Short Paper

Immunostimulatory effect of the aqueous leaf extract of *Phyllanthus niruri* on the specific and nonspecific immune responses of *Oreochromis mossambicus* Peters

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Summary

Plant derived immunostimulants are a promising alternative to chemotherapeutics and also perhaps vaccines. In the present study, we examined the immunostimulating properties of aqueous leaf extract of *Phyllanthus niruri*, an Indian traditional medicinal herb, on neutrophil activation and antibody response of *Oreochromis mossambicus* (Peters). Serial ten-fold diluted doses of *P. niruri* ranging from 0.002 mg to 20 mg were administered to two groups of *O. mossambicus* (n=8). One group of fishes was administered with sheep red blood cells and the primary and secondary antibody responses were estimated using direct haemagglutination assay. The other group of fishes was administered heat-aggregated BSA to assess the ability of plant extract to elicit neutrophil activation. Our results indicate a significant enhancement of both neutrophil activation and antibody response. Among the various doses tested, fishes administered 20 mg/kg body weight caused the maximal enhancement of both primary and secondary antibody response and 0.002 mg/kg showed higher neutrophil activation compared to that of the control group. This short study indicates that aqueous leaf extract of *P. niruri* has the potential to be used as an immunostimulant and after confirming its immunostimulatory properties by a battery of tests on other nonspecific and specific parameters and disease-protective property by challenging the fish with virulent fish pathogens, it can be used either as a routine feed supplement to activate the immune system of farmed fishes or as an adjuvant to enhance the efficacy of vaccines.

Key words: Antibody response, Aqueous extract, Immunostimulant, Neutrophil activation

Introduction

Aquaculture has been gaining importance over capture fisheries since 1990 and growing at a rate of 6% annually (Reverter *et al*., 2014). Poor culture practices, overcrowding the fish, poor water quality, etc. causes diseases and disease-outbreaks in aquaculture on a large scale (Reverter *et al*., 2014) resulting in substantial loss in production (Gabriel *et al*., 2015).

Plant derived immunostimulants are better alternatives to conventional chemotherapeutics and antibiotics (Vaseeharan and Thaya, 2014). Immunostimulation by plant extracts are better because they confer protection to fish against a wide range of infectious agents compared to vaccines that are specific to single pathogenic organism (Anderson, 1992).

Phyllanthus niruri is an important ethno-botanical species of India and widely used in Ayurveda formulations (Narendra *et al*., 2012). *Phyllanthus niruri* was shown to possess anti-hepatitis (Venkateswaran *et al*., 1987) activity and diuretic properties (Boim *et al*., 2010). The phytochemicals present in *P. niruri* and their pharmacological effects were reviewed elsewhere (Bagalkotkar *et al*., 2006). In the present study, we demonstrate the efficacy of aqueous extracts of *P. niruri* leaves in positively modulating specific and nonspecific immune responses of *O. mossambicus*.

Materials and Methods

Oreochromis mossambicus of both sexes weighing 25-30 g were collected from a local fish farmer. All the experiments were carried out in circular plastic tanks of 70 L capacity at ambient temperature with daily renewal of water; fishes were fed *ad libitum* with a balanced diet prepared in this laboratory (Table 1).

Leaves of *P. niruri* were purchased from a local traditional medicinal plant vendor. Aqueous extract of *P. niruri* was prepared according to our earlier protocol (Logambal *et al*., 2000).

Sheep red blood cells (SRBC) were used as the antigen for the studies on antibody response. Blood was collected from the jugular vein of a sheep and SRBC was prepared according to our earlier protocol (Logambal *et al*., 2000). Heat aggregated-bovine serum albumin (HA-BSA) was used for neutrophil activation assay and prepared according to the protocol of Nakano (1976).

Fishes were administered intraperitoneally with 0.2

ml of saline containing five different doses of the aqueous extract of *P. niruri* with 10-fold dilution that corresponds to 20 mg to 2 µg (w/v) dose range. All the injections were made with 1 ml tuberculin syringes fitted with 24 gauge needle. Control group received 0.2 ml saline.

Table 1: Preparation of balanced fish diet. The ingredients are dried and powdered separately, sieved through fine strainers and autoclaved. Finally, 10 g vitamin mix was added to mixture and a dough was prepared using 300 ml double distilled water. The dough is then pressed through fine pored plate, dried, and stored in air-tight container at 15°C

Ingredients	Quantity in gram
Dried fish	420
Groundnut oil cake	200
Blood meal	50
Tapioca	150
Wheat flour	150
Mineral mix	20

Two days after the administration of plant extracts, experimental fishes were primed with 0.1 ml of 5% SRBC intraperitoneally. After three days, a booster dose of 0.1 ml of 25% SRBC was administered. To investigate secondary response, the same priming and booster doses were administered intraperitoneally after sixty days post primary challenge.

Fishes were bled (0.1-0.2 ml) repetitively from common cardinal vein with an interval of five days for 85 days after antigen priming (Michael *et al*., 1994). Serum was separated and complement was inactivated as described elsewhere (Logambal *et al*., 2000) and stored at -20°C until used. Primary and secondary antibody responses were measured according to our earlier protocols (Logambal *et al*., 2000).

To estimate the number of activated neutrophils, another set of six groups (n=8 per group) of fishes were intraperitoneally administered the same doses of the aqueous extract as that of the previous experiment, two days prior to antigen challenge. Untreated control group received 0.2 ml of saline. One hundred µL of prepared HA-BSA (5 mg) was injected intraperitoneally to elicit neutrophil activation. 0.5 ml of blood samples were withdrawn from the common cardinal vein using 1 ml tuberculin syringe containing 0.5 ml heparinized saline (40 IU heparin/ml) at 2 days intervals after the challenge. Activated neutrophils were counted using the method described previously (Venkatalakshmi and Michael, 2001).

