ISSN 0975-6299 Vol 2/Issue 1/ Jan-Mar 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIOTECHNOLOGY

EVALUATION OF IMMUNOSTIMULANT POTENTIAL OF *SOLANUM NIGRUM* L. USING FISH, *ETROPLUS SURATENSIS* CHALLENGED WITH *APHANOMYCES INVADENS*

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ABSTRACT

In the present investigation found immunostimulant potential plants being an alternative for preventing fish diseases. Six groups of experimental fishes (*E. suratensis*) were immunized with 0.2ml (4ppm) of five different extracts of *Solanum nigrum* through intra-peritoneal injection and challenged with heat killed *Aphanomyces invadans*. Blood collected from immunized and normal fish were analyzed such as, radial immunodiffusion, antibody titration, Nitro Blue Trtrazolium Assay, determination of IgG concentration and host Resistance Test. In both control and the experimental groups the peak antibody response was on day 21 after immunization and decreased towards 28th day. The Methanol extract treated group, the antibody response was significantly enhanced on the day 14 and day 21 ($p<0.05$). The highest IgG level was on day 21 and decreased towards day 28. In Chloroform extract treated group the neutrophil activity was significantly enhanced on day 6 ($p<0.05$). In Toluene extract treated group the neutrophil activity was significantly enhanced on day 6 ($p<0.05$). The ethanol and methanol extract treated group showed less mortality rate when compared to chloroform toluene and water extract treated group. From the results it can be concluded that plant extracts have great potential as immunostimulant against microorganisms and that they can be used in the treatment of infectious diseases caused by microorganisms.

KEYWORDS

Solanum nigrum, Aphanomyces invadans, Etroplus suratensis, Immune response and diseases.

INTRODUCTION

Fish is an important part of the diet for a large proportion of the people living in the developing world. Fish food represents the primary source of animal protein for a billion people in 58 countries worldwide (FAO, 2007). The contribution of fisheries in India is very promising and important for creating job opportunities for unemployed people, earning foreign exchange, alleviating poverty and improving nutritional status of the people (Subasinghe, 2005). By 2030, the addition of 2 billion more people to the world population will mean that aquaculture will need to produce nearly double that, 85 million tones of fish per year, just to maintain current consumption levels (FAO, 2007).

Etroplus suratensis is a freshwater fish also known as the pearlspot found in southern India. The most severe impact of EUS has probably been on small-scale, mixed species fisheries and aquaculture activities in rice-fields and rural waterways. At present there are no systemic treatments for use against EUS in fish. The humoral and cellular immune response of fish is known to be supressed at low temperatures (Avtalion *et al.*, 1980), which may explain why mortalities from EUS occur when water temperatures are low. Naturally and artificially infected snakeheads have been shown to produce an antibody response against *A. invadans* (Thompson *et al.*, 1997), and the cellular macrophage response is also considered to be important in enabling fish to resist infection (Wada *et al.,* 1996). To tackle the problem of disease it is essential to vaccinate the fishes. The test plant, *Solanum nigrum* L. (Plate 1) posses high antiulcerogenic and ulcer healing effect were studied by Jainu *et al*., (2006). Plant based drugs to be used for immunotherapy against variety of diseases. Use of plant extracts for controlling microbes had be a major option worldwide. Many intermediate metabolites produced by plants possessed antibiotic properties. Common endemic plant species were not often properly analyzed for the presence of effective metabolites (Dhasarathan *et al.,* 2010). Hence, in the present study to investigate immunostimulation are being used as an alternative for preventing fish diseases.

MATERIALS AND METHODS

Collection and acclimatization of test fish, E. suratensis: Thirty numbers of juvenile fishes (25 ± 5 g), *E. suratensis* were obtained from three different locations (Tamirabarani, Bhavanisagar and Kottayam) of South India. The experimental fishes were kept in plastic tubs (30 Liter capacity). The fishes were acclimatized for two weeks and fed with balanced nutritious fish feed. The animals are sensitive to accumulation of ammonia and hence the tub water had to be changed frequently by the method of Sailendri (1973).

