PHYSIOLOGICAL AND IMMUNOLOGICAL EFFECTS OF BASIL *IN VITRO* AND *IN VIVO*

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Biology at Purdue Fort Wayne

Fort Wayne, Indiana

Spring 2020

THE PURDUE UNIVERSITY GRADUATE SCHOOL

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Dedicated to

Trishna Modak

You were the brightest among us, in the whole school, or maybe the whole world. I never saw anybody smile so innocently like you. I believe in myself, because you once told me to do so! When you passed the medical school, we all knew you were going to be an amazing doctor. The bus that crushed you with your unborn child also crushed our souls that day. Rest in peace, Trishna. We love you.

Always…

ACKNOWLEDGMENTS

First, I would like to thank my mentor, Dr. Ahmed Mustafa. He guides and cares so much for me which only a father does. I also want to thank my committee members- Dr. Elliott Blumenthal and Dr. Robert Gillespie. They discussed their concerns with my research and helped me to think like a scientist.

The team of Mustafa lab has been of tremendous support. My research design required intense work in shortest possible time on sampling days, which had not been possible for me without the team. I want to give special thanks to the team- Kenneth Saillant, Destin Furnas, Lindee Mason, Ashley Baum, and Asif Mortuza. I also thank Jesse Rinard for his technical help operating the aquaponics system.

Being in Department of Biology at PFW was the ideal graduate life for me. I am grateful to my teaching instructors- Mrs. Marylin Shannon, Dr. Connie Kracher, Mrs. Karen McLellan, Mrs. Regina Trout and Dr. Arturo Villalobos. Their guidance and suggestions made me confident to teach. I would like to thank Mrs. Arlis LaMaster, Mr. Bruce Arnold and all the graduate students for their continuous help, no matter how odd the time was.

I am grateful to Mrs. Darlene Bender and Mrs. Colleen Krohn. Their helps and efficiency made my academic life so much easier that I did not have to worry about any official matter, and I could focus on my study and research.

Being in Purdue Fort Wayne was not all about academic for me, it was also organizational. I am thankful to Dr. Ahmed Mustafa (advisor of Bangladesh Students' Association) and Dr. Jordan Marshall (advisor of Green Action Club) to support and guide me to run the offices. I would also like to acknowledge Ms. Gail Lugo of Student Life and Leadership for helping me in organizing different organizational activities.

I cannot describe in words how thankful I am to my single father and grandmother, who raised me facing all odds, and always stand beside me. I thank all other family members, specially my husband and in-laws, who keep supporting me from the day they became a part of my life. I thank my friends in Bangladesh, who makes me smile. It is hard to stay so far away from family, but having these extraordinary people in my life, I never felt alone.

Thank you all, my life is not life without you.

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ABSTRACT

In this research we observed the effects of basil on stress modulation and immune response in vertebrates *in vitro* and *in vivo*.

The *in vitro* study was done on mice spleen cell cultured with Holy basil and Thai basil to observe their proliferation stimulation to spleen cells and spleen T cell. The study was done in presence and absence of Concanavalin A (Con A)- a mitogen known as a T cell proliferation stimulator. A single cell suspension of mice spleen cells was incubated with four different dilutions of Holy and Thai basil (1:1, 1:5, 1:25, and 1:125). No significant differences have been found between control groups (cells without factor) and treatments suggesting the basils did not show any stimulation to spleen cell or spleen T cell proliferation.

The *in vivo* study was conducted with tilapia reared in aquaponics system to observe the stress modulating property of Holy basil. The physiological and immunological responses and growth performances of stressed and basil fed tilapia have been observed. There were four groups of fish- Control (unstressed) fish fed with commercial feed, Stressed fish fed with commercial feed, Control (unstressed) fish fed with basil-supplemented commercial feed, and Stressed fish fed with basil-supplemented commercial feed. The experiment was six weeks long. The parameters which have been recorded were- serum cortisol, packed cell volume, plasma protein, hepatosomatic index, spleen-somatic index, lysozyme activity, macrophage phagocytic capacity, length, weight, and condition factor. The results did not suggest any effects of basil on stress response. But the harmful effect of stress on fish growth and immune response was evident, as the stress groups showed significantly lower length, weight and condition factor.

The aquaponics system used in this study was also investigated for its production of the crops. Total fish production after eight weeks of placing the fingerlings in the aquaponics system were as follows- Control group: 6.00 g/L, Stressed group: 3.98 g/L. It suggests that stress can result in far less profit in aquaponics production.

Total plant production after 5 months is 6,521.10 g/sq. m (leaf with stem of marketable size) for Holy basil and 7219.73 g/sq. m for Thai Basil. This result suggests that basils are a viable crop in aquaponics and Thai basil would be more productive as the plant crop than Holy basil when grown on aquaponics.

CHAPTER 1. GENERAL INTRODUCTION

The word ‘nutraceuticals’ is a hybrid term between nutrients and pharmaceuticals. These are functional food or food additives containing one or more bioactive constituents that is physiologically beneficial (Espín et al., 2007). According to Zeisel (1999), “Nutraceuticals are dietary supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods.”

Some plant derived foods containing non-nutrient secondary metabolites (phytochemicals) have shown to reduce the risk of chronic diseases (Espín et al., 2007). These phytochemicals have several health beneficial roles such as cofactors or inhibitors of enzymatic reactions, undesirable toxic chemicals remover, enhancing the absorption or stability of nutrients, having selective factors that help the beneficial microbiota and inhibits the harmful microbiota, and so on (Dillard & German, 2000). These are also often used as nutraceuticals.

