EFFECTS OF HOLY BASIL (*Ocimum sanctum* Linnaeus) ON THE MODULATION OF STRESS IN NILE TILAPIA (*Oreochromis niloticus* Linnaeus)

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Abstract

Fish in aquaculture face stress, which is a major concern due to its effects on the overall well-being of the fish. To modulate the stress responses, researchers are turning to the use nutraceuticals rather than chemical drugs. In a six-week long study, holy basil (*Ocimum sanctum*) was used to observe its effects on stress in Nile tilapia (*Oreochromis niloticus*). Stress was induced by adding cortisol with the feed as a supplement. Three different treatments (control, stress, stress- basil), each with two replicates, were used to measure serum cortisol, lysozyme activity, macrophage phagocytosis, spleen somatic index, and condition factor. Basil had no significant effect on any of the parameters when comparing the stress and stress basil treatments. But the result showed significant imperative effect of stress due to cortisol supplement in both stress and stress-basil groups. The results suggest that basil may have potentials to modulate the stress response in Nile tilapia.

Key words: Aquaculture; Nutraceuticals; Cortisol supplement; Stress response.

INTRODUCTION

Stress is a response of an organism to a stressor that alters the organism's homeostasis (Tort 2011). Aquaculture practices either remove the fish from their natural habitat so they can live in raceways, aquaria, tanks, etc., or confine them in cages in oceans, ponds, etc. Since these fish are either no longer in their natural habitat or confined within it, the change in their environment causes stress. In nature, severe stressors that are detrimental to fish health are rare since these challenges are often acute. In aquaculture, however, fish are exposed to more chronic stressors than in nature. These chronic stressors are due to human interactions with the fish (Pickering 1989), such as handling, sorting, grading, transportation, crowding, etc. Chronic stressors affect fish health in many ways, but the most important one is the suppression of the immune system which makes fish susceptible to infectious diseases (Barton and Iwama 1991).

Fish, when exposed to pathogens, have an increased rate of mortality due to cumulative effects of stress (Barton and Iwama 1991, Mustafa *et al.* 2000, Stickney 2005). In aquaculture, where the focus is to grow healthy fish for consumption, sick and dying fish can cause major economic loss. To help modulate this loss, farmers may treat diseased fish with antibiotics and various other synthetic chemicals (Logambal *et al.* 2000, Limbu *et al.* 2021). This practice is known to induce oxidative stress to fish (Limbu *et al.* 2021) and leads to the build-up of antibiotic resistant bacteria within the intestinal flora in consumers' bodies. Since these bacteria are resistant to antibiotics, the bacteria continue to grow and spread infection. Ingested antibiotics are excreted through urine and stool (Done *et al.* 2015, Lulijwa *et al.* 2020, Vignesh *et al.* 2011). These excretions expose the environment's microbiome to growth-inhibiting agents that alters the environmental ecology since the number of resistant microorganisms surpasses the susceptible bacteria (Ventola 2015). To lower the use of antibiotics and to prevent contamination of the environment with artificial chemicals that are used in fish farming, researchers have started to investigate the use of nutraceuticals as an alternative. Nutraceuticals are food or food additives, which can be beneficial to health (Espín *et al.*

2007). Nutraceuticals are being used to lessen the maladaptive responses to stressors and to enhance the immune response.

One of the nutraceuticals that seem promising is holy basil, *Ocimum sanctum* (Linneaus) (Das *et al.* 2015). It is widely used in Asia due to its medicinal properties (Miller and Miller 2003). In several studies done on mice and rats, holy basil has been shown to modulate stress (Gholap and Kar 2004) and have antioxidant (Kim *et al.* 2010), anti-inflammatory (Singh *et al.* 1996, Singh and Majumdar 1996), hypoglycemic (Gholap and Kar 2004, Grover *et al.* 2002), hepato-protective (Lahon and Das 2011), and anticancer (Joseph and Nair 2013, Karthikeyan *et al.* 1999) properties. It has also been shown to increase growth, disease resistance, and immune response in Nile tilapia, *Oreochromis niloticus* (Linnaeus), when exposed to *Streptococcus agalactiae* (Lehmann and Neumann) (Panprommin *et al.* 2016) while basil nanocomposite showed to improve immune response of Nile tilapia against *Aeromonas sobria* and *Candida albicans* (Rahman *et al.* 2022).

