Comparative Analysis Of Growth And Survival Of *Aeromonas Hydrophila* Challenged *Poecilia Sphenops* Fed With Herbal Extract Diet.

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ABSTRACT
The objective of the current study is to compare the growth and survival rate of *Aeromonas hydrophila* challenged *Poecilia sphenops* fed with *Psidium guajava* leaves isopropyl alcohol extract and *Azadirachta indica* isopropyl alcohol extract supplemented diet for 30 days. The *Poecilia sphenops* fishes weighing an average of 2.85 ± 0.06 g were fed diets with varying percentages of *P. guajava* leaf -isopropyl alcohol extracts (GD1-5%, GD2- 10%, GD3- 15%, GD4- 20%, and GD5- 25%) and *A. indica* leaf – isopropyl alcohol extracts (ND1-5%, ND2- 10%, ND3- 15%, ND4- 20%, and ND5- 25%) The growth parameters such weight gain and specific growth rate (SGR) of the molly fish (*P. sphenops)* were assessed after 30 days of herbal diet feeding. After 30 days fishes were challenged with fresh water bacteria *A. hydrophila*. This 10-days challenge test was conducted to determine the survival rate of bacterial challenged fishes which were fed with herbal diets. The results were comparatively analysed, from that we found that the molly fishes (*P. sphenops*) of GD5 diet group showed higher weight gain (1.56 ± 0.02 g) and specific growth rate [SGR] (1.46 %) than the weight gain (1.25 ± 0.03 g) and specific growth rate [SGR] (1.23 %) of molly fishes (*P. sphenops*) of ND5 diet group. The molly fishes of GD5 diet fed group showed maximum survival rate of 90 % than the survival rate 70 % of molly fishes (*P. sphenops*) of ND5 diet fed group. According to our findings, the molly fish *P. sphenops* fed with *P. guajava* leaves isopropyl alcohol extract supplemented diet showed higher growth performance and survival rate than the molly fishes fed with *A. indica* leaves isopropyl alcohol extract supplemented diet.

Keywords: *P. guajava, A. indica, P. sphenops, A. hydrophila*, Growth, Survival

1. Introduction

Ornamental fish farming is a rapidly increasing industry because of its popularity and extremely profitable exports to a diverse variety of countries (Ahilan *et al*., 2013). The outbreak of several bacterial infections results in significant fish mortality, which results in huge economic losses for ornamental fish farming (Austin *et al*., 2007). The major bacterial diseases like red fin disease, motile areomonas septicemia, and hemorrhagic septicemia are caused by a gram-negative fresh water bacteria *A. hydrophila* (Bisht *et al*., 2016). Numerous synthetic drug types are used to treat these bacterial diseases, but they are costly and unsuccessful. These synthetic drugs are also harmful to the environment because they promote biomagnification and the emergence of resistant bacterial strains (Citarasu *et al*., 2002). Antibiotics that are spread in aquatic habitats will have disastrous impacts on other animals (Kim *et al*., 2009).
Herbal plants are the best source of antibiotic alternatives for common aquaculture infections (Van Hai and Ngo, 2015). Numerous herbal plant by-products aid in the improvement of fish immune systems (Giri *et al.*, 2015).
P. guajava is widely available throughout Asia, particularly in India, and is consumed for its exquisite flavour, health benefits, and traditional treatment of a range of diseases (Gutierrez *et al.*, 2008; Joseph and Priya, 2011). Guava leaves are utilised as a natural antibiotic because they contain active components that serve as anti-bacterial substances (Afifi and Erlin, 2017).

Azadirachta indica, is used as traditional medicine, and every component of the tree has therapeutic benefits (Vinoth *et al.*, 2012). Leaf, seed, and bark extracts from *A. indica* have a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacteria (Biswas *et al.*, 2002).