Usage of nutraceuticals has become a leading trend in recent times to health conscious customers as studies have shown a direct relationship between eating habit and disease susceptibility (Espín et al., 2007). In last 20 years, there has been a growing interests of researchers on health benefits of nutraceuticals focusing on their specific natural compounds, beneficial properties and their benefits against specific diseases (Figure 1.1) (Bernal et al., 2011).

The usage of nutraceuticals also can solve the issues with the side effects of using chemical drugs and antibiotics to cure diseases, as drugs and antibiotics can get released into the environment from human and other animal body and can cause severe problems (Kummerer, 2001). These chemicals can destroy natural microbiome, give rise to drug resistant strains of pathogens and cause severe harm to wildlife (Kummerer, 2001). On the other hand, nutraceuticals do not interfere with natural process of biological system, it only has beneficial properties, and it does not have any negative impact on the natural environment including microbiome, flora or fauna.

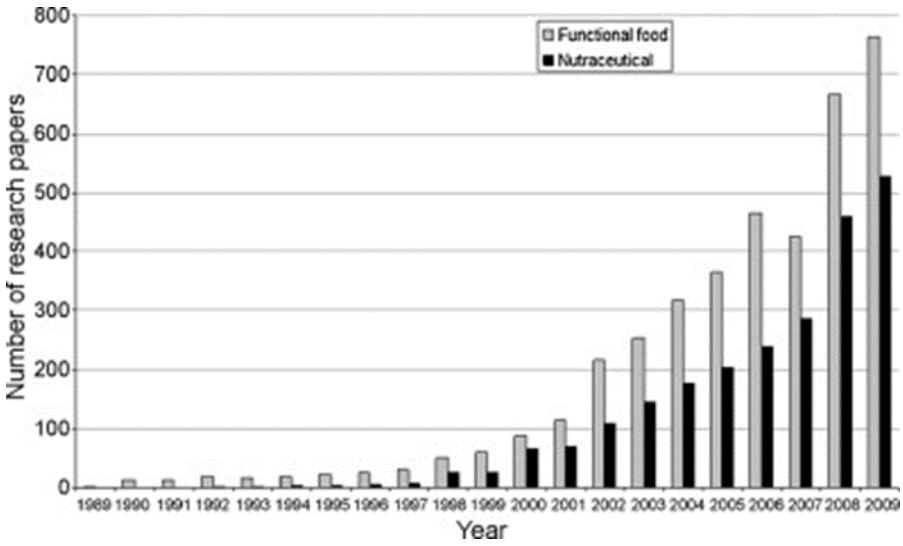

Figure 1.1. Research articles on nutraceuticals published from 1989 to 2009 (Source: ISI Web of Knowledge, © 2010 Thomson Reuters).

Basils belongs to plant Family Lamiaceae and Genus *Ocimum*. There are about 150 species of basils found in tropical and sub-tropical regions. They have been widely used in foods and as ornamental plants in gardens for their flavors and fragrances, and most importantly as medicines for their therapeutic usage (Datta et al., 2010). More than 50 species among them have been thoroughly studied for their different properties in various animals including human. Different species of basils have shown to modulate metabolic and psychological stress, to have antioxidative (Juntachote & Berghofer, 2005), anxiolytic and anti-depressant properties, antimicrobial activity (Cohen, 2014), and many more.

The study was conducted on basils both *in vitro* and *in vivo* to observe their effects on immune cell proliferation and stress modulation. Another part of the study was conducted to measure the production of fish and two species of basils in a laboratory scale aquaponics system.

1.1 References

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CHAPTER 2. EFFECTS OF HOLY BASIL AND THAI BASIL ON MOUSE SPLEEN CELL PROLIFERATION *IN VITRO*

2.1 Introduction

Nutraceuticals are food or food additives which can be beneficial for health (Espín et al., 2007). The phytochemicals in plant based nutraceuticals showed several roles in reducing inflammation and the risk of disease, promoting digestion and absorption of nutrients. (Dillard & German, 2000). Because of the promise of nutraceuticals in promoting health without having any negative impact, scientists are becoming more interested in conducting research about the effects, mechanisms, and use of nutraceuticals (Bernal et al., 2011).

Basil is a well-studied plant-based nutraceutical. There are various types of basil in the world. Two of the most commonly used basils are Holy basil (*Ocimum sanctum*) and Thai basil (*O. basilicum* var. *thyrsiflora*). Holy basil is popular as a medicinal plant whereas Thai basil is mostly used in spices for its aroma. However, studies have shown that both Holy and Thai basils to have health benefits (Miller & Miller, 2003; George, 2014). The active ingredients of both plants are given in Table 2.1.

Table 2.1. Active ingredients of Holy and Thai basil

Holy basil (Kelm et al., 2000)	**Thai Basil** (Wesolowska et al., 2012)
Eugenol	Eugenol
Linalool	Linalool
Carvacrol	Methyl- chavicol
Beta-caryophyllene	1, 8-Cineole
Rosmarinic acid	Alpha-bergamotene
Oleanolic acid	
Ursolic acid	

In vertebrate, the spleen is of vital importance to maintain the immunity against pathogens. It is a storehouse of macrophages, B cells and T cells and a unique organ which combines both innate and adaptive immune response (Mebius & Kraal, 2005).

One of the major cellular components of spleen is T cell. There are two major subpopulation of T cells- T helper cell (T_H) and T cytotoxic cell (T_C). T_H cell stimulates macrophages, B cell, and T cell itself to respond to antigens. On the other hand, T_C cells monitor abnormal cells (possibly infected by a pathogen or tumor cells) and eliminate them (Kindt et al., 2007). Thus, T cells play a vital role in body's adaptive immune response.