Due to all the negative effects antibiotics and other chemicals have, this study looks at holy basil's potential as an alternative to combat the effects of stress via physiological and immunological parameters. Stressed and unstressed Nile tilapia were used as the animal model for the study since it is the third most farmed fish species in inland aquaculture in 2020 (FAO 2022). The stress condition was confirmed by the serum cortisol level in fish groups. The objective of this study was to provide evidence on the efficacy of nutraceuticals, such as holy basil, on improving health and immunocompetence. It was hypothesized that holy basil will be able to help modulate the stress response in Nile tilapia by improving the lysozyme activity, spleen somatic index (SSI), macrophage phagocytic capacity, and condition factor of stressed fish fed with a basil supplemented diet when compared to stressed fish without the supplemented diet.

MATERIAL AND METHODS

Fish acquisition and management

The aquaponics sector of aquaculture is becoming more popular amongst various kinds of farmers, i.e. hydroponic growers, fish farmers, and greenhouse growers. This is due to aquaponics' ability to add up production (seafood and plants), ease the maintenance of water quality (Diver and Rinehart 2000), and for its ecological merits and extra economic advantage (Liang and Chien 2013). This sector of aquaculture is promising for future sustainable fish farming and fighting pollution in both global and urban food production (Goddek *et al.* 2015). Hence, to maximize the amount of space in the lab facility, and to adequately use our resources (recycling water and the production of natural fertilizer), the fish were grown in an aquaponics system. Fingerlings (average total length 11.92 ± 0.21 cm and average weight 33.22 ± 2.3 g) were obtained from Troyer Farms located in Geneva, IN. They were housed in a recirculating-coupled aquaponics system, located at Purdue University Fort Wayne (PFW). Upon acclimation, while all fish were fed with regular commercial diet over a two-week period as suggested by Kutty (1972). The fish were divided into three experimental treatments. Each treatment had two replicates that contained three fish each making a total of six fish per treatment. The first treatment was the control treatment in which these fish were fed with only a commercial diet. The second treatment was the stress treatment in which these fish were fed with a commercial diet that was supplemented with hydrocortisone. The final treatment was the stress basil treatment; these fish were fed with a commercial diet that was supplemented with both *O. sanctum* and hydrocortisone. Hydrocortisone was used to induce stress (Barton *et al.* 1987)

and *O. sanctum* was used as a nutraceutical to modulate stress. The dosage of these supplements is described in the next section.

The water quality was maintained throughout the study as described as ideal for aquaculture by Ostrander (2000) and monitored weekly, late in the evening as suggested by Shoko *et al.* (2014). The water temperature ranged from 21.1-26.7°C, dissolved oxygen was > 4.7 ppm, pH ranged from 7-7.6, and ammonia was kept <0.05 mg/L. The fish were taken care of following an animal care protocol approved by the Purdue University's Institutional Animal Care and Use Committee (IACUC).

Fish diet preparation

All fishes were fed using a commercial diet (Purina® Aquamax® Fingerling Starter 300 that contained 50% protein, 16% fat, 3% fiber, 2.35%-2.85% Calcium, 1.30% Phosphorous, and 0.60% Sodium). This commercial feed was used throughout the experiment and modified based on the experimental treatments. The unmodified commercial feed that was fed to the fishes in the control treatment was prepared by mixing 1 kg of the commercial feed with 500 mL of 100% ethanol. Ethanol was used to dissolve the hydrocortisone powder; hence, the same concentration of ethanol was also added to the control feed to keep the feed ingredients consistent. Tilapia fishes, in general, are very hardy, so they usually do not respond to mild stress factors, such as hypoxia and handling stress. To ensure the animal was stressed, stress was induced by supplementing the commercial feed with hydrocortisone powder (Acros Organics, NJ) to prepare the diet given to the stress treatment. The 100 mg of hydrocortisone powder were mixed with 500 mL of 100% ethanol. The commercial feed was mixed with the ethanol-hydrocortisone mixture. The hydrocortisone made up 0.01% (100 mg/ kg) of the feed (Barton *et al.* 1987).

