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Research Article

BIOENCAPSULATION OF *ARTEMIA* NAUPLII WITH HERBAL EXTRACT FOR PROMOTING GROWTH OF FISH FRY *POECILIA SPHENOPS* VAL.

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Abstract

The present investigation aimed to evaluate the bioencapsulation of *Artemia* nauplii with *Vitex negundo* leaf extract for promoting growth and survival of *Poecilia sphenops* fish fry was carried out. *Artemia* cyst were collected from Kelambakkam saltpan and cultured in the laboratory. Cyst processing and hatching were made in the laboratory. The assessment of cytotoxic bioassay of leaf extracts of *V. negundo* (aqueous, methanol, ethanol) were used *Artemia* nauplii. The LC_{50} value of *Artemia* nauplii was observed in 2.5, 1.0 and 1.0 mg/ml of aqueous, methanol and ethanol extracts respectively. After optimization, *Artemia* nauplii were enriched with 6 and 12 hrs were fed to fish fry. The experimental fish fry were assessed for their survival, specific growth rate (SGR) and mortality compared with the control. The Maximum SGR was observed in experiment I (6 h enriched *Artemia*) 2.70% and the minimum of 1.13% in Control. The maximum survival rate was observed in Experiment I value of 95.83% (6 h) *Artemia* nauplii enriched with aqueous leaf extract and the minimum of 89.58% observed in control. The maximum mortality rate was observed in control value of 10.41% (6 h) *Artemia* nauplii unenriched with aqueous leaf extract and the minimum mortality rate was observed in experimental I (4.16%) enriched *Artemia* nauplii. The above result clearly indicated that *V. negundo* leaf extract could be used as nutritional and enriches feed for *Artemia* nauplii.

Keywords**:** *Artemia*, Cytotoxicity, Enrichment, Survival, Bioencapsulation

INTRODUCTION

The ornamental fish sector is a widespread and global component of international trade, fisheries, aquaculture and socio-economic development. Since 1985, the international trade in exports of ornamentals, which usually takes place in the majority of developing countries, followed an increasing trend with an average growth rate of approximately 14% per year. Successful rearing of larval stages of aquatic organisms is a challenge for aquarium hobbyists, an aim and a necessity for the success of the aquaculturist. All these specialists will agree that the primary problem in any type of larval rearing is that of food. Ideally, one would prefer to feed larvae their natural diet, which is characterized by a wide diversity of nutritious live organisms. Live feed is an essential food source for the fry of cultured species, especially those without a fully developed digestive system. In the freshwater ornamental fish culture, *Artemia* nauplii are used as the live feed. Two major concerns of aquaculturists are: (i) providing organisms appropriate to the size of the feed to the first feeding stage and (ii) supplying adequate number of feed organisms to ensure higher survival and faster growth (Arulvasu and Munuswamy, 2009).

The continuous, nonselective feeding behavior of on-grown *Artemia* also makes the organism an ideal booster diet, as its nutritional quality could be tailored to suit the fish requirements through bioencapsulation. Bioencapsulation is defined as the process by which live food organisms are enriched with specific nutrients or

drug molecules (for example, vaccines) and fed to the target organisms. Enrichment or "boosting" of the fatty acids of both food organisms has become incorporated into the larval-rearing protocols for many fish species (Sorgeloos *et al*., 1991). Tamaru *et al.,* 1999 reported that those live feed contain only a small amount of essential fatty acids and DHA has not been reported in any live feed organism while little EPA level has been reported in all live feed varieties.

The importance of medicinal plants and traditional health systems in solving the health care problems of the world is gaining increasing attention. Because of this resurgence of interest, the research on plants of medicinal importance is growing phenomenally at the international level, often to the detriment of natural habitats and other populations in the countries of origin. Historically, all medicinal preparations were derived from plants, whether in the simple form of raw plant materials or in the refined form of crude extract mixtures etc. In order to study the toxicity of these medicinal plants we performed brine shrimp lethality bioassay which based on the ability to kill laboratory cultured brine shrimp (*Artemia* nauplii) the brine shrimp assay was proposed by Michael *et al.* (1956) and latter developed by (Vanhaecke *et al.*, 1981; Sleet and Brendel, 1983). The assay is considered a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity (Harwig and Scott, 1971). The Method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the micro well scale. Bioencapsulation method is now being developed for oral delivery of vitamins, chemotherapeutic vaccines, essential PUFAs, pigments sterols and carotenoids through *Artemia* nauplii (Touraki et al., 1996). Encapsulation of herbal extract was achieved by using two approaches i.e., dripping, gelation and absorptive encapsulation method (Chan *et al*., 2009). The aqueous extract of *Piper sarmentosum* (furley, Malaysia) was used as the model herbal fluid (Yim *et al*., 2010).