One part of this study has been conducted on the proliferation of spleen T cells with basil extracts with the presence of Concanavalin A (Con A). Con A is a lectin which is commonly collected from the jack-bean, *Canavalia ensiformis.* It is a plant mitogen which is known to have stimulatory effects on T cell proliferation and response and on T cell differentiation to its subsets (Dutton, 1972; Dwyer & Johnson, 1981).

present study focused on the mouse spleen cell proliferation *in vitro* with different dilutions of Holy basil and Thai basil, separately, with and without Con A.

More specifically the objectives were-

- To see if the basil extracts could enhance the immune cell proliferation compared to controls with or without Con A
- To determine which dilution of each factor would have the maximum effect
- To observe the difference between the proliferative effect of Holy basil and Thai basil

2.2 Materials and Methods

2.2.1 Plant extract preparation

Leaves (0.5g) were obtained from plants which were grown in the aquaponics system of Purdue University Fort Wayne (PFW) biology greenhouse and ground using mortar and pestles with 10 ml distilled water (Holy basil and Thai basil separately). The mixtures were shaken for 15 min with a rotating shaker mixer. The extracts were then centrifuged (3000 rpm, 10^0C, 10 min) and the supernatants were filtered with filter paper. The extracts were sterilized by passing through a Sterile Millex Syringe Filter (mesh size: 0.2 micron). All the extracts were stored at 4^0C as 1:1

(volume) stock until use (7 days maximum). The plant extracts were diluted with sterile water inthe following dilution factors 1:5, 1:25, and 1: 125 compared to the stock extract.

2.2.2 Treatment groups and variables

1. Thai basil vs Holy basil
2. Dilution 1 (1:1) vs Dilution 2 (1:5) vs Dilution 3 (1:25) vs Dilution 4 (1:125)
3. With Con A (w/Con A) vs without Con A (w/o Con A)

The groups were the set up as the following (Figure 2.1):

Thai (Dilution 1:1) w/ Con A	Holy (Dilution 1:1) w/ Con A
Thai (Dilution 1:1) w/o Con A	Holy (Dilution 1:1) w/o Con A
Thai (Dilution 1:5) w/ Con A	Holy (Dilution 1:5) w/ Con A
Thai (Dilution 1:5) w/o Con A	Holy (Dilution 1:5) w/o Con A
Thai (Dilution 1:25) w/ Con A	Holy (Dilution 1:25) w/ Con A
Thai (Dilution 1:25) w/o Con A	Holy (Dilution 1:25) w/o Con A
Thai (Dilution 1:125) w/ Con A	Holy (Dilution 1:125) w/ Con A
Thai (Dilution 1:125) w/o Con A	Holy (Dilution 1:125) w/o Con A

Controls: Mice spleen cell w/ Con A and w/o Con A, no factors were added to the control wells.

Each sample was run in triplicate. The whole assay was done three times with three separate mouse spleens. The process was conducted according to Ottenweller et al. (2004)

2.2.3 Spleen collection

BALB/c mice were used as model in this study. For spleen collection, mice were sacrificed by cervical dislocation and dissected following approved protocol. Spleen was removed by using aseptic technique. This was then used for the cell culture right away.

2.2.4 Cell culture

A suspension of single spleen cells was prepared, in sterile media using a screen wire mesh. The spleen was placed on top of the mesh and macerated by sterile plunger. Cells were collected

using sterile syringe and 1 mL RPMI was used as cell media. The cells were suspended in 1ml of RPMI 1640 (Mediatech Inc, Herndon, VA) and collected with sterile syringe.

Solution containing an isotonic buffer (295 µL), Trypan Blue (100 µL), and RBC Lyse Buffer (100 µL) was added to 5 µL of cell suspension to remove red blood cells. 10 µL of this solution was placed on a hemocytometer and cells were then counted under a microscope.

96 microwell plates were used for cell culture (Figure 2.2). Each well was filled with $1x10^6$ cells, 100 µL of media consisting of RPMI 1640+ 10% Fetal Bovine Serum (FBS) (Sigma, St. Louis, MO). Each treatment was performed in triplicate.

2.2.5 Spleen Cell Proliferation Assay

To observe the effect of different dilutions of the plant extracts, 1:1, 1:5, 1:25, and 1:125 dilutions of both Holy basil and Thai basil were used. Each three wells were added with 10 µL of one of these concentrations of either Holy basil or Thai basil (Figure 2.1). In the control wells, everything was added except for the plant extracts; in place of plant extracts, sterile water was added. For the spleen T cell proliferation assay, concanavalin A (Con A) (Sigma, St. Louis, MO) was used as a mitogen (Figure 2.4) which has shown to act as a stimulator of T cell proliferation (Coutinho et al., 1973). 10 µL of Con A were added to each '+' marked well (Figure 2.1).

The plates were incubated for 48 hours into CO_2 incubator at 37°C with 95% humidity. After incubation, 10 µL (stock: 0.0375 µCurie/µL) of radioactive isotope (^3H Thymidine) (Moravek Biochemicals, stock: 1 mCi/mL) were added to each well and the plates were incubated for additional 24 hours at same condition. The spleen cells uptake thymidine as a pyrimidine nucleotide when they undergo mitotic cell division. Thus, the radioactive thymidine incorporated inside the cells will be proportional to cell proliferation.