The diet for the stress basil treatment was made using *O. sanctum* powder obtained from Alka Ayurvedic Pharmacy, India (GMP certified). Thirty grams of the basil powder were dissolved in 200 mL of distilled water. The mixture was filtered using a sterile vacuum filter (Thermo Fisher Scientific, NY) with 0.20 µm pore size to obtain basil extract. The dry weight of basil in the extract was calculated as 15% of the crude powder. The extract was mixed at 0.2% (200 mg dry weight/ kg feed) of the total feed weight which previously showed improved health in Rohu fish (Das *et al.* 2015). The extract was mixed with a portion of the hydrocortisone-supplemented diet to prepare the stress basil diet.

Once the diets were mixed to get the desired mixture, they were dried at room temperature (25°C) for 24 hours and then placed in a 4°C refrigerator until use. The Fish were fed twice daily at 3% of their body weight (1.5% each feeding). The amount of feed was adjusted every two weeks.

Sampling

Three fishes from each replicate per treatment were sampled (a total of six fishes per treatment) once every two weeks for six weeks. All fishes were starved for 24 hours prior to euthanization for sampling. The fishes were euthanized using Tricaine mesylate (MS-222) (250 mg/L) (Western Chemicals, WA) within two minutes of catching (Strange 1983) and kept in MS-222 for about 10 minutes until opercular movement stopped before collecting data and samples. This dose is adequate to cause rapid immobilization (Leary *et al.* 2013) and does not allow cortisol to increase due to handling stress. For analyzing the condition factor, the total length and weight of each fish was

recorded. To show the level of serum cortisol over time, serum samples were collected every two weeks for six weeks. Data from six weeks were collected for lysozyme activity, macrophage phagocytic capacity, and spleen-somatic index.

Blood was collected via the caudal vein using one mL heparinized syringes (BD, NJ). The collected blood was kept on ice until its use. The collected blood was first transferred to sterile Eppendorf tubes and then centrifuged for ten minutes at 5000 RPM to collect the serum following Stahl *et al.* (1992). After centrifugation, the supernatant (serum) was transferred to a sterile Eppendorf tube. 50 µL of serum were removed and placed into another sterile Eppendorf tube (used for lysozyme assay). The tube containing the serum to measure cortisol was stored at -80ºC until analyzed. After all the samples from the whole study were collected, they were analyzed using a Cortisol ELISA Kit (Cayman Chemical, MI) following manufacturer's instructions.

To prepare a sample for lysozyme activity analysis, a suspension of 0.2 mg of *Micrococcus lysodeikticus* (Fleming) in 0.05 M (pH = 6.2) 1 mL of sodium phosphate buffer solution was made. *M. lysodeikticus* is a gram-positive bacterium that was obtained from Sigma (St. Louis, MO). This suspension was vortexed before use. One mL of the suspension was transferred to a sterile Eppendorf tube. 50 µL of fish serum were added to an Eppendorf tube containing the bacteria suspension and vortexed. Absorbance was taken at 540 nm using a Spectronic 601 spectrophotometer (Milton Roy Company, PA). The absorbance (abs) was recorded at one minute and at five minutes after mixing. These readings were used to calculate the lysozyme activity (LA) using the method adopted by Parry *et al.* (1965) as follows:

$$LA = \frac{Absorbance\ (Final) - Absorbance\ (Initial)}{Total\ Time\ Elapsed\ (minutes)}$$

After the blood collection, the fish were dissected and spleen was collected and weighed. This weight was used to calculate the spleen somatic index (SSI), following the method described by Hadidi *et al.* (2008). The values in the figures for the spleen-somatic index and the lysozyme activity are shown as percentages. The value for the control treatment was considered as 100%. Stress and stress basil were calculated as a percentage of the control value.