Vitex negundo Linn. (Family: Verbenaceae), vernacular name nochi in Tamil, is a small evergreen, much branched shrub and ascending up to an altitude 1100-1400 ft, is found almost throughout India (Chopra *et al.,* 1956; Anonymous, 2001). Although all parts *V. negundo* are used as medicine in the indigenous system of medicine, the leaves are the most potent for medicinal use. A number of pharmacological activities have been attributed to *V. negundo*, such as analgesic and anti-inflammatory activity (Dharmasiri *et al.,* 2003), Enzymes inhibition (Azhar-Ul-Haq *et al.,* 2006), Nitric oxide scavenging activity (Jagetia *et al.,* 2001), Snake venom neutralization activity (Alam *et al.,* 2003), Anti-feeding activity (Chandramu *et al.,* 2003), Anti-radical and anti-lipoperoxidative (Munasinghe *et al.,* 2001), CNS activity (Gupta *et al.,* 1999), Hepatoprotective activity (Avadhoot *et al.,* 1991), antibacterial activity (Permalsamy and Ignacimuthu 1998), Anti-fungal (Damayanthi *et al.,* 1996), Larvicidal activity (Pushpalatha *et al.,* 1995), Anti-androgenic effects (Bhargava, 1989), Mosquito repellent activity (Hebbalkar *et al.,* 1992) and Anti-diabetic effect (Manikandan *et al.,* 2009). The main aim of the present study is to examine the bioencapsulation of *Artemia nauplii* with *V. negundo* leaf extract for promoting the growth and survival of *Poecilia sphenops* fish fry.

MATERIALS AND METHODS

Animal collection and maintenance

The white molly *Poecilia sphenops* fish fry were obtained from a commercial ornamental fish farm (Golden galaxy) Kulathur near Chennai and their fish fry were collected and maintained in the culture tank. The fish fry were acclimatized for 5 days. After measuring the weight of the fish fry, they were stocked at a density of 16 fry in plastic troughs. (Capacity 5L) with 3L of mildly aerated freshwater. Three replicates were maintained for every experiment trial. The experimental period was restricted to 20 days.

Plant identification and preparation of extracts

The Medicinal Plant used in this study was collected from Velachery, Chennai, Tamil Nadu. The plant was examined and identified by Centre for Advanced Studies (CAS) in Botany, University of Madras, Guindy Campus, Chennai, Tamil Nadu, India.

Aqueous extract

The leaves were collected, garbled and dried under shadow. The dry leaves were powdered coarsely. The powdered leaves of *V. negundo* were weighted (25g). The leaves crushed to powder with a mortar and pestle. A 5% (w/v) suspension was prepared in a flask by adding hot boiled water and kept in a shaker at 200 rpm for 4 h and the temperature was maintained at 37°C. After being shaken, the suspension was brought to room temperature.

Then the suspension was filtered through Whatman No. 1 filter paper and finally passed through a 0.22 µm filter paper (Millipore, Billerica). The filtered aqueous extract was freeze dried and powder was stored at -20°C until for further experiments.

Ethanol and Methanol extract

The leaves were collected, garbled and dried under shadow. The dry leaves were powdered coarsely. The powdered leaves of *V. negundo* were weighted (25g). The leaves crushed to powder with a mortar and pestle. The powdered sample was soaked in ethanol and methanol. About 5% (w/v) suspension was kept for 4 h on shaken condition. The solvent was evaporated at room temperature in Petri dishes. The dried material was retrieved and stored in test tubes at 20°C. The filtered extract was stored at –20°C until for further experiments.

