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Original Article

Effect of *Annona squamosa* linn. Leaf Extraction *Clarias batrachus* Challenged with *Aeromonas hydrophila*

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ABSTRACT

Objectives:.The current work was to determine the efficacy of aqueous extract of leaf on growth status and survival of *Clarias batrachus* after 15 and 30 days and after 15 days of infection with *Aeromonas hydrophila*, as bath treatment. **Experimental approach**:- Investigation was done toanalyse the effect of different concentration of aqueous leaf extract (0, 25, 50 and 100% in 15 litre of water)and supplemented diet added in group I, II, III and IV on survival and growth status of freshwater *Clarias batrachus* fish after 15 and 30 days. To study inhibitory effect for infection caused by pathogenic bacteria, fishestreated with aqueous bath, were infected with *Aeromonas hydrophila* and was rechecked for their survival and growth status. The antibacterial activity of leafextract was studied using agar well diffusion method. Growth (Weight and Length) and survival rate was observed in pre and post challenged groups. **Findings and discussion:** -The weight in pre-challenged fishes after 15 days and 30 days were 122.9±1.2 (Mean±SD) gm, 125.2±12gm in group I and IV while it was 125.5±15gm, 130.4±12 gm after 30 days and post challenged fishes in group I and IV were 125.57 ±10 and 130.8±10.9 after15 days. The length of group I and IV was16.8±1.3 cm, 17.2±1.3cmafter 15 days and17.5±1.2, 17.9±1.1 after 30 days and post challenged fishes in group I and IV were 17.57±1.6 and 17.94±1.38 after 15 days. Survival rate was higher in Group IV. It was 100%as compared to group I (66.6%).
Conclusion: -It was observed that 100% aqueous leaf extract showed good antibacterial activity against *Aeromonas hydrophila* as well as enhancement in growth status and survival rate.
Keywords –*Aeromonas hydrophila, Annona squamosa, Clarias batrachus,* Supplemented feed

1. INTRODUCTION

Aquaculture represents one of the fastest growing food producing sectors. Fish diseases constitute one of the most important problems and challenges fish culturists. Hence, aqua-culturists are forced to undertake good management

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practices, so that they can ensure a healthier fish[1]. Fishes not only play an important role in the demand of food for humans but they are widely used for various biological experiments[2]. Bacterial infections are the major reasons for fish mortality in aquaculture industry[3]. *Aeromonas hydrophila*is a gram negative opportunist bacterium associated with aquatic animal disease[4]. *Aeromonas hydrophila*causes mass mortalities in several species including Carps, Snake heads, Gouramies and Cat fishes and are considered as an etiological agent of several diseases such as emaciation, haemorrhagic, septicaemia, asymptomatic septicaemia, ulcerative infection, tail rot and fin rot [5].In India no commercial vaccine or recommended immunotherapy is currently available for catfish culture[6]. The plant products are generally regarded as harmless and can be used as novel methods of minimizing disease risk and as a good substitution for antibiotics in aquaculture[7]. *Annona squamosa*, sugar apple is native to the tropic America and is widely grown throughout the tropics in India[8]. Phytochemical studies on *Annona squamosa* showed many active compounds which are having many pharmacological activities such as anti-inflammation, anti-tumour activities[9]. The leaves of *Annona* species are used as a vermicide, for treating cancerous tumors and are applied to abscesses, insect bites and other skin complaints. Scrapings of root-bark are used for toothache. The leaves and bark of custard apple contains alkaloids and the fruit contains iron, calcium, fiber, amino acids, vitamins, carotene, thiamine, riboflavin, niacin and ascorbic acid[10].*Clarias batrachus*is well adapted virtually in all India aquatic aquaculture system specially West Bengal and Tripura where it is considered to be a medicinal fish and it is also considered to be a favourite diet for pregnant women, lactating mothers, elderly and the children and also for anaemic persons. Intensive culture of *Clarias batrachus*is done in many states, since it requires no special treatment and growth factors for culture, as such work on immunostimulatory aspects in this fish will serve to increase the fish production and help in solving the national food problem to some extent[11]

2. MATERIALS AND METHODS

Collection of plant material and authentication of the plant

The leaves of the *Annona* specieswas collected from St. Aloysius College (Autonomous), Jabalpur garden then *Annona* species was identified and authenticated by a Professor of Botany Dr. ShailendraTiwari, Senior Scientist State Forest Research Institute (SFRI), Jabalpur. The leaves of *Annona squamosa* was collected and were washed thoroughly first in tap water and then rinsed with distilled water and dried completely in shade at room temperature for 30 days. The plant material was crushed and blended to fine powder.

