation of reactive oxygen species to fight against the bacteria pathogen and stimulate the non specific immune function compared with the control. In this study, the respiratory burst activity of phagocytes was quantified by the NBT assay, which measures the quantity of intracellular superoxide radicals produced by leucocytes (Siwicki & Studnicka, 2006). Giri et al., (2015) showed evaluation of the effects of Psidium guajava (guava) leaves on the growth and immune response of the fish species Labeorohita.

![Lysozyme Graph](image)

Fig. (1): Effect of different treatments of Guava leaves powder on Lysozyme values.
Effect of Guava on blood parameter of experimental fish:

In this experiment serum total protein and globulin showed significant increase in the 4th week at groups feed 1% guava powder when compared to control group (Fig. 4&5). Significant decreased of serum albumin and A/G ratio was observed in all treated groups and highest decrease were observed at 4th week in 1%, compared to control (Fig. 6&7). The total protein in the serum can be divided into two groups, albumin and globulin. They are the major proteins, which plays an important role in the immune response of fish (Kumar et.al., 2007). A rise in the total protein, albumin and globulin levels is thought to be associated with a
stronger non-specific immune response in fish (Wiegertjes et al., 1996). The leaf extracts used showed significantly enhanced serum total protein, albumin and globulin contents in the treatment groups compared with the control. This is consistent with the findings of Kumar et al. (2013), Basha et al. (2013) and Sahu et al. (2007) in L. rohita. Similarly, a significant increase in total protein, albumin and globulin was recorded in C. carpio fed diets containing 0.5% and 1% Chinese herbal medicine (Yuan et al., 2007). However, a significantly lower albumin-globulin (A/G) ratio observed indicates the presence of more amounts of globulin in the treatment groups compared with control. All treatment showed significant decrease of creatinine while first and second week show non significant increase of urea at 0.5 and 1.5% with significant decrease at 1% Guava supplemented diet while fourth week revealed significant increase at 0.5 and 1.5% with non significant increase at 1% compared with control (Fig. 8&9). There's no significant (P < 0.05) difference in urea and creatinine among all the treatments (Asmaa Abd El-Naby, 2014). Cuvelier et al. (1994) showed total lipid, urea, creatinine, AST and ALT were significantly decreased by increasing levels of S. officinal. Sage has been observed to have excellent properties in inhibiting lipid peroxidation and this activity is attributed principally to the presence of phenolic compound such as carnosic acid, carnosol and rosmarinic acid.

Fig.(4): Effect of different treatments of Guava leaves powder on Total protein
Fig.(5): Effect of different treatments of Guava leaves powder on Globulin

Fig.(6): Effect of different treatments of Guava leaves powder on Albumin
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**Fig. (7):** Effect of different treatments of Guava leaves powder on A/G ratio

**Fig. (8):** Effect of different treatments of Guava leaves powder on Creatinine
Effect of Guava on antioxidant enzymes:

At the feeding trials treatment 0.5% showed significant decrease GST at all period also 1 and 1.5% at 1st and 2nd weeks while 1 and 1.5% at 4th week showed non-significant decrease (Fig.10). SOD all treatments showed significant decrease except 1% at 1st week and 1.5% at 4th week non-significant decrease (Fig.11) and catalase activity showed significant decrease in all treatment except 1% at 1st week (Fig.12) while all treatments of MDA revealed significant decrease compared to control (Fig.13). Generally we can say that antioxidant enzymes (GST, SOD & CAT) and MDA showed significant decrease than control.

Superoxide dismutase is a class of enzymes that catalase the dismutation of superoxide into hydrogen peroxide (H₂O₂), and catalase convert the H₂O₂ to oxygen and water. They are an important antioxidant defense system in nearly all cells and offers a protection against the damaging effects of free radicals induced by oxidative stress. This oxidative stress can alter the antioxidant defense mechanism when fish are exposed to unfavorable condition like high or low temperature, toxicant, pathogen, etc., which may lead to cellular disruption, DNA damage, liver impairment and or immune suppression (Martinez-Alvarez et.al., 2005; Patra et.al., 2008). However, antioxidant containing compound can quench this harmful effect of free radicals by giving out their own electrons to stop the chain of reaction. Some studies had demonstrated that the antioxidant enzymes can serve as stress and immune–response biomarkers, quantified by the enzyme activity to evaluate the health of animal including fish (Sagstad et.al., 2007; Tovar-
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Ramirez et al., 2010; Tancredo et al., 2015). They are also said to scavenge free radicals generated by external stimuli such as pathogen, toxicant, etc. (Johnson 2002). The lower antioxidant enzymes activities recorded in the treatment groups is an indication of the leaf extracts capability to scavenge or neutralize the free radicals generated due to oxidative stress induced by pathogenic organism. This scavenging effects can be attributed to the phenolics, a secondary metabolite present in the leaf extracts which are powerful antioxidant agent (Liu et al., 2008; Fawole et al., 2013). Similar observation was made by Mamdouh and Abdel-Raheim (2003), who observed a significant reduction in SOD activities of rats fed garlic oil. MDA revealed that best result in concentration 1% of livelong attempt, also (5th and 6th) A better than B trials. The accumulation of MDA in tissues or biological fluids is indicative of the extent of free radical generation, oxidative stress and tissue damage (Gutteridge, 1995). Increase in MDA concentrations have been related to the amount of stress and well correlated with lipid membrane damage and deterioration of membrane integrity (Ekmekci & Terzioglu, 2005).