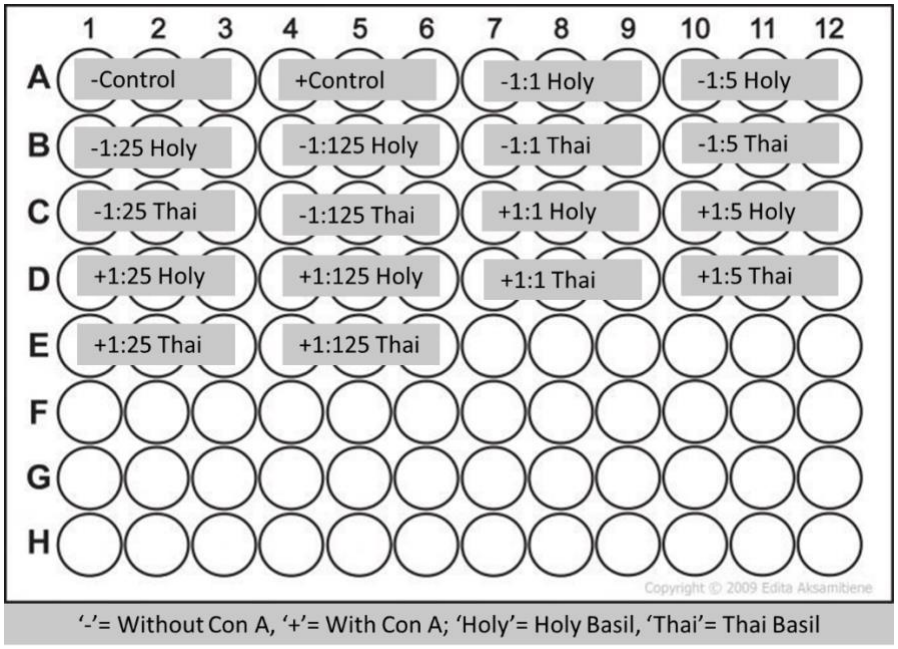

Figure 2.1 Template of 96 well plate for spleen cell proliferation assay with basil plant extracts

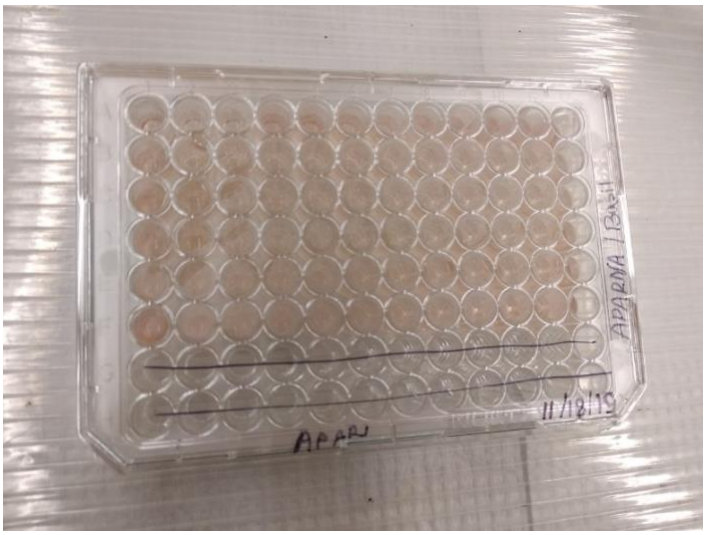

Figure 2.2. 96 well plate with spleen cells.

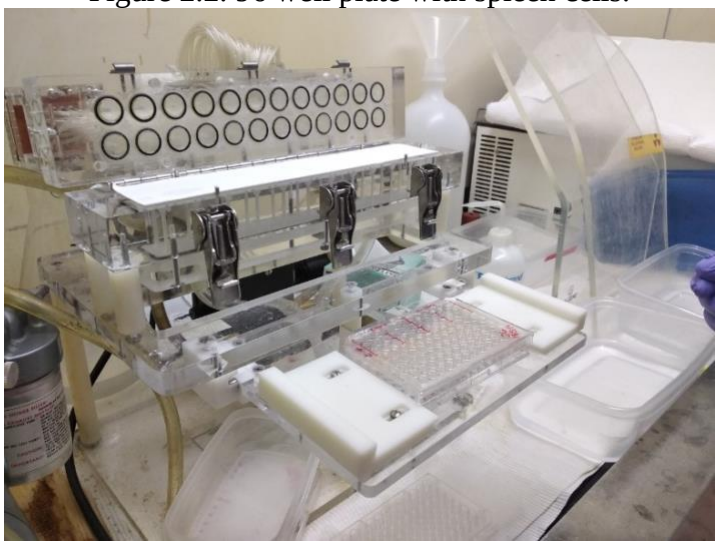

Figure 2.3. Cell harvest chamber

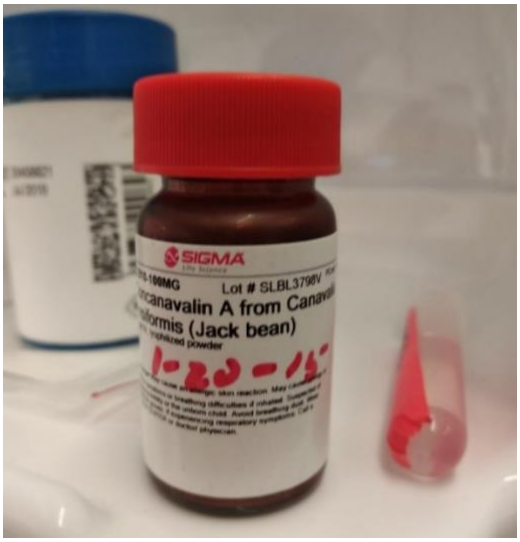

Figure 2.4. Concanavalin A (Con A) extracted from Jack Bean

Figure 2.5. Scintillation cocktail

Figure 2.6. Transferring the filter paper in the scintillation vial

Figure 2.7. Scintillation counter

2.2.6 DNA harvesting

After incubation, DNA of the cells were harvested onto filter paper strips (Whatman™) using a cell harvester (Brandel Cell Harvester, model M 24) by washing the wells twenty times with PBS (Figure 2.3). The strips were then treated with 10% trichloroacetic acid (TCA) solution for five times to rupture the cells. Then ethanol was passed through the filter five times to wash out the unincorporated ^{3}H Thymidine.