Collection acclimatization and maintenance

Clarias batrachus fishes of mixed sexes weighing about 110gm-160gm was used for the study which was purchased from the local fisherman of Jabalpur, Madhya Pradesh, India and was identified with the help of the plate XI fig.3(fig.140) pp. 305 of the book cat fishes of India by K.C Jayaram (2006). Fishes were disinfected with potassium per magnet. Fishes were kept in aquarium capacity of 100l and allowed to acclimatize to laboratory conditions for 15 days with continuous aeration. During the experiment the water was maintained at a temperature of 27^{o}C - 28^{o}C, pH at 7.5, D.O level of (6.8–7.2 mg/L), Alkalinity mg/L(85.4 -97.6), Hardness mg/L (44-56). Ammonia and nitrite levels in the water were 0.00 mg/L.

Quantitative screening of aqueous leaf extract of *Annona squamosa*.

For aqueous extraction, 5g of the leaf powder were taken in 500ml distilled water and boiled in water bath at 50-60°C for two hours. It was then filtered through Whatman filter paper no. 1. The crude extracts were then stored in screw capped bottles in refrigerator at 4°Cfor further use.

Thequantitative assays of prepared plant extracts were used to analysed for the presence of tannins, flavonoids and phenolic compounds.

Estimation of Total Phenol in leaf extract of *Annona squamosa*.

The total phenolic content was estimated by using FolinCiocalteu spectrophotometric method of with some modifications[12-13]

Leaf extract (0.5 ml)of different concentration(25,50, 75 and 100%) was mixed with 0.5ml of Folin-Ciocalteu reagent and the mixture was diluted by adding 7.5ml of distill water, the resultant mixture was mixed and shaken. Then the reaction mixture was kept undisturbed at room temperature for five minutes after that 5 ml of 7% sodium carbonate was added and incubated for 90 min at room temperature. After incubation blue coloured complex was formed and the absorbance was determined at 760 nm against the reagent blank prepared inthe similar manner without the gallicacid.The experiment was carried out in triplicate. The total phenolic content of the leaf extract was expressed as gallic acid equivalent (mg/g dry weight of leaf).

Estimation of Total Flavonoid in leaf extract of *Annona squamosa*.

The TFC was measured by spectrophotometeric method [14]

Leaf extract of 0.5ml of different concentration (25, 5075 and 100%) was mixed with 2 ml of distill water in 10 ml test tube. Add 5% of 0.15ml of sodium nitrite solution and the test tubes were allowed to stand for five minutes, after that 10% of 0.15mlaluminium chloride was added and left for five minute, on sixth minute 1ml of 1.0 M NaOH was added and mixed well, orange yellowish colour was developed. Absorbance of the resultant mixture was read at 510 nm against the reagent blank prepared in the similar manner without the quercetin.TFC was determined as quercetin

equivalent (mg/g of dry weight of leaf). The experiment was conducted in triplicate.

Estimation of Total Tannin in leaf extract of *Annona squamosa*.

Total tannin content was determined by using Folin - Ciocalteu Spectrophotometric method[15]. Leaf extract 0.1 ml of different concentration(25,50 ,75 and 100%)was added to a test tube containing 7.5 ml of distilled water, 0.5 ml of Folin-Ciocalteu reagent was added and after that 1 ml of 35 % Na_2CO_3 solution was added and the mixture was diluted with 10 ml distilled water. The mixture was shaken well and incubated at room temperature for 30 min. Absorbance of samples was measured against blank at 725nm in spectrophotometer. The reagent blank was prepared in the similar manner without the gallic acid. The tannin content was expressed in terms of mg of GAE /g of extract (mg/g of dry weight of leaf).

Challenged test and antibacterial activity of leaf extract of *Annona squamosa*

Bacterial strains *Aeromonas hydrophila* (ATCC 7965), Lyophilized cells of *Aeromonas hydrophila* was cultured in nutrient broth and was incubated at 37^{o}C for and maintained. The culture was centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant solutions were discarded and the pellet was resuspended in 1X phosphate buffer saline (PBS, pH-7.4) and the OD of the solution was adjusted to 0.5 at 456 nm which corresponded to 1x10^7 cells/ml. These bacterial suspensions were serially diluted with PBS used for experiment 16.