Results

Only the highest dose of 20 mg leaf extract was able to elicit significantly higher primary (Fig. 1) antibody titres compared to those of other doses and untreated control group. The secondary antibody response (Fig. 2) on the peak day (day 15) was significantly enhanced by different doses of *P. niruri* aqueous leaf extract (20, 2, and 0.002 mg) compared to untreated control group.

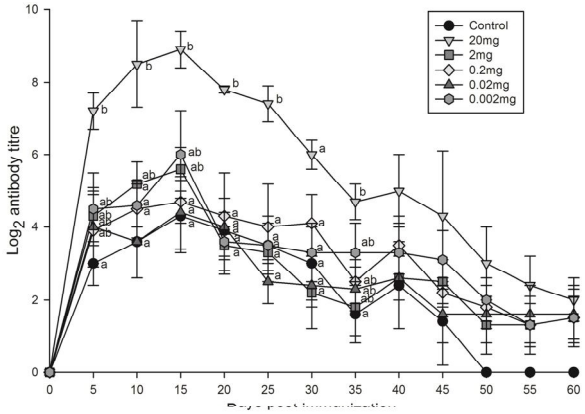

Fig. 1: Primary antibody response of *Oreochromis mossambicus* treated with various doses of *Phyllanthus niruri* aqueous leaf extract against SRBC. Each point represents mean±SE of eight fishes; a posteriori Tukey comparison of control and treated groups on particular days shown with different alphabets represents significant difference (P<0.05)

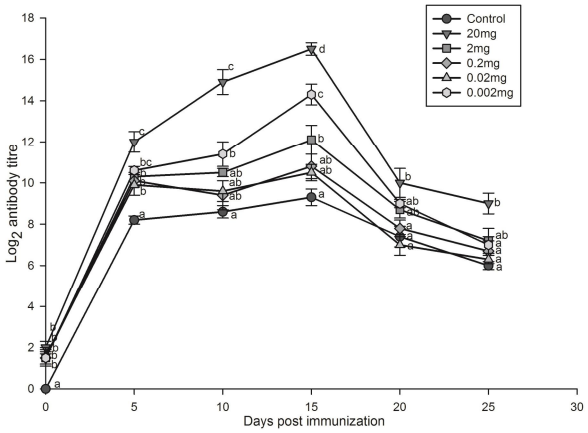

Fig. 2: Secondary antibody response of *Oreochromis mossambicus* treated with various doses of *Phyllanthus niruri* aqueous leaf extract against SRBC. Each point represents mean±SE of eight fishes; a posteriori Tukey comparison of control and treated groups on particular days shown with different alphabets represents significant difference (P<0.05)

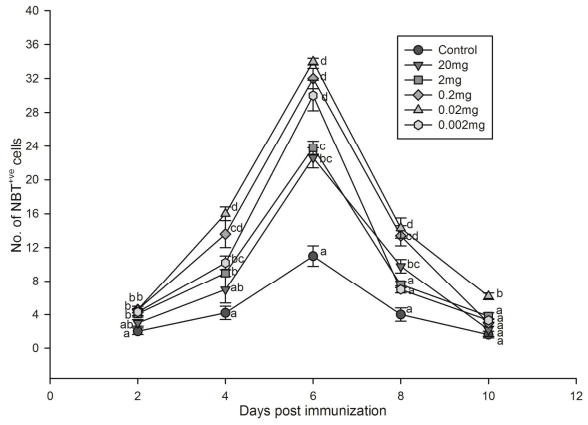

Fig. 3: Effect of *Phyllanthus niruri* aqueous leaf extract on neutrophil activity of *Oreochromis mossambicus* as evidenced by the number of NBT positive cells. Each point represents mean±SE of eight fishes; a posteriori Tukey comparison of control and treated groups on particular days shown with different alphabets represents significant difference (P<0.05)

Neutrophil activity was enhanced several fold by all doses of the extract tested (Fig. 3). The highest number of activated neutrophils was observed in fishes administered with 0.02 mg extract followed by 0.2 and 0.002 mg.

Discussion

Our study shows that the primary and secondary antibody responses of *O. mossambicus* were enhanced with aqueous extracts of *P. niruri*. This is in agreement with another study where the methanol extracts of *P. niruri* showed increased primary and secondary antibody titres of rats against SRBC (Eze, 2014). Aqueous extract of *P. niruri* is a potent murine lymphocyte mitogen (Nworu *et al*., 2010b) and improved the antigen presentation capability of dendritic cells (Nworu *et al*., 2010a). This may be an explanation of the increased production of antibodies by aqueous extract of *P. niruri*. *Phyllanthus niruri* also activated neutrophils at all the doses tested. Lower doses caused significantly higher activity than that of the higher doses which is similar to the study conducted with Levamisole (Kajita *et al*., 1990).

Though proved to be an immunostimulant in other species, serious studies on the effect of *P. niruri* on fish immunity are lacking. *Phyllanthus niruri* has been shown to possess antimicrobial activity against the dominant fish pathogen, *Vibrio harveyi* (Punitha *et al*., 2008). Preliminary studies of *P. niruri* in *O. niloticus* showed antihyperglycemic properties (Ibrahim *et al*., 2015) and improved haematological parameters in *Labeo rohita* (Annalakshmi *et al*., 2013).

This stimulation of immune mechanisms in fish by *P. niruri* most likely protects them from infection from pathogens. However, studies on other non-specific and specific parameters and also on functional immunity/ disease resistance involving pathogen challenge experiments may bring out the efficacy of this traditional medicinal plant and its applicability in aquaculture.

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