The leaves of *P. guajava* are capable of protecting against *A. hydrophila* infection. In this work, *P. guajava* leaf isopropyl alcohol extract and *A. indica* leaf isopropyl alcohol extract supplemented with a fish basal diet to prevent infection in *P. sphenops* caused by the freshwater fish disease *A. hydrophila*. Based on our findings, we believe that *P. guajava and A. indica* leaf supplemented diet contain a variety of bioactive compounds that are responsible for elevating the growth and survival of fishes their antibacterial activity against *A. hydrophila*.

2. Material and Methods

2.1. Preparation of plant powder

The leaves of *P. guajava* and *A. indica* were collected from the southern villages of Nagercoil, Kanyakumari district, Tamilnadu, India. The leaves were washed thoroughly with distilled water and then the washed leaves were shade dried at room temperature. The dried leaves were crushed to a fine powder and sieved through 75-micron mesh sieve and stored at room temperature.

2.2. Preparation of herbal extracts

For preparation herbal extracts, 10 g of *P. guajava* leaf powder and 10 g of *A. indica* leaf powder were taken separately in two conical flasks and mixed 100 ml of the isopropyl alcohol solvent each. To stop the solvent from evaporating, silver foil was placed over the mouth of the conical flasks. The concentration of leaf powder in extract was calculated using these formulae,

$$\text{Concentration} = \frac{Mass\ of\ solute}{Volume\ of\ solvent} = \frac{10\ gm}{100\ ml} = 0.1\ \text{gram}$$

The plant leaf powder solution had a concentration of 100 mg/ml when converted to milligrams. For 48 hours, this mixture was kept in a mechanical shaker for continuous mixing. The mixture was filtered through Whatman No. 1 filter paper after 48 hours, and the extract was then gathered in sterile containers. The plant extracts in these containers were kept open at room temperature for one day in order for the solvent to evaporate and leaving the crude extract remained. The crude herbal extracts were then stored in a 4° C refrigerator.

2.3. Basal diet preparation

The basal diet was prepared according to the methodology of Giri *et al*., 2012. The ingredients for basal diet were taken in appropriate quantity and were blended in a mixture and cooked and then pelletized, air dried and sieved into appropriate pellets, and were stored at -20º C for further use. The prepared basal diet was used as control diet. Appropriate quantity of basal diet was added with supplementary diet with prepared *P. guajava* leaf isopropyl alcohol extract at 5 different concentrations. i.e., BDC (Basal Diet Control without *P. guajava* leaf isopropyl alcohol extract), GD1 -5 %, GD2- 10 %, GD3- 15 %, GD4- 20 %, GD5- 25 % (Table 1). Appropriate quantity of basal diet was added with supplementary diet with prepared *A. indica* leaf isopropyl alcohol extract at 5 different concentrations. i.e., BDC (Basal Diet Control without *A. indica* leaf isopropyl alcohol extract), ND1 -5 %, ND2- 10 %, ND3- 15 %, ND4- 20 %, ND5- 25 % (Table 2).

Table 1. Formulation of experimental diet with *P. guajava* leaf isopropyl alcohol extract.

Ingredient	Composition	Diet (gm /100 gm)					
		BDC	GD1	GD2	GD3	GD4	GD5
Fish meal	Animal protein	30	30	30	30	30	30
Groundnut oil cake	Plant protein	30	30	30	30	30	30
Rice bran	Carbohydrate	20	20	20	20	20	20
Tapioca powder	Binder	20	20	20	20	20	20
P. guajava leaf isopropyl alcohol extract	Supplement	-	10	15	20	25	30
Total		100	110	115	120	125	130

(DC: Diet ControlGD1 to GD5: Guava Diet 1 to Guava Diet 5)

Table 2. Formulation of experimental diet with *A. indica* leaf isopropyl alcohol extract

