Determination of Effective Dosage of *Phyllanthus niruri* to Modulate Stress in Tilapia, *Oreochromis niloticus*

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Abstract A study was performed to determine the effects of supplementing *Phyllanthus niruri*, an herbal medicinal plant found worldwide and its optimal dosage to modulate stress in tilapia. *Phyllanthus niruri*-extract from three different sources (suppliers) was given to study fish through supplementation to commercial fish feed at the rate of 1%, 2%, 5%, and 10% concentration. Administration of 5% *Phyllanthus niruri* from Source B significantly reduced blood glucose in fish.

Keywords Tilapia, Stress, Nutraceutical, *Phyllanthus*, Glucose

1. Introduction

The world's population is rising and the subsequent demand for high protein sources of food is rising with it [1, 2, 3]. Aquaculture offers a solution to these issues by providing a high protein food that requires less space to farm, a faster harvest time, lower cost to produce, imparting minimum threat to the environment.

With the increase in intensification of aquaculture chances of fish getting diseases are also on the rise. Antibiotics, therefore, are excessively in use to cope with the disease prevalence and subsequent mortality. When stressed, fish become susceptible to diseases that can spread rapidly in close quarters [4, 5]. In order to provide solutions to these problems of farming and the needs of the people without the use of potentially harmful substances many researchers are looking into the use of nutraceuticals in feed in order to decrease stress responses, increase immune responses, increase growth, and increase the nutritional value of farmed fish.

Many researchers have been using herbal plants as nutraceuticals (feed additives) to modulate stress and boost the immune system [6, 7]. Natural plant products have been reported to have various properties, such as stress reduction, growth promotion, appetite stimulation, and immunostimulation in fish and shellfish due to their active

components, such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils [6, 7].

Phyllanthus niruri, also known as stone breaker, is an herbal medicinal plant under Euphorbiaceae family, available in tropical areas and Amazon. Human being has been using it to relieve pain and inflammation, cure viral and bactericidal diseases, and reduce glucose levels in blood [8]. From research, better growth and survival rate were also reported when Indian carp Rohu, *Labeo rohita*, [9] and Goldfish, *Carassius auratus* [10] were fed *Phyllanthus niruri* supplemented feed. Goldfish, *Carassius auratus* [10] also showed their resistance to *A. hydrophila* infections when given feed supplemented with *Phyllanthus niruri* [10]. But there was no research done on reducing stress using the same supplement.

Hyperglycemia, increased level of glucose, is a common indicator of acute stress for fish. So, any ingredients which can induce the reduction of glucose level in fish can be considered as stress reliever [11]. As it has been established that *Phyllanthus niruri* can reduce the blood glucose level in higher vertebrates [7], we want to investigate if it can also reduce the stress in lower vertebrates, such as fish, and thereby increase the immune response. Since, our study will be the first of a kind, we decided to run a pilot study to determine the right dosage of *Phyllanthus niruri* to produce the best response. We have chosen tilapia as the fish model since tilapia, *Oreochromis niloticus*, is widely used in aquaculture industries throughout the world.

2. Materials and Methods

2.1. Experimental Design

Disease free juvenile tilapia (*Oreochromis niloticus*) with average weight of 38 ± 2 g and average length of 13 ± 2 cm were obtained from a certified fish hatchery and kept under optimal conditions in a recirculating system for acclimation. Once, acclimated (over a 4 weeks period), fish were randomly assigned to four treatment groups, each group with two replicates: Control feed, Supplemented feed from

Source A, Supplemented feed from Source B, and Supplemented feed from Source C. Each treatment group was further divided into two rearing environments: Controlled environment (fish kept in optimal density; 5 g/l) and Stressed environment (fish kept in crowded density; 30 g/l). All fish were given controlled feed for first week and then controlled feed with supplemented nutraceutical (*Phyllanthus niruri*) for the second week at the concentration of 1%, 2%, 5%, and 10% to determine the best source and the best concentration (dosage).

Commercial feed (Aquamax Fingerling Starter 300) was purchased from PMI Nutrition International (MO, USA) and *Phyllanthus* extracts were purchased from three different suppliers. Extracts were mixed with the commercial feed at 1%, 2%, 5%, and 10% of total ingredients by weight thoroughly and homogenously. The feed was then allowed to dry for 24 hours before being stored in airtight containers. All groups of fish were fed once daily to satiation and the amount of food recorded by weight in grams. The trial was run for 2 weeks. Fish were sampled at day 0, day 7, and day 14 to measure the levels of stress namely, blood levels of glucose as glucose is known as the valid indicator stress in fish [16].

2.2. Fish Maintenance

Water quality (dissolved oxygen, pH, ammonia) was monitored and maintained at the optimal level regularly throughout the experimental period. All experiments were conducted following good laboratory practices and fish were cared for according to the guidelines of the Purdue Animal Care and Use Committee.