Filter paper with DNA were air dried behind radioactive shield for 3 hours. Dried discs of filter paper were placed in separate scintillation vials. 3 mL of scintillation cocktail (EcoLume ™ scintillation cocktail, MP Biomedicals) (Figure 2.5) was added to each vial (Figure 2.6) and then were placed in a scintillation counter (Beckman Coulter™, LS 6500 Multipurpose Scintillation Counter) that counts radioactivity in the vials as count per minute (CPM). The scintillation counter coverts the radioactivity to the number of cell divisions. (Figure 2.7).

2.2.7 Data analysis

Data were analyzed using SigmaPlot 14.0 (Systat Software Inc). The differences between cell proliferations by two plant extracts and the control were analyzed by two-way ANOVA. Following ANOVA, Tukey's test was performed for getting the significance of the differences between concentrations. Values are presented as average percentage where controls are calculated as 100%. The graphs are presented as mean (of percent values) ± standard error of means (SEM).

2.3 Result and Discussion

The results are graphically presented in Figures 3.1 and 3.2.

2.3.1 Spleen Cell Proliferation Assay

Adjusted percentage of cell proliferation of control was 100 ± 0.0. The adjusted values of cell proliferation of Holy basil dilutions 1:1, 1: 5, 1:25, and 1:125 were 202.529±94.090, 117.746±36.745, 110.663± 19.146 and 116.860±13.12, respectively (Figure 2.3.1). The adjusted values of cell proliferation of Thai basil dilutions 1:1, 1: 5, 1:25, and 1:125 were 72.768± 9.830, 87.682± 9.825, 74.270 ± 11.869 and 101.358 ± 27.230, respectively (Figure 2.3.1). No significant

differences were observed between control and the dilutions Holy basil or Thai basil extracts. It suggests the factors did not show any significant stimulatory effects on spleen cell proliferation.

2.3.2 Spleen T cell Proliferation Assay

Adjusted percentage of cell proliferation of control was 100 ± 0.0. The adjusted values of cell proliferation of Holy basil dilutions 1:1, 1: 5, 1:25, and 1:125 were 98.428± 12.324, 118.524± 8.397, 122.960 ± 8.282 and 121.120± 29.942, respectively (Figure 2.8). The adjusted values of cell proliferation of Thai basil dilutions 1:1, 1: 5, 1:25, and 1:125 were 129.061± 20.389, 83.113± 15.550, 99.437± 25.871 and 132.445± 24.511, respectively (Figure 2.8). No significant differences were observed between control and the dilutions Holy basil or Thai basil extracts. It suggests the factors did not show any significant stimulatory effects on spleen T cell proliferation.

There are no previous studies on spleen cells or spleen T cell proliferation of basils, hence it is difficult to compare this study. One study showed its inhibitory activity on macrophage phagocytic capacity *in vitro*, which suggests its anti-inflammatory property (Courrèges & Benencia, 2002).

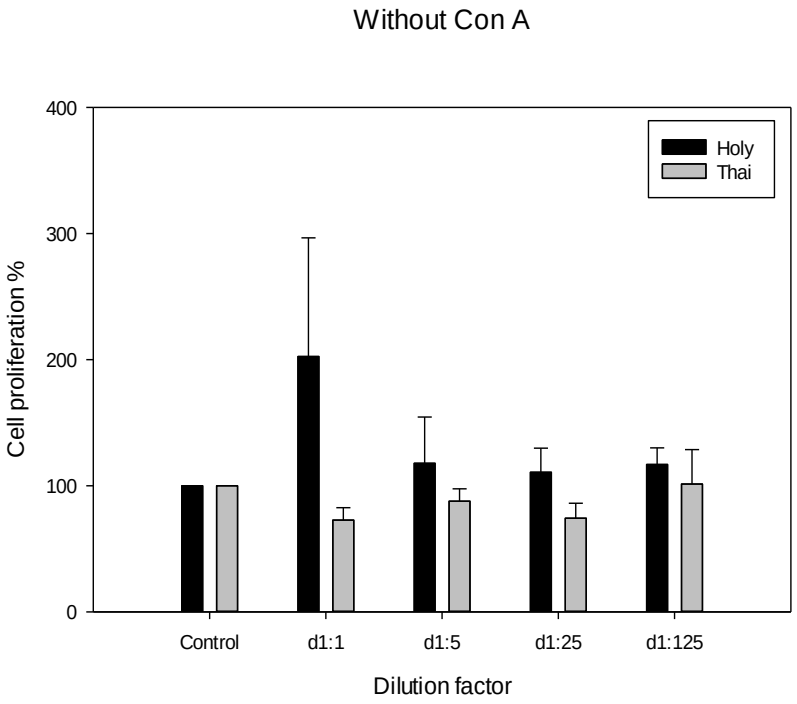

Figure 2.8. Percent proliferation of spleen cells with different dilutions of Holy basil and Thai basil