Ingredient	Composition	Diet (gm /100 gm)					
		BDC	ND1	ND2	ND3	ND4	ND5
Fish meal	Animal protein	30	30	30	30	30	30
Groundnut oil cake	Plant protein	30	30	30	30	30	30
Rice bran	Carbohydrate	20	20	20	20	20	20
Tapioca powder	Binder	20	20	20	20	20	20
A. indica leaf isopropyl alcohol extract	Supplement	-	10	15	20	25	30
Total		100	110	115	120	125	130

(DC: Diet Control, ND1 to ND5: Neem Diet 1 to Neem Diet 5)

2.4. Experimental setup

The ornamental fish *P. sphenops* 2.85 ± 0.06 g was brought from an aquarium shop in Nagercoil, Kanyakumari, Tamilnadu, India, and were transported alive to the laboratory in oxygenated plastic bags. The fishes were acclimatized in 4 food grade plastic tubs each of 50 liters capacity for 2 weeks in laboratory conditions. Basic water quality parameters like dissolved oxygen concentration 5.2-7.2 mg / l^{-1} (Winkler's method), pH 5.5 – 7.1, and temperature 22 ± 2°C were maintained during this period (Harikrishnan *et al.*, 2010).

After two weeks of acclimatization, the fishes were randomly divided into 6 experimental groups. Each experimental group had 10 fishes in 50 liters plastic tub.

2.5. Feeding of herbal extract diet

The grouped fishes were fed with *P. guajava* leaf isopropyl alcohol extract and (BDC, GD1, GD2, GD3, GD4, GD5) and *A. indica* isopropyl alcohol extract supplemented diet (BDC, ND1, ND2, ND3, ND4, ND5) for 30 days at 3 % of body weight for 2 times per day.

2.6. Growth performance parameters

The fish weight was recorded at the beginning and end of every week. Total fishes in a group were weighed and divided by the number of fish in each group to find average body weight of fishes in each group. The growth parameters are evaluated by using the following formulae (Mir *et al.*, 2017).

- Weight gain = FBW- IBW

Here,

FBW: Final Body Weight, IBW: Initial Body Weight.

- SGR (%) $= \frac{\ln(FBW) - \ln(IBW)}{(N)} \times 100$

Here,

FBW = Final Body Weight, IBW = Initial Body Weight, N = Number of days, ln = Natural log.

2.7. Preparation of bacteria for challenge trail

The bacteria *A. hydrophila* was isolated from the stock culture and cultured in tryptone soy agar (TSA) using streak plate method and incubated at 37º C for 24 hours. After 24 hours colony forming units of *A. hydrophila* were transferred to 5 ml tryptic soy broth and kept in shaker for 24 hours at 37º C. Then 10 µl of *A. hydrophila* ST1 suspension (bacterial density 1 x 10^6 CFU/ml) was taken for the challenge test.

2.8. Challenge test

After 30 days feeding period, the fishes of each diet concentration including control diet were injected with 10 µl of *A. hydrophila* suspension (bacterial density 1 x 10^6 CFU/ml) intraperitoneally. The fishes were observed for 10 days. Abnormal clinical signs and survival rate of fishes were calculated. Survival rate (%) = Number of live fish / Number of fish stocked x 100 (Opasola *et al.*, 2013).

3. Results

3.1. Growth parameter of *P. sphenops*

The growth parameters of *P. sphenops* fed with *P. guajava* leaf isopropyl alcohol extract is shown in table 3. The growth parameters of *P. sphenops* fed with *A. indica* leaf isopropyl alcohol extract is shown in table 4. The weight gain was higher in GD5 diet (1.56 ± 0.02 g), GD4 diet (1.43 ± 0.02 g), GD3 (1.29 ± 0.02 g), GD2 (1.18 ± 0.02 g) and GD1 (0.98 ± 0.01 g) when compared to the weight gain of ND5 diet (1.25 ± 0.03 g), ND4 diet (1.16 ± 0.01 g) ND4 diet (1.16 ± 0.01 g), ND3 (1.07 ± 0.03 g), ND2 (1.01 ± 0.02 g) and ND1 (0.93 ± 0.03 g). The specific growth rate (SGR) was higher in GD5 diet (1.46 %), GD4 (1.38 %) GD3 (1.25 %), GD2 (1.16 %) and GD1 (0.97 %) than SGR recorded in ND5 diet (1.23