***Isolation of Aphanomyces invadens from EUS affected fish*:** EUS affected gold fish (*Carassius auratus*) obtained from Kurinji Aquarium, Thisaiyanvillai (Plate - 2) was killed and pinned, with the lesion uppermost, to a dissecting board. Using aseptic technique, carefully excised up to 4 pieces of affected muscle, approximately 2 mm^3 and placed them on a Petri dish containing the isolation medium (GP agar medium). Inoculated media are incubated at approximately 25°C and examined under an inverted microscope within 12 hours. Emerging hyphal tips were repeatedly transferred to fresh plates of GP agar until cultures are free of bacterial contamination. They may then be sub-cultured on GP agar at intervals of no greater than 5 days.

Inducing sporulation in Aphanomyces invadans cultures: Sporulation was induced by placing an agar plug (3-4 mm in diameter) of actively growing mycelium in a Petridish containing GPY broth and incubated for 4 days at approximately 20ºC. After 12 hours, the formation of achlyloid clusters of primary cyst and the release of motile secondary zoospore was observed under microscope.

Identification of Aphanomyces invadans: Aphanomyces invadans does not produce any sexual structures and can not be diagnosed by morphological criteria alone. Fish was experimentally infected by injecting intramuscularly a 0.1mL suspension of motile zoospores in ESU susceptible healthy fish at 20 ºC. Autoclaved pond water injected to a group of fishes was served as a positive control. The lesions were developed after 16th day of injection.

Plant extracts: Fresh *solanum nigrum* leaves were collected during the month of December (2009) from the outskirts of Tirunelveli, South India. The leaf material was washed in distilled water. It was dried under shade and then coarsely powdered. 10 g of this powdered plant material was soaked in 200mL toluene for 2 days and then extracted in Soxhlet apparatus with five different solvents (Toluene, Chloroform, Ethanol, Methanol and Water) for 6 hours each. The dried extract was weighed and then kept at −4 °C until ready for use.

Experimental groups and Immunization: Six groups of experimental fishes were maintained. Five groups of fishes immunized with 0.2ml (4ppm) of five different extracts (Methanol extract, Ethanol extract, Chloroform extract, Toluene extract, Water extract) of *Solanum nigrum* through intra-peritoneal injection using 1mL glass syringe with 24 gauge needle. A group of fishes used as control had received same amount of saline. After two weeks all the fishes including control group were challenged with heat killed *Aphanomyces invadans* intra peritonealy.

Blood collection: The fishes were bleed serially using 1 mL tuberculin syringe with 24 gauge needle from the common cardinal vein situated just below the gills (Michel et al., 1994) at regular intervals of seven days for antibody response till 28th day and intervals of 2 days for lysozyme and neutrophil assay till 10th day. Blood collected from immunized and normal fish was kept at sterilized vials and stored in freezer at -20ºC until use.

Immunological assays

Radial immunodiffusion: 10 mL of 0.1% agarose was heat dissolved in 1X assay buffer, 120µL of antiserum was added to the 6mL of agarose. Wells were cut on the gel using gel puncher. Different concentration of antigen (*Aphanomyces invadans*) was added to the wells. The plate was kept in a moist chamber (box containing wet cotton) and incubated overnight at room temperature. A graph was plotted against diameter of the ring and the concentration of the antigen.

Antibody titration: Antibody titration was performed in 96 well “V” bottom microtitre plates. 25µL of serum was added to the first well and two fold serial dilutions were made with PBS. A volume of 25µL of heat killed bacterial cell suspension prestained with crystal violet was added to each well. The plate was hand shaken for effective mixing and incubated for overnight at 35ºC. The highest dilution of the serum sample which showed dectable macroscopic agglutination was recorded and expressed as $\log_2$ antibody titer of the serum.

Nitro Blue Trtrazolium Assay (NBT assay): The NBT assay followed was of Anderson 1992 except that distilled water instead of saline used to prepare NBT solution. 50µL of blood from common cardinal vein was mixed with 50µL of heparinised saline and placed on a coverslip for 30 minutes. The excess cells were washed out by PBS. The coverslip were turned upside down onto a drop of (50µL) the NBT solution in a glass microscope slide. The slides were incubated for 30 minutes. Then were examined under a

light microscope, 5 random fields of positive, dark blue stained cells were observed for each coverslip. Activated neutrophils in each field were added together to give a total number of cells per slide.