$$SSI = \frac{spleen\ weight\ (g)}{body\ weight\ (g)} \times 100$$

Fish's kidney was collected to determine the macrophage phagocytic capacity. The collected kidneys were placed in individual centrifuge tubes with 2 mL of L-15 Leibovitz medium containing glutamine (Sigma, At. Louis, MO, USA). The tubes were stored on ice until maceration. Each kidney was then macerated through a sterile metal sieve (80 mesh/190 µm) using a sterile glass plunger. Sieves were changed between each sample. Macerated kidneys were centrifuged at 1000 rpm for ten minutes. Supernatant was discarded and 2 mL of L-15 was added to the cell pellet. The tube was then vortexed until the pellet was resuspended. This centrifugation process was repeated two more times for a total of three centrifugations. After the third centrifugation, the supernatant was discarded and the pellet containing the cells were resuspended in 1 mL L-15.

For the macrophage phagocytic assay, 50 µL aliquot of the cell/ L-15 suspension was placed on both wells of double-etched microscope slides (on etched side) after vortex. Cells were then incubated for ninety minutes at 25°C. Cells were kept moist by placing the slides on a tray with

1XPBS-soaked paper towels. PBS was added as needed to keep the paper towels saturated. After incubation, 50 µL of heat killed bacteria (*Bacillus megaterium*) was added to the cell suspension on each well of the microscope slide. The slides were kept at 25°C for two hours keeping the cells moist. Next the slides were prepared for staining. Each slide was gently washed with PBS, then dipped into absolute methanol for one minute to fix the smears. The slides were then dipped in Wright-Giemsa stain solution for one minute. Finally, the slides were carefully rinsed in PBS and allowed to air dry.

The macrophage cells present on each slide were randomly counted from both wells on the slides. At least 50 cells were counted per well. The macrophages were counted as positive if they engulfed five or more bacteria spores. Phagocytosis was calculated by determining the proportion of positive macrophages among all the cells counted in each slide. This method was followed by Mustafa *et al.* (2000) with slight modification.

The weight and the length of each fish was also measured at each sampling. This data were used to determine the weight length relationship by calculating Fulton's condition factor (K) (Froese 2006). The formula used is depicted below:

$$K = \frac{weight\ (g) \times 100}{(length\ (cm))3}$$

Statistical analysis

All data obtained in this study were analyzed using Sigma Plot 14.0 Scientific Graphing and Statistical Analysis Software (Systat Software, Inc., San Jose, CA). If data were not found normal through a Shapiro-Wilk normality test ($P<0.05$), Kruskal-Wallis was used to determine significance ($P<0.05$) and Dunn's method was used for comparison ($P<0.05$). Normal data ($P<0.05$) were determined significant using one way analysis of variance (Brown-Forsynthe) ($P<0.05$) and Tukey test was used for comparison ($P<0.05$). All data presented are the means ± standard error of the means (SEM).

RESULTS AND DISCUSSION

Cortisol level was observed to see the effect of hormonal stress response of cortisol supplement in feed. Secreting cortisol is an important primary response or hormonal response of fish to stress (Mazeaud *et al.* 1977).

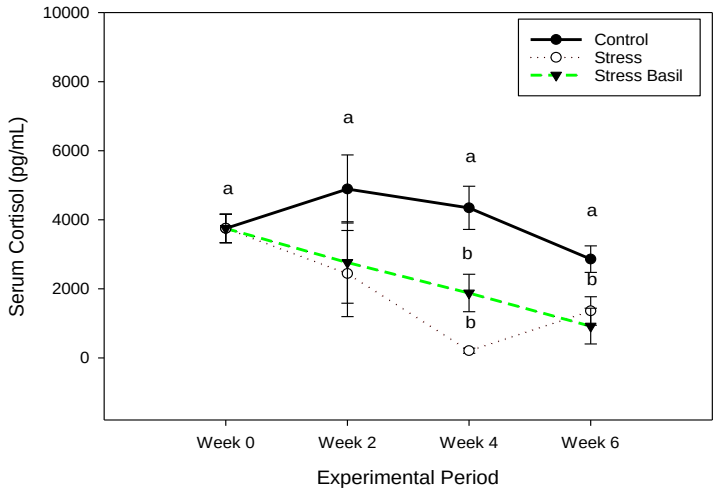

Fig. 1. Serum cortisol levels (pg/mL) of *Oreochromis niloticus*, reared in aquaponics system, throughout the experimental period. Results are presented as means ± SEM. Different letters (a,b) mean they are significantly different ($P < 0.05$).