%), ND4 (1.15 %) ND3 (1.07 %), ND2 (1.03 %) and ND1 (0.95 %). The basal diet control (BDC) of both groups showed lower weight gain (0.89 ± 0.06 g), (0.91 ± 0.05 g) and specific growth rate (SGR) (0.90 %), (0.93 %) than herbal diet fed groups. According to the earlier results of Giri *et al.*, 2015**,** the supplementary diet of *P. guajava* leaves showed increased weight gain and specific growth rate in *Labeo rohita*, than the control diet group. The silver carp fed with *A. indica* supplemented diet indicated increased growth parameters including specific growth rate (SGR) and weight gain than the normal diet fed fishes (Mona *et al.*, 2015).

Table 3. Growth performance of *P. sphenops* in different diet concentrations of *P. guajava* leaf -isopropyl alcohol extract.

S.No.	Diet Group	IBW (gm)	FBW (gm)	WG (gm)	SGR (%)
1	BDC	2.85 ± 0.15	3.74 ± 0.09	0.89 ±0.06	0.90
2	GD1	2.90 ± 0.14	3.88 ± 0.13	0.98 ± 0.01	0.97
3	GD2	2.81 ± 0.15	3.99 ± 0.13	1.18 ± 0.02	1.16
4	GD3	2.83 ± 0.14	4.12 ± 0.12	1.29 ± 0.02	1.25
5	GD4	2.78 ± 0.13	4.21 ± 0.11	1.43 ± 0.02	1.38
6	GD5	2.82 ± 0.15	4.38 ± 0.13	1.56 ± 0.02	1.46

(Data expressed as Mean ± SD, n=10. BDC: Basal Diet Control, GD1 to GD5: Guava Diet 1 to Guava Diet 5, IBW: Initial Body Weight, FBW: Final Body Weight, WG: Weight Gain, SGR: Specific Growth Rate).

Table 4. Growth performance of *P. sphenops* in different diet concentrations of *A. indica* leaf -isopropyl alcohol extract.

S.No.	Dietgroup	IBW (gm)	FBW (gm)	WG (gm)	SGR(%)
1	BDC	2.81 ± 0.15	3.72 ± 0.10	0.91 ± 0.05	0.93
2	ND1	2.82 ± 0.13	3.75 ± 0.10	0.93 ± 0.03	0.95
3	ND2	2.78 ± 0.13	3.79 ± 0.11	1.01 ± 0.02	1.03
4	ND3	2.80 ± 0.14	3.87 ± 0.11	1.07 ± 0.03	1.07
5	ND4	2.80 ± 0.13	3.96 ± 0.14	1.16 ± 0.01	1.15
6	ND5	2.78 ± 0.14	4.03 ± 0.11	1.25 ± 0.03	1.23

(Data expressed as Mean ± SD, n=10. BDC: Basal Diet Control, ND1 to ND5: Neem Diet 1 to Neem Diet 5, IBW: Initial Body Weight, FBW: Final Body Weight, WG: Weight Gain, SGR: Specific Growth Rate)

3.2. Challenge test

The dietary supplementation of *P. guajava* leaf isopropyl alcohol extract at higher concentration (GD5) fed *P. sphenops* fishes showed the highest percentage of survival rate 90 % when compared with survival rate of *A.* indica leaf – isopropyl alcohol extract 70 % at higher concentration (ND5) fed *P. sphenops* fishes; the GD4 diet fed group showed 80 % survival rate than the ND4 diet fed group survival rate 60 % ; the GD3, GD2 and GD1 showed higher survival rate of 50 %, 30 %, 10 % respectively than the survival rate of ND3, ND2 and ND1 (30 %, 20 % and 0 % respectively). The basal diet control fed molly fishes (*P. sphenops*) showed 0 % survival rate (Figure 1 and figure2). The *P. guajava* ethanol extract supplemented diet indicated higher survival rate in *Oreochromis mosambicus* challenged with *A. hydrophila* (Gobi *et al.*, 2016). The supplementation of *A. indica* leaves with normal fish diet has improved the immunity and survival rate of bacterial challenged Asian seabass fishes (Talpur and Ikhwanuddin, 2013).