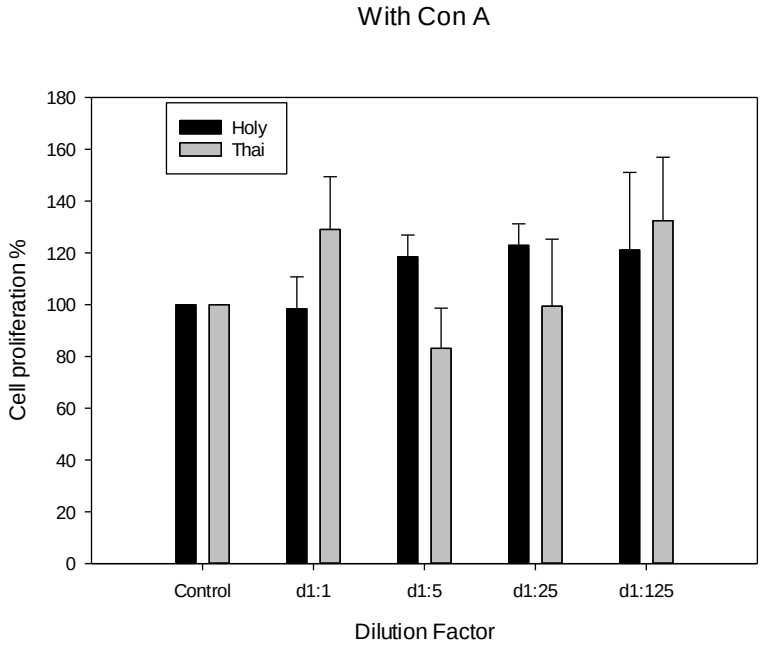

Figure 2.9. Percent proliferation of spleen T-cell with different dilutions of Holy basil and Thai basil

2.4 Conclusion

Nutraceuticals can be a solution for different physiological and immunological problems as they showed properties to enhance health in different previously done studies. As several studies showed, basil is one promising nutraceuticals (Miller & Miller, 2003). As no study was done on effects of basils on spleen cell proliferation till the date of this experiment, our aim was to observe the stimulatory property of two different kinds of basils to spleen cells.

The results did not show any significant stimulatory property of any of the basils on spleen cells or spleen T cells. Further research is needed to observe the basils' stimulatory effect on B cells, Interleukins, Interferons, and other cytokines as well as inhibitory properties against tumor cells or macrophages to determine the immunological properties of it. Also, different methods of plant extraction, different dilutions, mixing with other nutraceuticals and extraction of bioactive compounds should also be considered. If nutraceuticals such as basils can show significant improvement of health and can lessen the use of chemical drug to any extent, it will be beneficial both for humankind and the environment.

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CHAPTER 3. EFFECTS OF HOLY BASIL ON THE MODULATION OF STRESS OF TILAPIA RAISED IN AQUAPONICS SYSTEM

3.1 Introduction

3.1.1 World population and food security

The world population is 7.8 billion as of March 2020 (Worldometers, 2020) which is expanding at an alarming rate (Figure 3.1). About 2 to 4 billion more people will be added to this population by 2050 (Cohen, 2003). This rapid growth in population challenges us to combat hunger and malnutrition along with many other things. In 2018 across 53 countries worldwide, more than 113 million people have faced acute hunger and additional 143 million people were living in stressed condition who were at a high risk of facing acute hunger (Global Report on Food Crises, 2019). Sufficient diet is unavailable to more than 1 billion people (Barrett, 2010). Among the people facing acute hunger, 29 million were pushed to acute food insecurity in 2018 (Global Report on Food Crises, 2019). Since 2014, the number of people facing malnutrition is increasing which have reached to 821 million in 2017. This trend will continue to drive the world hunger in future and harshly affect some regions (Global Report on Food Crises, 2019).

Published results also indicate that the impacts of climate change are significant for world hunger (Schmidhuber & Tubiello, 2007). It will negatively affect the crop production in developing countries due to water unavailability, poor vernalization and disruption of crop growing period, which in turn will worsen the world nutrition situation (Parry et al., 1999).

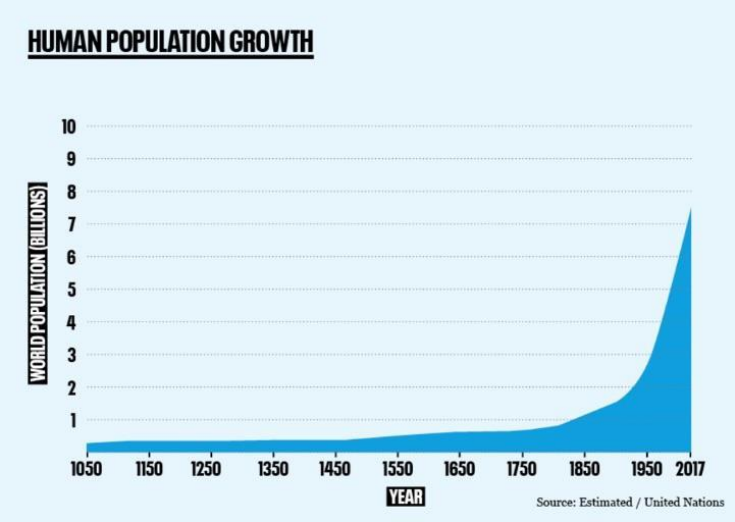

Figure 3.1. Human Population Growth Chart (Source: United Nations)