***Artemia* cyst collection and hatching procedure**

Cysts were collected from (100 µm scoop net) Kelambakkam saltpan, Tamil Nadu. Cyst washed with fresh water at five times rapidly (400 µm scoop net) and transfers to brine solution (separation of heavy debris and sand particles) remaining floating cyst were collected and transfer to fresh water. Bottom settled cyst were collected and dried hot air oven at 37°C. Completely dried cyst was sieved using magnetic sieve shaker (300, 250, 210 and 150 µm mesh) and stored as described by Sorgeloos *et al*. (1986). *Artemia* cyst was hatched following standard procedure of Sorgeloos *et al*. (1986). One gram of dried cyst was hydrated in freshwater for one hour in a conical flask with vigorous aeration. The cyst were collected after one hour and rinsed with tap water and cyst transferred into the decapsulating solution (4% sodium hypochlorite). After 5 to 10 min, the entire cyst turned to orange-pink in color indicating decapsulation. The cyst were collected in a 100 µm bag and rinsed with fresh water to remove all traces of the hypochlorite solution. The treated decapsulated cyst were then transferred to 1L seawater and kept under 1000 lux light intensity, vigorously aerated at room temperature for 24 h. the hatched nauplii from the decapsulated cyst (more by wrapping with instar-I) were siphoned out by exposing them to light by wrapping with dark cloth at the basal region of the flask and discarded unhatched and empty shells. The nauplii were washed thoroughly in seawater and used for the enrichment experiments.

Cytotoxicity bioassay

Brine shrimp cytotoxicity bioassay was carried out using standard procedure of krishnaraju *et al*. (2005). Ten brine shrimps were transferred to the 12 well plate. Each well containing 4.5 ml of brine solution and 0.5 ml plant extracts with different concentration (0.5, 1.0, 2.5, 5.0 mg/ml) of the leaf extracts along with control (vehicle treated) with the help of a pipette. Each test consists of exposing groups of 10 *Artemia* nauplii (instar-II). They were provided sufficient light and aeration for 24 hours. The number of survivors was counted and percentages of mortality were calculated after the time period. The percentage toxicity was calculated after comparing with the untreated control (sea water) and compare to positive control (Gallic acid).

Enrichment of *Artemia* nauplii

Artemia nauplii enrichment was carried out by following the standard procedure of Sorgeloos and Kulasekarapandian (1984). After 24 h of incubation, the first instar nauplii appeared; they did not feed as their anus was still closed. After 12 h the larva molts into the second stage the nauplius (instar-II) started feeding on small particles (<50 µ) and this stage of the nauplius was enriched with aqueous leaf extract of *V. negundo*. The second-instar *Artemia* nauplii were separated from the hatching container using 120 µm sieves and transferred into a glass enrichment container at a density of 120 nauplii/ml of one liter filtered sea water, at the temperature of 25 ± 1.5°C. They were enriched for 6, 12 hrs separately with selected aqueous leaf extract of *V. negundo* at the concentrations of 2.5 mg/ml of sea water. Strong aeration was provided to keep the O_2 level at 5 ppm. Each enrichment test was performed in triplicate. The *Artemia* nauplii were collected from the enriched media. They were washed thoroughly with tap water and stored at -20°C for further use. As the nauplii were transparent, the presence of the leaf extract molecules could readily be assessed by the yellowish gut. The time required for filling the gut with phytomolecules in the nauplii was observed periodically under light microscope (Figure1).

Experimental design and feeding schedule

After measuring the weight of the fish fry, were transferred into experimental tank containing 3 liter of filtered fresh water. The experimental fish fry were fed

with 6 and 12 h enriched *Artemia* nauplii (2.5 mg/ml herbal extract/L) and the control fish fry with unenriched *Artemia nauplii*. Both the experimental and control fish fry were fed at a density of 4 nauplii per fry for, two times a day at (7 and 16 h). The unfed dead nauplii were removed from the culture tank. The experimental duration was restricted to 20 days. At the end of experiment, both control and experimental fish fry were randomly selected and the total body weight was measured. The remaining fish available at the end of experiment were counted and relative percentage survival was calculated.

Figure 1: Photomicrograph showing the gut region *Artemia* nauplii enriched with *V. negundo* leaf extract.