Figure 1. Survival rate of *P. sphenops* in different *P. guajava* leaf -isopropyl alcohol extract diet concentrations infected with *A. hydrophila*.

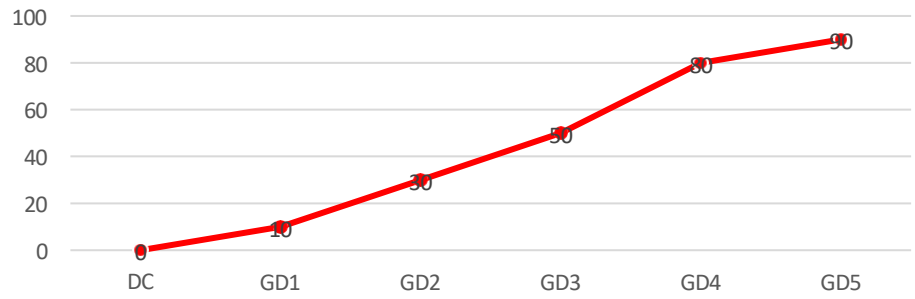
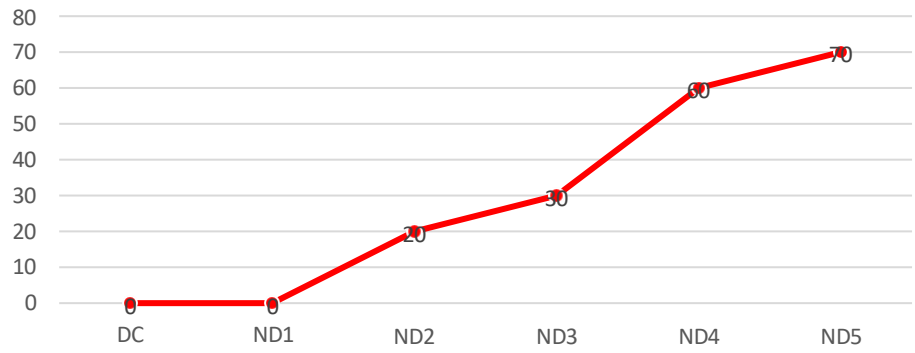


Figure 2. Survival rate of *P. sphenops* in different *A. indica* leaf -isopropyl alcohol extract diet concentrations infected with *A. hydrophila*.



4. Conclusion

The infection caused on by *A. hydrophila* can be prevented by the antimicrobial principles of *P. guajava and A. indica* leaves. In order to prevent the infection of *P. sphenops* by the aquatic fish pathogen *A. hydrophila*, fish basal diet was supplemented with *P. guajava* and *A. indica* leaf – isopropyl alcohol extract. Our research findings revealed that the molly fish (*P. sphenops*) fed with *P. guajava* leaf isopropyl alcohol extract supplemented diet fed group showed higher growth parameters than the molly fish (*P. sphenops*) fed with *A. indica* leaf isopropyl alcohol extract. The *P. guajava* leaf isopropyl alcohol extract supplemented diet fed group showed higher survival rate of the *A. hydrophila* challenged molly fish (*P. sphenops*) than *A. indica* leaf isopropyl alcohol extract diet fed group. Hence, *P. guajava* leaves and its extracts may act as potential growth promoter for enhancing the growth and survival rate of molly fishes (*P. sphenops*) infected by freshwater fish pathogen *A. hydrophila.*

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