![GST ng/ml](Fig.(10): Effect of different treatments of Guava leaves powder on GST ng/ml)
Fig. (11): Effect of different treatments of Guava leaves powder on SOD µ/L

Fig. (12): Effect of different treatments of Guava leaves powder on CAT ng/ml
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**Bacterial challenge test:**

Fish mortalities due to the experimentally infection with A. hydrophila in fish fed with basal diet, 0, 0.5, 1 and 1.5% guava powder, it was 70, 30, 20 and 30 % respectively during 14 dayes after inoculation (table 1). **Fawole et.al., (2016)** and **Sahu et.al., (2007)** showed after challenges with A. hydrophila, the highest percentage survival was recorded in the group fed mango followed by guava leaf extracts and least in the control group. This is due to the enhancement of the nonspecific immunity of the fish by the leaf extracts, reveal that the supplementation of the leaf extracts had a positive influence on the survival of L. rohita fingerlings by resisting the A. hydrophila infection. Similar results were also reported after feeding L. rohita fingerlings with M. indica kernel and challenged with A. hydrophila. **Basha et.al., (2013)** reported that L. rohita fed dietary andrographolide (EC 50%) showed significantly enhanced resistance against A. hydrophila infection. Similarly, **Pachanawan et.al., (2008)** reported an increased survival in Oreochromis niloticus fed P. guajava after experimentally infected with A. hydrophila.

**CONCLUSION**

In this study, the guava (P. guajava) leaves powder enhance the non-specific immunity and increase resistance of Oreochromis niloticus against Aermonous hydrophila infection.
Table(1): Mortality rate in treated *Orechromis niloticus* due to challenge with *Aermonous hydrophila*

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<tr>
<th>Groups</th>
<th>No. of inoculated fish</th>
<th>1st Day</th>
<th>2nd Day</th>
<th>3rd Day</th>
<th>4th Day</th>
<th>5th Day</th>
<th>6th Day</th>
<th>7th Day</th>
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<th>9th Day</th>
<th>10th Day</th>
<th>11th Day</th>
<th>12th Day</th>
<th>13th Day</th>
<th>14th Day</th>
<th>No. Mortality fish</th>
<th>% of Mortality rate</th>
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<td>Control</td>
<td>30</td>
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<td>8</td>
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<td>21</td>
<td>70</td>
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<td>0.5%</td>
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Kumar S., Raman R.P., Pandey P.K., Mohanty S., Kumar A. & Kumar K. (2013). Effect of orally administered azadirachtin on non-specific immune parameters of goldfish Carassius auratus (Linn. 1758) and resistance against Aeromonas hydrophila. Fish Shellfish Immunology 34, 564–573.


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كفاءة استخدام أوراق الجوافة على الاستجابة المناعية غير المتخصصة في أسماك البلطي النيلى

جيهان إبراهيم عبد البر شجر
قسم صحة وأمراض الأسماك المعمل المركزي لبحوث الثروة السمكية بالعباسة - أبو حماد - شرقية - مركز البحوث الزراعية - جمهورية مصر العربية

المملوئ العربي

الهدف من هذا الالك هو دراسة أثار الجرعات المختلفة (0, 0.5, 1, و 1.5 جرام) من مسحوق أوراق الجوافة للإستدلال على الأداء المناعي ومحاولة للتحقيق من النشاط المضاد للأكسدة من الجوافة في دم أسماك البلطي النيلى لمدة 30 يوما.

تم الحصول على أسماك البلطي النيلى من أحواض مزرعة المعمل المركزي لبحوث الثروة السمكية بالعباسة، وتم نقل الأسماك إلى المعمل مع ملاحظة فحص الحالة الصحية للأسماك وتسجيل وزنها وكان متوسط وزن السمكة (45 ± 2 جم / سمكة).

أثبتت الدراسة أن إضافة مسحوق أوراق الجوافة له تأثير إيجابى على رفع المناعة، و ذلك من خلال قياس (الليزوزيم، والأكسجين النشط وأكسيد النيتروجين)، وكان أعلى زيادة في الأسبوع الرابع حيث سجلت مع المجموعة التي تم تغذيتها بمسحوق الجوافة بنسبة 1٪.

أظهرت التجربة زيادة معنوية في نسبة البروتين الكلي والجلوبولين في الدم وذلك في الأسبوع الرابع من تغذية المجموعات بالعلف المضاف إليه مسحوق أوراق الجوافة بنسبة 1٪ عند مقارنتها ب группа الضابطة، و لوحظ انخفاض معنوي في نسبة الزلزال في الدم ونسبة الزلل / الجلوبولين في جميع المجموعات المعالمة في الأسبوع الرابع التي تم تغذيتها بالعلف المضاف إليه مسحوق أوراق الجوافة بنسبة 1٪ مقارنة بالجروعة الضابطة، وأظهرت جميع المعاملات انخفاضا معنوي في الكرياتينين في الأسبوعين الأول والثاني، وأظهرت زيادة معنوية في اليوريا عند 0.5 و 1.5٪ معدل معنوي عند 1٪ مقارنة بالجروعة الضابطة.

لوحظ انخفاض معنوي للإنزيمات المضادة للأكسدة (جوليناز، سوبر أوكسيد ديسيمتواز، وكاتالاز) ومايونيرادتان من المجموعة الضابطة.

وقد أجريت العدوى الإصطناعية بالحقن البروتون للأسماك التي تم تغذيتها بالأعلاف المعالجة بمسحوق أوراق الجوافة لمدة أربع أسابيع ب뮤كروب الإيروموناس هيندوفيلا الممرضة مع إبقاءها تحت الملاحظة لمدة 14 يوما بعد الحقن مع تسجيل معدل النفوق في المئة.