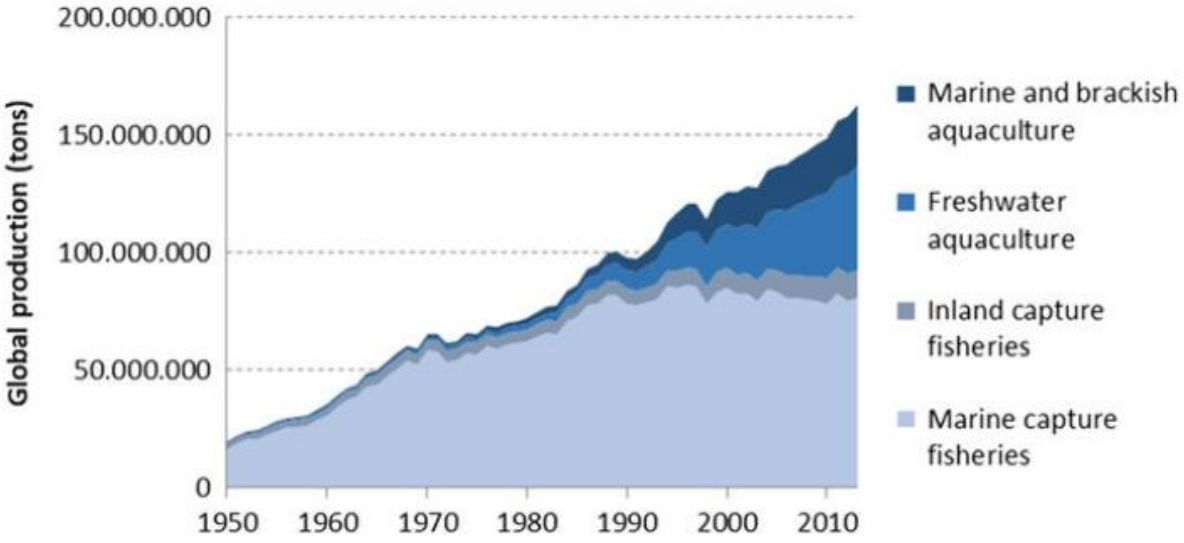

Figure 3.2. World capture fisheries and aquaculture production (Lewandowski et al., 2018)

It has been estimated that by 2080, at least 5 million (can be as high as 170 million depending on the socio-economic condition of their countries) of additional people will be at risk of hunger due to the climate change alone (Schmidhuber & Tubiello, 2007). The continent of Africa alone will face a crisis with around 60 million additional people at this risk by this time because of greenhouse gas effect only (Parry et al., 1999).

A major component in addressing malnutrition in many parts of the world is protein-based diet. Protein is required for growth, reproduction and optimal performance of animals including humans. There are some dietary essential proteins (more specifically amino acid) which cannot be made in body, so must be present in the diet. Consumption of protein depends on average income and livelihood. The people who are suffering from hunger and malnutrition are more likely in a crisis or are low income people with low standard of life, so it is obvious that they do not get enough protein in their diet. Moreover, based on the trend in increasing protein demand, it has been estimated that it will be even higher in future due to rapid growth of both population and income (Boland et al., 2013). There is even higher protein demand in developing countries as there is a trend of eating high protein containing diet to reduce or control weight and for better muscle function. Study suggests that there will be a 82% increase in demand of protein by 2050 globally as a result of a combination of all factors if the trend continues, means an additional demand for 233 Metric tons of meat (Boland et al., 2013).

Not only human, even the livestock has a requirement for protein which also contributes to the high protein demand. The main protein ingredient in animal feed is soy. As the climate change is affecting mostly the plant crop production, the source of soy protein for animal feed will also be scarce.

Considering all the factor of protein demand, scarcity and farming problem, an action based adaptive plan is highly necessary.

3.1.2 Problems of protein production and aquaculture

In the near future, the global agriculture will face challenges to meet both population growth and climate change. Climate change has vast impact on crop production, fisheries and livestock, also it will change the crop pests' prevalence (Campbell et al., 2016) and we can already measure some of the impacts. Impacts of some slow changes like temperatures and sea level will be a critical problem in near future but farmers are already facing problems due to

some relatively rapid changes such as weather patterns. Studies suggests there will be 3% to 10% decrease of main plant crops as well the quality because of increased CO_2 and temperature (Campbell et al., 2016). Therefore, adaptation actions to reduce farming risks and finding the alternatives are urgent.

There are two sources where we get the aquatic food, capture fisheries and aquaculture. Until 1890s, capture fisheries sector was a dependable resource for human demand, but since 1990s, as a combination of both population explosion, and wild population and habitat depletion, capture fisheries alone has been unable to meet the aquatic food demand. There has been an increase of production of aquaculture which is increasing by an average of 8.8% every year (Figure 3.2) making it the fastest growing food producing sector (Lewandowski et al., 2018), especially in Asia and it supplies huge amount of fish for human consumption along with other products of various purposes.

In 1974, aquaculture provided only 7% of the total fish for human consumers throughout the world, which became 50% in 2014 and it shows the rapid growth of this sector (FAO, 2018). Globally, aquaculture employed more than 19.3 million people in 2016, which increased from 17 to 32 percent in last 26 years (1990-2016) (FAO, 2018). In the future, the contribution of this sector to food security and nutrition is expected to increase. The advancement of science and technologies is the key behind this improvement (Lewandowski et al., 2018).

According to Stickney (2005), 'aquaculture is the rearing of aquatic organisms under controlled or semi-controlled conditions.' In simple word, aquaculture is *underwater agriculture*. There is a great variety of aquatic organisms including plants, animals (both vertebrate and invertebrate) which are used as human food, medicine etc., hence are of particular human interest (Stickney, 2005). Aquatic food products are highly important to provide healthy diet and to secure balanced nutrition whether it is rich or poor people. Richer people are increasingly taking more and more fish and other aquatic product meal as it is a healthier source of vitamins and minerals, also, they play a vital role to fight food risk and malnutrition in developing countries (Lewandowski et al., 2018).