Determination of IgG concentration: The protein A column was washed with 1mL of 1X equilibration buffer and the flow rate was adjusted to <0.25mL/miunte. The serum with equal volume of equilibration buffer was added to the column. 500µL of equilibration buffer was passed through the column and discarded then 1mL of elution buffer was added to the column and elute containing IgG was collected in tube containing neutralizing buffer (25µL/mL). This process was repeated for 2-3 times. The optical density was read at 280nm.

Host Resistance Test: Group of fishes were administered with different extracts of *Solanum nigrum* and challenged on 21st day by intra peritoneal injection of live *Aphanomyces invadens*. Earlier the challenge dose was standardized to give 90% mortality in control group. 15 fishes were used for each test and 96 hours mortality rate of each group was recorded. The relative percentage survival was calculated using the following formula

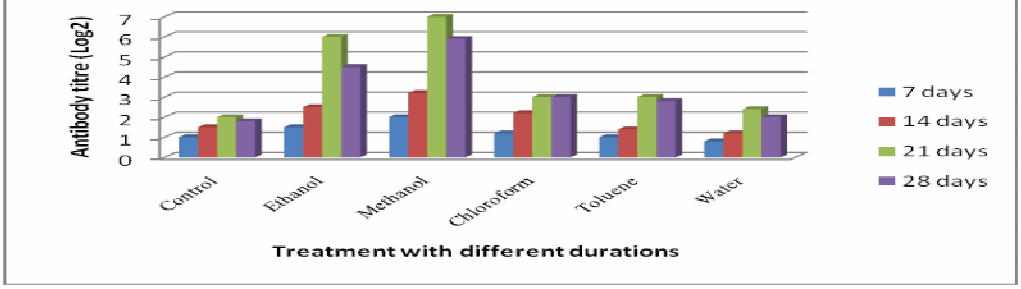
Relative % survival = {1 – (% of experimental mortality/ % of control mortality) X 100}

RESULT AND DISCUSSION

Isolation of EUS pathogens: Fish injected with the two *Aphanomyces invadans* isolates started to develop clinical lesions by day 16 and by day 20, fungus was seen erupting from lesions at the site of injection. Lesions progressed from small (1-2 cm) dermal ulcers with associated haemorrhaging, to large necrotic ulcers, with fungal growth penetrating deep into the musculature. Lesions were occasionally present near the tail and on the opercula of infected fish. Fish injected with autoclaved pond water did not develop lesions and no significant pathological signs were observed. The similar findings also observed by Willoughby *et al*., (1995) and the current work showed to support that *Aphanomyces invadans* is the major causative agent of epizootic ulcerative syndrome. *Aeromonas hydrophila* generally considered to be a secondary invader in red sore disease, in which the primary etiological agent was believed to be the fungus *Aphanomyces invadans* (Rogers, 1971).

Antibody response in E. suratensis: In both control and the experimental groups the peak antibody response was on day 21 after immunization and decreased towards 28th day. The Methanol extract treated group, the antibody response was significantly enhanced on the day 14 and day 21 ($p<0.05$). In Ethanol extract treated group, the antibody response was significantly enhanced on day 14 and day 21 ($p<0.05$). In Chloroform extract treated group, the antibody response was significantly enhanced on the day 14 and day 21 ($p<0.01$). In Toluene extract treated group, the antibody response was significantly enhanced on the day 14 and day 21 ($p<0.01$). In water extract treated group, the antibody response was not significantly enhanced (Figure 1).

Figure 1: Effect of *Solanum nigrum* on the antibody response in *E. suratensis*



The mechanisms of innate resistance and adaptive immunity are interdependent. Cellular interactions through cytokines, antibodies, complement and their corresponding surface receptors represent communication elements in the cross-talk between the two types of pathogenic resistance. Fungal growth of spores in the lung or the skin produces fungal particles and soluble antigens that are presented by appropriate APCs as a consequence of phagocytosis. This activates specific T-cells and cytokine secretion, leading to antibody production by B-cells. Thus, cellular and humoral immune responses are generated (Romani, 1997).