Agar well diffusion method was used to detect antibacterial activity of aqueous leaf extract of different concentration(25, 50 and 100%)against *A.hydrophila.* All tests were conducted in replicates and zone of inhibition of eachextract was measured. The wells 10 mm in diameter were created using sterile agar borer and the wells were filled adding 25 µl of different aqueous concentration of leaf of *Annona squamosa* extracts and was incubated at 37°C for 24 hr. One pair was kept as control. Three replicates were prepared from each concentration. The clear zones were measured in millimeters. Observations were analyzed applying standard deviation and data presented in table with ±SD.

Experimental Design

Fishes of mixed sexes were divided into four groups (n = 21) and kept in glass aquarium containing 15 litre of water. The control and experimental groups I, II & III were exposed to 5,10 and 15ml of dose in 15Litre of different concentration of (25%, 50%,100%) aqueous leaf extract of *Annona squamosa* in it every alternate day till 30 days. The control group and experimental fishes were fed with pelleted commercial food and worms at 2% of body weight and water was changed daily after 24 hours of feeding. Growth rate were determined after 15 and 30 days. After 30 days of immersion in different concentration of aqueous extract of leaf, all experimental fishes were taken out and were kept in water devoid of leaf extract and then all experimental groups were given only control feed mentioned above at the rate of 2% of body weight. At the 30th day the fishes of experimental groups were challenged with *Aeromonas hydrophila* intramuscularly in the caudal region at a dose of 0.5ml of 1.0×10^{7}cells/ml. After 15 days of infection, studies were carried to monitor the growth rate and survival rate of fishes. The weight and length of nine randomly selected fishes from each group were recorded after 15, 30 days and after 15 days of infection, the weight and length of the fishes in control and experimental groups was calculated according to the formula given below.

i. Length gain = Final length of the fish – Initial length of the fish

ii. Weight gain = Final weight of the fish – Initial weight of the fish.

Mortality

Fish of control and experimental groups were challenged with an intramuscularly infection of 0.5 ml of 1x10^{7}cell/ml. *Aeromonas hydrophila* after 30 days of treatment. Mortality was recorded after 15 days of infection. Percentage of survival fish was calculated as per [17].

RPS=Total no. of dead fishx 100

Total no. of challenged fish

3. RESULTS

The quantitative estimation of phenols, tannins, andflavonoids contents in the aqueous extract of various concentration of leaf has been estimated as per given methods and the results were reported in Table 1. The highest phenols, flavonoid and tannin are as follows-: (674.2µg/ml, 1076 and 29.5µg/ml) was found in aqueous leaf extract of 100% concentration as compared to other concentration of leaf.

Table 1: Content of phytochemicals in the leaf extracts of *Annona squamosa*

S.No	Plant Part	Various concentration of leaf extract %	Phytochemicals in µg/ml		
			Phenol	Flavonoid	Tannin
1	Aqueous extract of leaf	25	188.9	507.3	6.7
2		50	209.6	586.7	10.3
3		75	496.9	752.1	15.2
4.		100	674.2	1076	29.5

Table 2: Antibacterial activity of different extracts of *Annona squamosa* by agar well diffusion method

S.No.	Doses of extract %	Inhibition Zone of *A. hydrophila*(mm)	Inhibition Zone of standard (mm)
1	25	No clear zone seen	8.1±0.36
2	50	No clear zone seen	9.16±0.57
3	75	No clear zone seen	10.33±0.47
4	100	16.5 ± 0.91mm	11.67±1.5

In aqueous leaf extract of *Annona squamosa*Linn.of this study however we found that the aqueous extract of leaf has also reported to inhibit the growth of gram negative bacteria strain (*Aeromonas hydrophila*). Aqueous extract of *Annona squamosa* in 100% concentration showed highest antibacterial activity against the fish pathogen *A.hydrophila*

with the inhibition zone of 16.5 ± 0.91mm and no inhibition zone was found at 25% and 50% of extract respectively. The positive control Linezolid disc showed the highest inhibition zone of 11.67±1.5mm, distilled water was used as negative control, no inhibition zone was observed in control. Our investigation showed that the antibacterial activity shown by the aqueous leaf extracts of the *Annona squamosa* Linn. plants is possibly due to the presence of phytochemicals i.e tannins, phenols and flavonoids.

Effect of different concentration of extracts on growth performance–(Pre challenged &Post challenged).