Assessment of growth and survival

Growth measurements such as weight of the *Poecilia sphenops* fish fry were recorded individually. At the end of the experimental period, specific growth rate (SGR) (Ricker 1979) and percent survival of fish fry fed with different groups were calculated as given below

SGR (%) =100× (1n final weight – 1n initial weight) / total duration of the experiment

where, 1n = logarithm.

Statistical analysis

Data obtained in various experimental trails i.e. growth, survival and specific growth rate were subjected to standard statistical analysis by using statistical package for social sciences (SPSS, version 17.0 for Windows, SPSS Inc., Chicago, USA) to find out the variation between the control and experimental fish fry.

RESULTS

Lethality concentration determination

After enumerating the number of *Artemia* nauplii surviving after 24 h the percentage inhibition was evaluated. The lethal concentration (LC_{50}) of the standard, gallic acid was found to be 1.0 mg. The results obtained for the bioassay with aqueous extract of *V. negundo*, the lethal concentration was found to be 2.5 mg. There was a gradual increase in the percentage inhibition with the increase in concentration of aqueous extract of *V. negundo* and from the (T_1), the lethal concentration of the methanol extract of *V. negundo* was found to be 1.0 mg (T_2), the lethal concentration of the ethanol extract of *V. negundo* was found to be 1.0 mg (T3) (Figure 2). Among the extract, aqueous extract is low toxic when compared to methanol and ethanol extract. Optimized 2.5 mg/ml aqueous leaf extract only have been selected and enriched with *Artemia* nauplii. Aqueous extract of *V. negundo* leaves, resembled a dark brown colored paste and powdered which was found to be highly soluble in water, methanol and ethanol. The plants may be considered as a biosynthetic laboratory, not only for primary chemical compounds such as carbohydrates, proteins and lipids that are utilized as food, but also for a multitude compounds like glycosides, tannins, alkaloids, volatile oils etc., that exert a physiological effect. The systemic study of the crude drug embrace through consideration of primary and secondary metabolites derived as a result of plant metabolism.

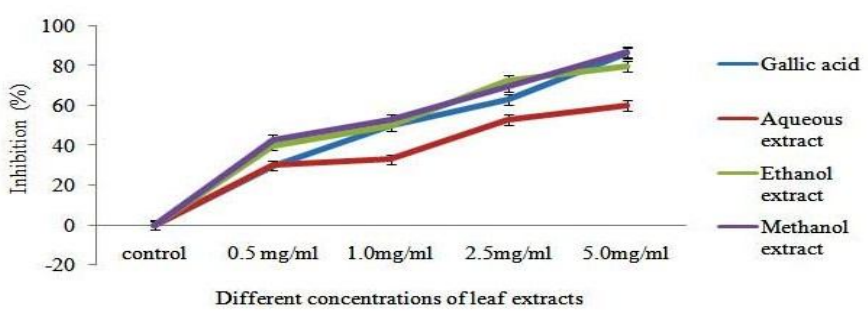


Figure 2: Cytotoxic effect of the different concentration of *Vitex negundo* leaf extracts and Gallic acid (positive control).

Growth, survival and mortality of fish fry

The present study was carried out to determine whether herbal extract of *V. negundo* leaf could induce growth of *P. sphenops* fish fry. The weight of all the experimental fish fry fed with herbal extract (6 and 12 hrs enriched nauplii at 2.5 mg/ml concentration) were observed to the higher than the control fry. The trends observed for incorporation of aqueous leaf extract into the nauplii at selected concentration against the selected durations of exposure. It is clear that exposure duration of 12 h at 2.5 mg/ml concentration aqueous leaf extract of *V. negundo* was optimum. The fish fry fed with nauplii enriched for duration of 12 hrs of aqueous leaf extract

ensured higher survival and faster growth of *P. sphenops* fish fry.