Only methanol and ethanol extracts have shown significant antibody production when compared to chloroform extract and toluene extract. The water extract didn't show a significant response. These results shows that the leaf extracts of *S. nigrum* stimulated the antibody production against *Aphanomyces invadans* therefore against epizootic ulcerative syndrome.

Determination of IgG concentration in antiserum: The highest IgG level was on day 21 and decreased towards day 28. This results shows that the antibody production with increased IgG concentration. These results proved that the antibody production increases with the increasing IgG production (Table 1). It has been shown that the great majority of patients suffering from fungal infections, as well as many normal, healthy individuals, develop serum IgG against fungal antigens (Saral, 1991).

Table 1

Serum IgG level in E. suratensis administered with test plant extracts.

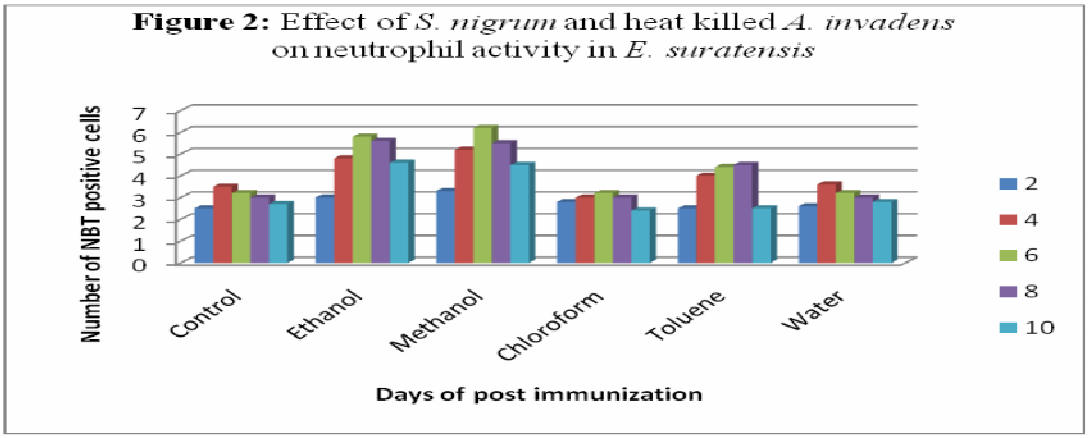
S.no	Sample	Day	OD at 280nm	IgG level mg/ml
1	Methanol extract	7	1.022	15.12
2	Ethanol extract	7	1.005	14.87
3	Chloroform extract	7	1.000	14.80
4	Toluene extract	7	0.654	9.67
5	Water extract	7	0.329	4.86
6	Control	7	0.258	3.81
7	Methanol extract	14	2.358	34.89
8	Ethanol extract	14	2.547	37.69
9	Chloroform extract	14	2.142	31.70
10	Toluene extract	14	1.245	18.42
11	Water extract	14	0.243	3.59
12	Control	14	0.257	3.80
13	Methanol extract	21	3.458	42.29
14	Ethanol extract	21	3.528	37.41
15	Chloroform extract	21	2.478	36.67
16	Toluene extract	21	2.682	27.85
17	Water extract	21	1.568	23.20
18	Control	21	1.257	18.60
19	Methanol extract	28	2.168	32.08
20	Ethanol extract	28	2.895	42.84
21	Chloroform extract	28	1.358	20.09
22	Toluene extract	28	1.247	18.45
23	Water extract	28	0.259	3.88
24	Control	28	0.235	3.47

The effect of Solanum nigrum (leaf extract) on neutrophil activity in E. suratensis: Nonspecific cellular immunity, mediated by macrophages and neutrophils, is considered to provide the first two lines of defence against fungi and is part of the innate resistance. Macrophages and neutrophils activated by Th1 cells products appear to be the major cell involved in clearing up fungal infections (Ranjithsingh *et al.,* 2004).

The super oxide anion is the first product to be released from the respiratory burst. The reduction of NBT is used to measure the intracellular oxygen produced by the activated neutrophils which will show the blue halo surrounding them. Thus this method allows the enumeration of NBT positive cells.