Effect of different concentration of extracts on body weight and body length after0,15 and 30 days were conducted . Before conducting the experiments the initial body weight and body length of fishes from each group (Control, Gr.I, Gr II and Gr.III) were recorded and they were considered as control and the readings of both were as given below: body weight: 120±12gm, 121±12.3gm, 121.8±12.3gm and 122.1±13.7gm and body length of the fishes were measured are as follows: 16.8±1.3 cm, 17.1±1.4cm, 17.1±1.3cm and 17.2±1.3 respectively. After experimental treatment of 15 days the weight of the fishes were recorded again from each group (Control, Gr.I, Gr II and Gr.III) and the readings are mentioned below: 122.9±12gm, 123.4±14gm, 124±13 gm and 125.2±12gm, body length: 17.4±1.5 cm, 17.4±1.3cm, 17.4±1.4cm and 17.6±1.5 respectively. After 30 days of treatment the final body weight of each group (Control, Gr.I, Gr II and Gr.III) were recorded and they are given below: 125.5±15gm, 126±13gm, 127.5±14gmand 130.4±12 gm and body length: 17.5±1.2 cm, 17.6±1.3cm, 17.7±1.1cm and 17.9±1.1respectively. The growth status (Length and weight) analysed after 0, 15 and 30 days showed an increasing trend in the entire three experimental groups as compared to control group.

Table 3: Effect of *Annona squamosa* leaf extract on the body weight (gm) and length (cm) of *Clarias batrachus* fish after 0, 15 and 30days.

Groups/Treatment	Doses of extract %	Body weight (gm) 0 days	Body length (cm) 0 days	Body weight (gm) 15 days	Body length (cm) 15 days	Body weight (gm) 30 days	Body length (cm) 30 days
Control	0	120±11.9	16.8±1.3	122.9±1.2	17.4±1.5	125.5±15	17.5±1.2
Group I/Bath	25	120.95±12.3	17.1±1.4	123.4±14	17.4±1.3	126±13	17.6±1.3
Group II/Bath	50	121.8±12.3	17.1±1.3	124±13	17.4±1.4	127.5±14	17.7±1.1
Group III/Bath	100	122.1±13.7	17.2±1.3	125.2±12	17.6±1.5	130.4±12	17.9±1.1

Table 4: Effect of *Annona squamosa* leaf extract on the body weight (gm) and length (cm) of *Clarias batrachus* fish post challenged with *A.hydrophila*.

Groups/Treatment	Doses of bacteria (ml)	Body weight (gm) 15days (Post challenged)	Body length (cm) 15 days (Post challenged)
Group I	0.5	125.57 ±10	17.57±1.6
Group II/Bath	0.5	126.28±7.2	17.64±1.6
Group III/Bath	0.5	127.6±10.9	17.7±1.7
Group IV/Bath	10.5	130.8±10.9	17.94±1.38

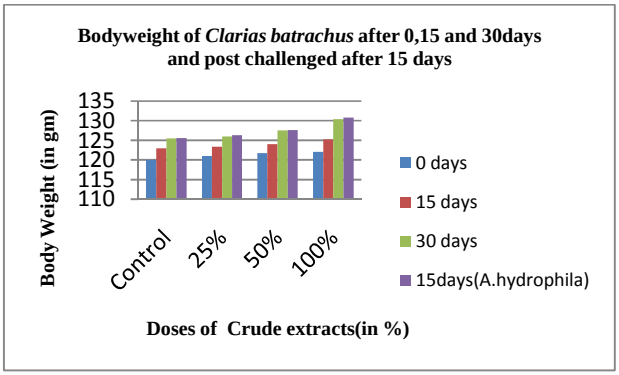

Fig 1: Bodyweight of *Clarias batrachus* after 0,15 and 30days and post challenged after 15 days

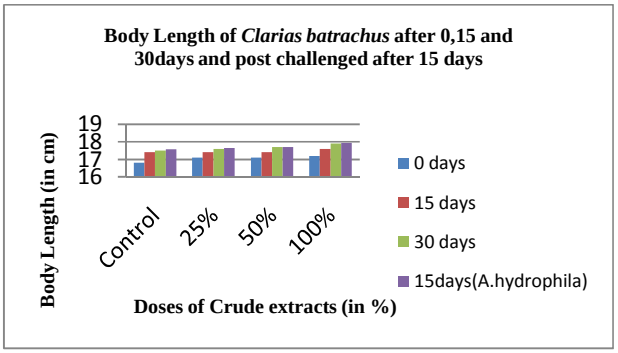

Fig 2: Body Length of *Clarias batrachus* after 0,15 and 30days and post challenged after 15 days

Effect of different concentration of extracts on survival rate after 15 days challenge with *A. hydrophila.*

In the current study, when the fish were intramuscularly challenged with *Aeromonas hydrophila* after 30th days of treatment the mortality percentage was found highest (33.3%) in the control group I (infected) and lowest (0%) in group IV. The relative percentage survival was found highest (100%) in group IV and lowest (66.66%) in control group I group. This effect may be due to the enhancement of the nonspecific immune system of the fish by aqueous leaf extract.