In this study, there were no statistically significant differences observed for the serum cortisol values among the experimental groups at week 0 (P=1) and week 2 (P=0.2). Statistically significant differences were observed among the unstressed and both stressed groups at week 4 (P=0.003) and week 6 (P=0.017). At week 4 and 6, the fishes of both groups fed with cortisol supplemented feed (stress and stress basil) showed significantly lower serum cortisol level when compared to the control group (Fig. 1). Lysozyme activity (Fig. 2) and macrophage phagocytic capacity (Fig. 3) both showed no significant difference between any of the treatments (P=0.794; P=0.134, respectively).

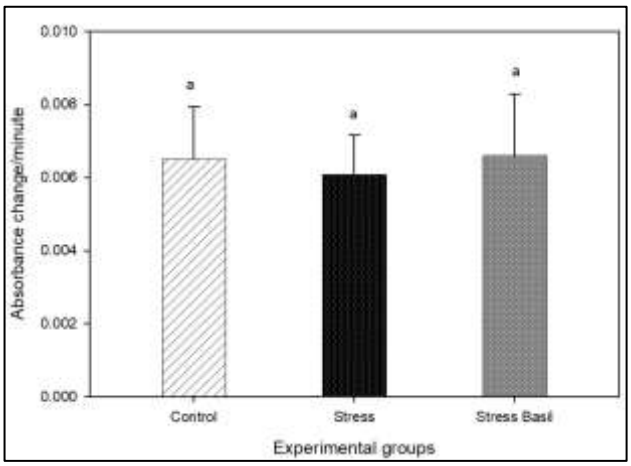

Fig. 2. Serum lysozyme activity (absorbance change/ minute) of three fish groups of *Oreochromis niloticus*, reared in aquaponics system, at week 6. Results are presented as means ± SEM.

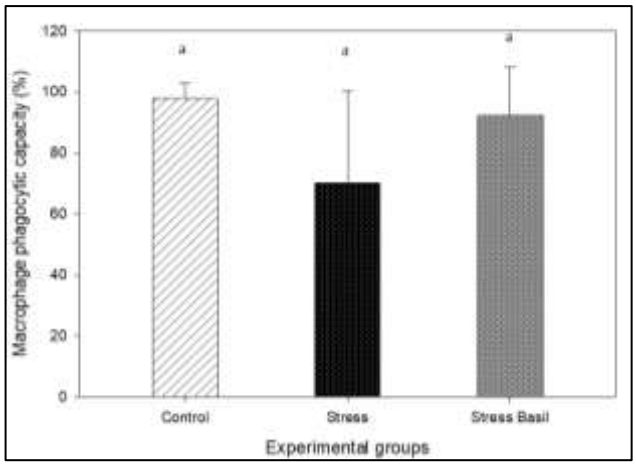

Fig. 3. Macrophage phagocytic capacity (%) of three fish groups of *Oreochromis niloticus*, reared in aquaponics system, at week 6. Results are presented as means ± SEM.

The control group was significantly different from the stress and stress basil treatments for SSI (P=0.033; P=0.043, respectively) (Fig. 4) and K (P<0.001 for both treatments) (Fig. 5). No significant difference was observed between the stress and stress basil treatments for SSI (Fig. 4) or K (Fig. 5) (P=0.952; P=0.294, respectively). From the macrophage phagocytic capacity (Fig. 5) it is evident that the stress basil treatment is closer to that of the control treatment than the stress treatment.

The data suggest that holy basil was unable to modulate the stress response of stressed Nile tilapia. While some of the results are promising, further research on holy basil's effect on stress is needed to determine this capability more effectively. The difference in serum cortisol levels was what we were expecting to see; this confirms that the fish were stressed. Since there is no significant difference between the stress and the stress basil treatments it can be concluded that *O. sanctum*, at least at the concentration that was used in this study, is not effective in modulating the serum cortisol levels in Nile tilapia, but it does show that a hydrocortisone supplemented diet can stress Nile tilapia. This may also be the reason why there was no significant difference between the treatments for the lysozyme activity and macrophage phagocytic capacity. It is possible that the concentration of holy basil that was used to supplement the diet was not sufficient. However, this does not explain why significant difference between the stress and the control treatments for these parameters was not observed. It was expected that fish within the stress treatment to have hindered responses when it came to lysozyme activity and macrophage phagocytic capacity.