2.3. Sampling

A sample of six fish from each group was taken and euthanized using Tricane Methane Sulphonate (MS222) @ 200 mg/l within 2 minutes of catching to reduce handling stress. Blood glucose concentration was determined using a glucometer (Freestyle, Abbott Laboratories; Abbott Park, IL) as validated by Wedemeyer et al. [12].

2.4. Data Analyses

The data obtained was analyzed using SigmaPlot® 12.5, 2013. The means and standard errors of the means were calculated for each assay by one way analysis of variance (ANOVA), and differences were considered significant when $P < 0.05$. Tukey's comparison of multiple means test was performed post-ANOVA to determine any differences between groups. The graphs included present means $\pm$ standard errors of the means.

3. Results and Discussion

Surges in blood glucose concentrations within stressed fish are hallmark indicators of the stress response within fish [12, 13, 14]. Elevated plasma glucose levels have been associated with reduced coronary blood flow [15] and impaired immune response [16]. In our study, supplementation of *Phyllanthus niruri* to commercial fish feed attenuated blood glucose concentrations to levels similar to the glucose concentrations seen in day 0 samples is concurrent with prior research on glucose homeostasis [17].

While blood glucose concentrations showed significant decline by different concentrations of different sources, indicating *Phyllanthus niruri*'s antihyperglycemic properties, 5% concentration worked best over the study period. We believe the supplemented *Phyllanthus niruri* enhances plasma glucose reuptake within the cells of the fish; regardless of stress. A reduction in blood plasma can lead to greater energy expenditure by fish to pump blood to their tissues, therefore leading to elevated plasma glucose levels and in extreme cases, exhaustion [13, 14]. Furthermore, stress induced hyperglycemia can lead to insulin-resistance which can reduce blood flow within skeletal tissues [18]. Studies on other animal models, along with its antihyperglycemic properties, *Phyllanthus niruri* has been shown to induce vascular relaxation in arteries while enhancing greater blood flow through induction of the synthesis of endothelial nitrous oxide synthase within arteries, and offered protection against hypercholesterolemia within mice [19].

Based on these finding, we are now conducting a detailed study on the effects of *Phyllanthus niruri* on the modulation of stress and immune response in tilapia raised in aquaculture conditions.

Table 1. Effects of different concentrations of *Phyllanthus niruri* extracts obtained from three different suppliers on blood glucose of tilapia reared in two different environments (controlled with optimal density and stressed with high density). Column 7 indicates % changes in blood glucose concentrations (+/-) (compared between Day 7 and Day 14) incorporated into 'heat-maps' (smaller bar indicates bigger reduction) and Column 8 indicates P values of the same.

Supplement Type	Supplement Conc.(%)	Fish Culture Environment	Conc. Of Blood Glucose (mg/dl)			% change in Blood Glucose (Day7 to 14)	P-value
			Day 0	Day 7	Day 14		
Control	0	Control	29	33.33	34.78	4.35	0.009
Control	0	Stressed	29	34.05	40.66	19.41	0.024
Source A	1	Control	29	34.33	35	1.95	0.561
Source A	1	Stressed	29	25.33	32.67	28.98	0.137
Source A	2	Control	29	30	28	-6.67	0.07
Source A	2	Stressed	29	40.67	29.33	-27.88	0.09
Source A	5	Control	29	34	26	-23.53	0.0006
Source A	5	Stressed	29	44.67	28.33	-36.58	0.0009
Source A	10	Control	29	36	27	-25.00	0.0003
Source A	10	Stressed	29	63.33	37.33	-41.05	0.169
Source B	1	Control	29	33.33	26	-21.99	0.002
Source B	1	Stressed	29	43.33	50.33	16.16	0.62
Source B	2	Control	29	29.67	32.33	8.97	0.099
Source B	2	Stressed	29	26.33	28	6.34	0.796
Source B	5	Control	29	37	21.67	-41.43	0.00002
Source B	5	Stressed	29	40	42.33	5.83	0.857
Source B	10	Control	29	24	29.66	23.58	0.0058
Source B	10	Stressed	29	47.33	70.33	48.59	0.6
Source C	1	Control	29	31	27	-12.90	0.008
Source C	1	Stressed	29	78.33	51	-34.89	0.487
Source C	2	Control	29	25	25.33	1.32	0.768
Source C	2	Stressed	29	44	38.33	-12.89	0.497
Source C	5	Control	29	26	28	7.69	0.077
Source C	5	Stressed	29	49.33	31	-37.16	0.341
Source C	10	Control	29	30.67	28	-8.71	0.467
Source C	10	Stressed	29	53.33	60.67	13.76	0.399

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