In Methanol extract treated group the neutrophil activity was significantly enhanced on day 4 and day 6 ($p<0.05$). In ethanol extract treated group the neutrophil activity was significantly enhanced on day 4 and day 6 ($p<0.05$). In Chloroform extract treated group the neutrophil activity was significantly enhanced on day 6 ($p<0.05$). In Toluene extract treated group the neutrophil activity was significantly enhanced on day 6 ($p<0.05$). In water extract treated group the neutrophil activity was not significantly enhanced ($p>0.05$) (Figure 2).

Figure 2: Effect of *S. nigrum* and heat killed *A. invadens* on neutrophil activity in *E. suratensis*



There is experimental evidence that neutrophilic granulocytes provide reliable protection against hyphae. Indeed, neutrophil is considered the strongest predisposing factor for invasive fungai (Saral, 1991).

Host resistance test: The ethanol and methanol extract treated group showed less mortality rate when compared to chloroform toluene and water extract treated group. The water extract treated group showed the mortality rate almost similar to the control group. This showed the water extract did not stimulate the immune response whereas the ethanol and methanol extract treated group showed good results (Figure 3).

Figure 3: Effect of *S. nigrum* on disease resistance mortality in *E. suratensis* challanged with *A. invadens*

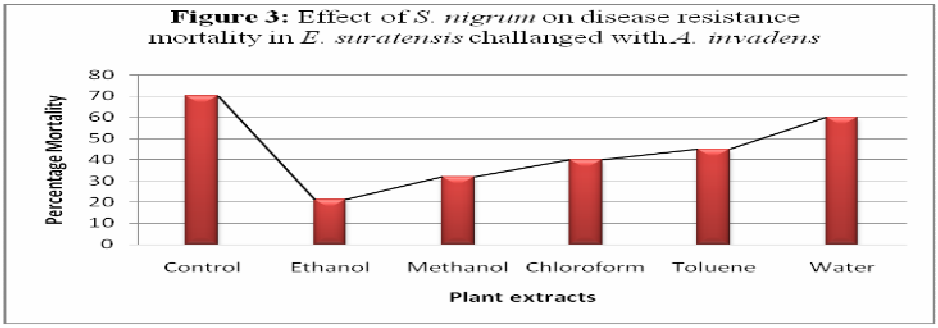


Plate 1
Test plant - Solanum nigrum

Plate 2
EUS affected Gold fish

Plate 2a
Experimentally infected Fish

Studies conducted on the immunostimulatory properties of Solanum *nigrum* showed that the various extracts of *Solanum nigrum* stimulated both the specific and non specific defence mechanism. They also had shown to give protection against *Aphanomyces invadans.* The ability of macrophages to kill microbes is a very important mechanism of protection against disease (Secombes, 1994). Enhancement of the immune response by Solanum nigrum may be correlated to its capacity to stimulate number of activated neutrophils thereby enhancing the non specific immune response.

The immune system was significantly enhanced by Solanum nigrum leaf extract. The methanol and ethanol extracts showed significant Immunostimulation whereas the chloroform and toluene extract showed comparatively less effect and the water extract did not show significant response. Immunostimulators such as *Solanum nigrum* induce both specific and non specific immune response which in turn helps in preventing epizootic ulcerative syndrome in fishes. More research and developmental work on immunostimulation by plant products is needed if immunostimulation would contribute to the development of the aquaculture industry.

Immunostimulation is one element in a strategy to achieve microbial control. Direct stimulation of nonspecific immunity and stimulation of specific defence mechanism and seem to be the most promising methods for preventing microbial diseases. Based on available knowledge, it is concluded that although this technique is still in its infancy, it

may be a potential immunostimulant. Immunostimulation of fish has a considerable potential for reducing losses in aquaculture, during both larval and on-growing stages.

From the above results it can be concluded that plant extracts have great potential as immunostimulant against microorganisms and that they can be used in the treatment of infectious diseases caused by microorganisms. This plant can be used to discover bioactive natural products that may serve as leads for the development of new pharmaceuticals that address hither to unmet therapeutic needs.

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