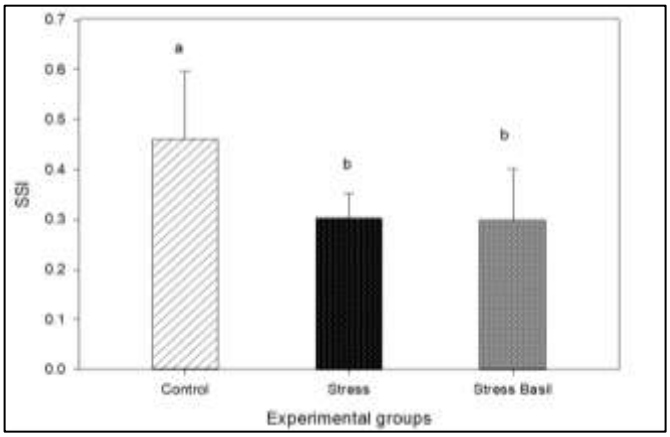

Fig. 4. Spleen-somatic Index (SSI) of three fish groups of *Oreochromis niloticus*, reared in aquaponics system, at week 6. Results are presented as means ± SEM. Different letters (a.b) mean they are significantly different ($P < 0.05$).

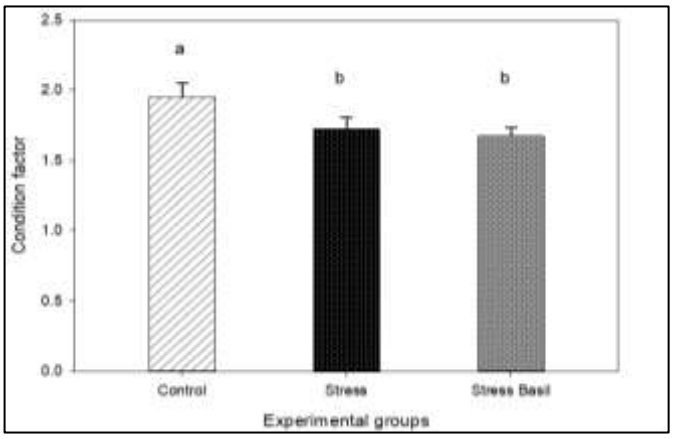

Fig. 5. Fulton's Condition Factor of three fish groups of *Oreochromis niloticus*, reared in aquaponics system, at week 6. Results are presented as means ± SEM. Different letters (a.b) mean they are significantly different ($P < 0.05$).

Measuring innate immunity in fish, which includes lysozyme activity and macrophage phagocytic capacity, is a powerful tool to understand overall fish health and environmental impact on fish population, especially the impact of toxicants (Bols *et al.* 2001). As fish does not have a well-developed adaptive immune response, innate immune response is more important for them than it is for mammals. Lysozyme is an important component of innate immunity of fish (Grinde 1989). It is an antibacterial protein that works against gram positive and some gram-negative bacteria (Düring *et al.* 1999) by breaking glycosidic bonds present in the cell wall of bacteria (Fearon and Locksley 1996). Fish has lysozyme in gill, skin, serum, brain, kidney and liver (Saurabh and Sahoo 2008) and Nile tilapia has the highest lysozyme activity in serum (Panase *et al.* 2017).

Following chronic stress, *O. niloticus* shows lower lysozyme activity (Caruso and Lazard 1999). Rainbow trout, *Oncorhynchus mykiss* (Walbaum), also show significantly lower levels of lysozyme and decreased lysozyme activity following acute stress and it takes two weeks for them to return to normal stage (Möck and Peters 1990). Fig. 2 shows that the stress treatment (93%) does have a slightly lower lysozyme activity and the stress basil treatment (101%) has a slightly higher lysozyme activity than that of the control treatment (100%). This suggests that the stress basil treatment had a slightly enhanced lysozyme activity than the control group, but the difference between them was not significant.