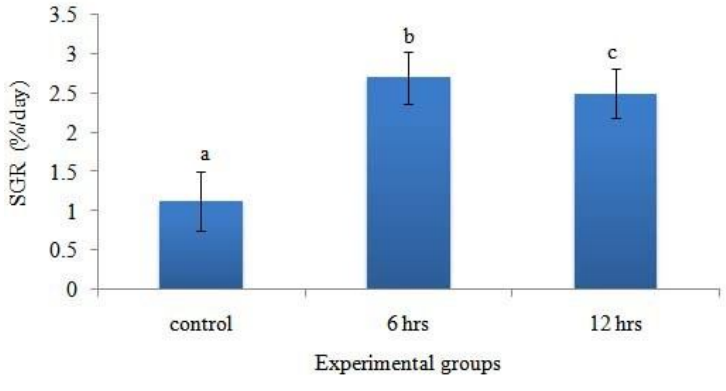
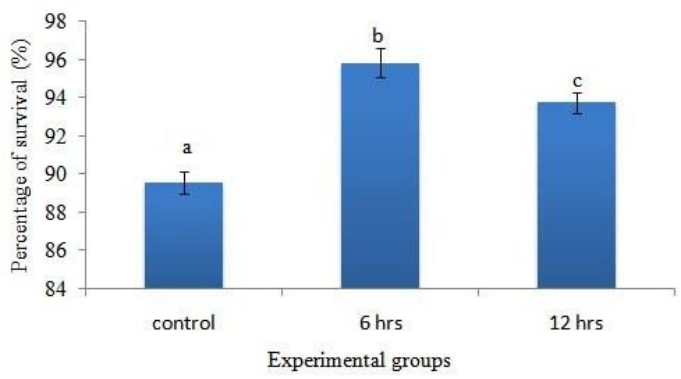


Figure 3: Graph showing specific growth rate of the fish fry *P. sphenops* fed unenriched and enriched *Artemia* nauplii with aqueous leaf extract for 20 days. Data represented as mean ± SD (P<0.05).

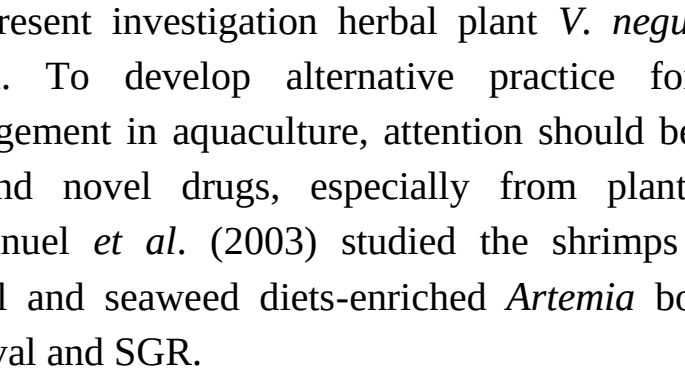
All fishes grow normally and all diet were accepted equally well by the fish, when fish was fed with selected concentration of aqueous extract. The maximum SGR was observed in experiment I (6 h) at 2.70%. The minimum SGR was observed in control as 1.13% (Figure 3). Generally, there is an increase in body weight of the fishes in control and experimental groups. Fish fry SGR gain has showed a significantly ($p<0.05$) increased in experimental I and II (6 and 12 h enriched nauplii) when compared to control (i.e. unenriched nauplii).

The maximum survival rate was observed in Experiment I value as 95.83% (6 h) *Artemia* nauplii enriched with aqueous leaf extract, whereas the minimum survival rate was observed in control as 89.58% unenriched nauplii. *P. sphenops* fish fry survival rate has showed a significantly ($p<0.05$) increased in experimental I and II (6 and 12 h enriched nauplii) when compared to control. The maximum mortality rate was observed in control value of 10.41% (6 h) *Artemia* nauplii unenriched with aqueous leaf extract. The minimum mortality rate was observed in experimental I (4.16%) enriched nauplii. *P. sphenops* fish fry mortality rate has showed a significantly ($p<0.05$) decreased in experimental I when compared to control (Figures 4 and 5).



Data represented as mean ± SD (P<0.05).

Figure 4: Survival rate of the Fish fry *Poecilia sphenops* fed unenriched and enriched *Artemia* nauplii with selected herbal extract (*V. negundo*) for 20 days.



Data represented as mean ± SD (P<0.05).