Table 5: Survival rate of *Clarias batrachus*fish after 15 dayschallenge with *A. hydrophila.*

Group	Doses of extract %	Total no. of challenged fish	Total no. of dead fish	Mortality (%)	Survival (%)
Group I/Control	0	9	3	33.3	66.66
Group II	25	9	1	11.11	88.89
Group III	50	9	1	11.11	88.89
Group IV	100	9	0	0	100

4. DISCUSSION

Natural immnuostimulants are biocompatible, biodegradable and safe for the environment and human health [18]. These products can be used as novel methods of minimizing disease risk and as a good substitution for antibiotics in aquaculture[7].The current study

was to investigate the effects of a queous leaf extract of *Annona squamosa* along with the supplemented feed given according to 2% of body weight on the growth performance and survival rate of fish. The outcomes of the present study showed that the fish bathed in various concentration 25, 50 and 100% in 15l of aqueous leaf extract of *Annona squamosa* after 15, 30 days and after 15 days of infection showed increase in the growth status (Weight and Length) at higher concentration of leaf extract as compared to the control fish which may be considered as a sign of growth enhancement. The weight of control fishes and in group IV after 15, 30 days and after 15 days of infection was 122.9±1.2gm, 125.2±12gm and 125.5±15gm, 130.4±12 gm. The length of control fishes and experimental Group IV was16.8±1.3 cm, 17.2±1.3cm and 17.5±1.2, 17.9±1.1.Survival rates of the *Clarias batrachus* fish bathed in different concentration of aqueous extract of *Annona squamosa* and fed the supplemented diets according to 2% of body weight were higher in Group IV (100%) as compared to the control group (66.6%).The current study agrees with the results of [19] , that inclusion of 5 grams of different plant ingredients in fish feed resulted in superior growth performance in *Oreochromismos sambicus* for 45 days and the results showed that for every 15 days once that the fishes fed with *Moringa oliefera* supplemented feed showed maximum increase in weight (0.96%, 1.33%, 1.78%) and maximum length was observed in the fishes that were fed *Ocimum basilicum* supplemented feed (1.2%, 1.6%, 2.0%).

Similar study was carried out and it was found that effect of some Chinese herbs (*Lonicera japonica and Ganoderma lucidum*) in Tilapia (*Oerochromis niloticus*) for three weeks and and infected with *A. hydrophila*. They found that the fish mortality after infection was found to be 55% in the control fishes, where as the fishes fed with Ganoderma showed the mortality of 21% , thus herbs extract added to the diet acted as immunostimulant and improved the survival rate and disease resistance in fish[20].Samework was done on *Cyprinuscarpio* by the researchers found that at a dose of 250mg /kg feed for seventy days levamisole treatment was able to enhance significantly the growth rate. The survival rate was also increased in fish challenged by *Aeromonas hydrophila* and also increases resistance to infection and reduces mortality and enhances the growth of fish in carp fingerlings[17].The immunostimulatory effect of the leaves extract of a medicinal plant *Solanum torvum* (Turkey Berry) on common carp *Cyprinus carpio* was studied [21]. They found the maximum growth in 80mg *S. torvum* fed fish and decreased growth was in 0.8mg and 800mg of herbal diet fed fish *(P*<0.05) compared with non-supplemented control fish. [22]studied the dietary onion and ginger was administered orally for 12-weeks enhance growth and disease resistance in brown-marbled grouper, *Epinephelus fuscoguttatus* against *Vibrio Harvey* infection.

5. CONCLUSION

1. The results of current study indicated the potential of *Annona squamosa* leaf extract as the growth promoters and disease resistance in fresh water *Clarias batrachus* fish with no adverse effects observed.
2. The study shows that fishes immersed in leaf extract of *Annona squamosa* at 100% concentration and supplemented feed increased the weight, length and survival rate of the experimental fishes when compared to control.
3. Survival rates of the *Clarias batrachus* fish bathed in different concentration of aqueous extract of *Annona squamosa* and fed the supplemented diets according to 2% of body weight were higher in Group IV (100%) as compared to the control group (66.6%). is due to presence of phytochemical constituents, supplemented diet and antibacterial activity of leaf. This concludes that these can help in formulation of drugs .

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