Previous research showed that basil has a significant effect on lysozyme activity when the herb is paired with other herbs. When basil is mixed with peppermint, *Mentha piperita* (Linnaeus) and English Walnut, *Juglans regia* (Linnaeus), it has been shown to enhance the lysozyme activity in common carp, *Cyprinus carpio* (Linnaeus) (Abasali and Mohamad 2010) and in rohu, *Labeo rohita* (Hamilton) (Das *et al.* 2015). This supports the hypothesis that holy basil may rely on a synergistic relationship to exhibit its full therapeutic potential.

A larger decrease for the stress treatment (72%) when compared to the control treatment (100%) is seen for the macrophage phagocytic capacity (Fig. 3), but again this decrease is not significantly different. The stress basil does have a higher capacity (94%) than that of the stress group, but it is not significant. Holy basil has been previously shown to improve phagocytosis when administered to

human alveolar cells (Suresh *et al.* 2022), but the same effect was not evident in this study on tilapia *in vivo.* In the current study we used macrophage cells collected from the kidney. We were certain that macrophages were present in our samples since we could visibly see them on the prepared cells. It could be possible that holy basil is more effective on cells collected from different parts of the body.

Spleen-somatic Index or SSI is the ratio of weights of spleen to the whole body and it is an indicator of spleen health. Spleen is an important part of the immune system and a healthy spleen in necessary for an active immunity against pathogens. It also has an erythrocyte reservoir which supplies additional erythrocyte to compensate for the oxygen demand in certain circumstances as stress (Pearson and Stevens 1991). This is specifically important to maintain healthy spleen in cultured fish as the aquaculture operating processes cause stress which makes them vulnerable to diseases.

The spleen somatic index also shows similar results (Fig. 4) as macrophage phagocytic capacity, but the stress (66%) and stress basil (65%) treatments are significantly lower than the control (100%). Holy basil has been previously shown to improve spleen cell proliferation in mice (Biswas *et al.* 2021), but that was not observed here in fish. Fulton's condition factor also shows similar results (Fig. 5). The stress (88%) and stress basil (86%) treatments are significantly different from the control (100%), but are not significantly different from each other. This result from the condition factor is not surprising since basil does not provide a substantial amount of calories or protein to the diet. It has been previously observed in tilapia, supplementing with rice bran does not affect the growth while nutritional diet is available. Fulton's condition factor takes in the weight vs length relationship, so while basil did not change the condition factor, stress can hinder the growth of the fish (Leal *et al.* 2011, Wedemeyer *et al.* 1990). This is what is shown in Fig. 5, control has the highest condition factor and the two stressed treatments have a lower condition factor. If holy basil was able to modulate the stress response though, it was expected to stop or slow down the effect stress has on the growth of the fish.

While it cannot be concluded that holy basil was able to successfully modulate the stress response, *O. sanctum* has been shown to have the ability to elevate the immune response in fish, such as *O. niloticus* in other studies. Our results suggest that *O. sanctum*, alone, is not sufficient in modulating stress and the immune response in *O. niloticus* that are reared in an aquaponic setting. It may be possible that the dose/concentration of *O. sanctum* may need to be adjusted, resulting in our results.

Further investigation needs to be done to find an effective dosage/concentration of *O. sanctum.* Further investigation should also look at trying different dosages using different stressors. This will determine if hydrocortisone/cortisol interferes with the compounds in *O. sanctum* or if the basil just did not work at the given concentration. It also may be interesting to isolate and test the different constituents within *O. sanctum*. The active compounds within the basil may need to be isolated to work, or perhaps paired with another compound for it to be therapeutic. Different combinations of herbs should also be tested for synergistic effects. These investigations will help produce a conclusion on the effects of *O. sanctum* on the modulation of stress and immunity in *O. niloticus*, reared in a farming environment, especially in recirculating and/or aquaponics systems. If future research determines that holy basil is a good alternative to antibiotic use in aquaculture, farmers will

be able to make more profit, produce a better-quality protein for human consumption and produce less pollution.

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