Figure 5: Mortality rate of the Fish fry *Poecilia sphenops* fed unenriched and enriched *Artemia* nauplii with selected herbal extract (*V. negundo*) for 20 days.

DISCUSSION

The research in the use of plant extracts for aquatic animals is increasing with the demand for eco-friendly and sustainable aquaculture (Lewis and Ausubel, 2006). Ayurvedic herbal compounds are having potential effect on growth and survival properties of aquatic organisms. In the present investigation herbal plant *V. negundo* were tested. To develop alternative practice for disease management in aquaculture, attention should be deviated to find novel drugs, especially from plant sources. Immanuel *et al*. (2003) studied the shrimps fed with herbal and seaweed diets-enriched *Artemia* boosted the survival and SGR.

In this study, the LC_{50} values of the brine shrimp cytotoxic effect of the different concentration of *V. negundo* leaf extracts. LC_{50} of aqueous extract was observed value of 2.5 mg/ml, LC_{50} of methanol and ethanol extracts was exhibited as same concentration of 1.0 mg/ml. Similarly results were obtained by Krishnaraju *et al.* 2005, who reported the LC_{50} values of the brine shrimp obtained for extracts of 120 medicinal plants and that of the positive control, podophyllotoxin. Alcoholic extract of *Pistacia lentiscus* showed most prominent activity with LC_{50} 2.5 µg. Michael *et al.* (1956) Vanhacecke *et al.* (1981) and Sleet and Brendel (1983) have also used the brine shrimp cytotoxicity assay.

The nutritional quality of *Artemia* nauplii is often unpredictable, thus making strain costly. Hence, several enrichment diets such as micro-algae, highly unsaturated fatty acid (HUFA)-modified yeast, compound diets coated micro particles, oil-based emulsions and microencapsulated preparation have been successfully used (Leger *et al.*, 1986). There are different approaches to present the amount of a drug in live feed. The concentrations of drugs in *Artemia* nauplii are often presented as either mg drug/mg protein or mg drug/mg dry weight (Verpraet *et al.*, 1992; Touraki *et al.*, 1995; Touraki *et al.*, 1996; Robles *et al.*, 1998; Touraki *et al.*, 1999; Mejia *et al.*, 2007). However, this is rather impractical for a fish farmer and in order to ease the calculation of correct doses of a drug to fish, prawns and shellfish larvae the use of ng or µg per nauplius is recommended (Gomez-Gil *et al.*, 2001).

Hence, in this study we recommend the concentration of aqueous leaf extract of *V. negundo* in *Artemia* nauplii as 2.5 mg/ml. Interestingly, *P. sphenops* fry fed with enriched *Artemia* nauplii with optimized dose herbal extract showed an overall increase in SGR and survival when compared to that of unenriched control. Maximum SGR were recorded in fish fry fed with enriched *Artemia* nauplii with aqueous leaf extract of 2.70 %. Similar observation was also made by Jones *et al.* 1984 with *Artemia* fed on gelatin-acacia microcapsules containing either cod liver oil or pollack oil, which significantly supported faster growth rate and higher survival in post-larval gobies than feeding with unenriched *Artemia*. Arulvasu and Munuswamy, 2009 also reported that the fish fry fed on the enriched nauplii with various oil emulsions showed an overall increase in total weight, survival and SGR, compared to those of the control. Compared to *Moina*, the use of *Artemia* nauplii for feeding would result in significant improvement in the growth performance of guppy adults and fry, and better survival rate in the adult fish (Lim et al., 2003). Therefore, the nutritional quality of *Artemia* nauplii is prime importance in aquaculture, especially at the hatchery level and accessibility of the on growth *Artemia* would offer ornamental fish farmers and exporters the possibility to apply the bioencapsulation technique to improve their fish performance and quality. In addition, the effective bioencapsulation characteristics of on-grown *Artemia* also make the organism a useful tool for larval nutrition study on fresh water ornamental fish. Hence, the present study aqueous leaf extract of *Artemia* nauplii before and after enrichment, prior to feeding the fry. Because the large amount phytomolecules present in leaf extract of *V. negundo* such as primary and secondary metabolites. So, *V. negundo* leaf extract most promisingly used as nutritional as well as enrich feed of *Artemia* nauplii.

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