At present, there are three types of aquaculture- extensive, semi-intensive, and intensive aquaculture. In extensive aquaculture, organisms are solely fed on natural sources (algae, plankton etc.). In this system, no external food or fertilizer is given. Although, it plays important role to counter eutrophication and preserve biodiversity in some cases, its production rate is low. And its

use in future will be limited because of the decreasing availability of water resources. In semi intensive aquaculture, the organisms are fed on both natural and external supplements in a natural waterbody. The production of this system is higher than extensive aquaculture and integrated semi intensive aquaculture. It is popular in developing countries, but the scarcity of suitable waterbody is a problem, and its production is not enough to meet the global demand. The major supply of the aquatic food demand in future is expecting to come from intensive aquaculture. In this system, organisms usually are kept in high density and are grown solely on supplemented feed. The environment is highly monitored, regulated and maximally utilized (Lewandowski et al., 2018). It has very high productivity hence is promising sector to meet the global demand.

There are two major types of intensive aquaculture- flow-through system and recirculating aquaculture systems (RAS). RAS recycle the water and make the same water available to the organisms through filtering it. One major function of the filtration is to convert the toxic ammonium to nontoxic nitrate. Fish in aquaculture system produces ammonia, which is toxic, and is detrimental to fish health if present in water even in small amount. As RAS reuses the water in the system, it is crucial to filter out the ammonia or convert it to non-toxic nitrate/nitrite before reusing it back to fish tank. In nature, the conversion is done by nitrifying bacteria and used up by plants. Following this principle, the design of aquaponics was formulated which not only solves the filtration problem, but also adds up production.

3.1.3 Aquaponics

The most recent form of intensive aquaculture is aquaponics. Aquaponics is a combined system of intensive aquaculture (of fish) and hydroponics (production of plants like herbs, flowers and vegetables) (Lewandowski et al., 2018). It uses the same water to grow both plants and animals hence it is a form of polyculture (Stickney, 2005). It is a soilless system that recycles the water. It converts the ammonia produces by the animals to nitrate/nitrite by the nitrifying bacteria present in the system which are taken up by the plants as nutrients. Thus, it meets the challenge of filtering the ammonia from the water and makes it suitable for fish health, also provides essential nutrients to the plants.

3.1.4 Aquaculture and Stress

In aquaponics, like other forms of aquaculture, several practices result in stress in fish. There are biological, physical as well as chemical stress factors. In nature, severe stressor that is detrimental to fish health is rare, as most of the natural challenges are acute that involves a fight-or-flight response which are not persistent. Chronic situations occur very often in aquaculture due to human activities (Pickering, 1989). These activities include handling, sorting, grading, transporting from hatchery to main aquaponics system, crowding etc. Chronic response results in depression in immune system in fish which make them more susceptible to infectious diseases (Barton and Iwama, 1991) and the effect of stress increases exponentially (Stickney, 2005). In recent times, several studies have been conducted on stress and their effects as it can harm fish health, reproduction and overall production.

3.1.5 Stress

Stress is the reaction by organisms to a stress factor which can alter organisms' homeostasis. The stimulus can be any factors that elicit responses in fish and these responses can be physical, biological or behavioral which extent beyond the normal state of fish. The reaction to stress varies depending on severity and persistence of the factor (Tort, 2011). The responses can be adaptive or maladaptive and the extension of responses can greatly reduce the chances of survival of fish (Barton & Iwama, 1991). There is a natural capacity in fish to overcome the stress causing situation to a certain stage, but when the range goes beyond limit, it becomes maladaptive which is detrimental to its health. Adaptive responses are beneficial to fish as they help to overcome the stress e.g., elevation of plasma glucose, branchial blood flow, muscular activity etc. On the other hand maladaptive responses are negative to the survival or reproduction including depletion of circulating lymphocytes, capacity of growth, immunocompetence etc. (Barton & Iwama, 1991). As stress causes a reorganization of metabolism to cope with stress condition, it affects other metabolic functions such as the immune system as the signaling between immune system's messengers gets delayed or reduced (Tort, 2011).

According to the body organization, stress responses can be classified in three major levels-primary, secondary and tertiary. The primary response in fish is elevation of plasma levels of

catecholamines (primarily epinephrine i.e., adrenaline) and corticosteroids (cortisol), can be also called as hormonal response (Figure 3.3, Table 3.1).

Table 3.1. Different properties of adrenaline and cortisol in fish

Criteria	**Hormones**	
	Adrenaline	**Cortisol**
Source in fish	Chromaffin tissue of head kidney	Interrenal tissue of head kidney
Elevation time	Quicker	Slower
Neuroendocrine pathway in fish (Figure 3.3)	Stress factor-Sympathetic nerves-Chromaffin cells-Adrenaline	Stress factor-Hypothalamus (CRF)-Pituitary (ACTH)-Interrenal cells-Cortisol
Stress it is responsible for	Acute	Acute and chronic

Researches on fish stress have been mostly done on cortisol because of its significance on fish's physiological responses, responsiveness to acute and chronic stress, and the ease of measurement. Also the suppressive effects of stress responses are mostly associated with cortisol (Tort, 2011). Hence, except for some cases, stress presence and severity in fish can be measured by cortisol level. If the stress stays longer, the effects of primary responses i.e., endocrine changes lead to secondary response of stress (Mazeaud et al., 1977). The effects from secondary responses can be metabolic, hematological, hydromineral or structural and most of them are usually adaptive. If the stress persists, it causes a whole body response called the tertiary response which is a change in metabolic rate that causes decrease of growth rate, disease resistance, reproductive capacity etc. (Barton & Iwama, 1991).

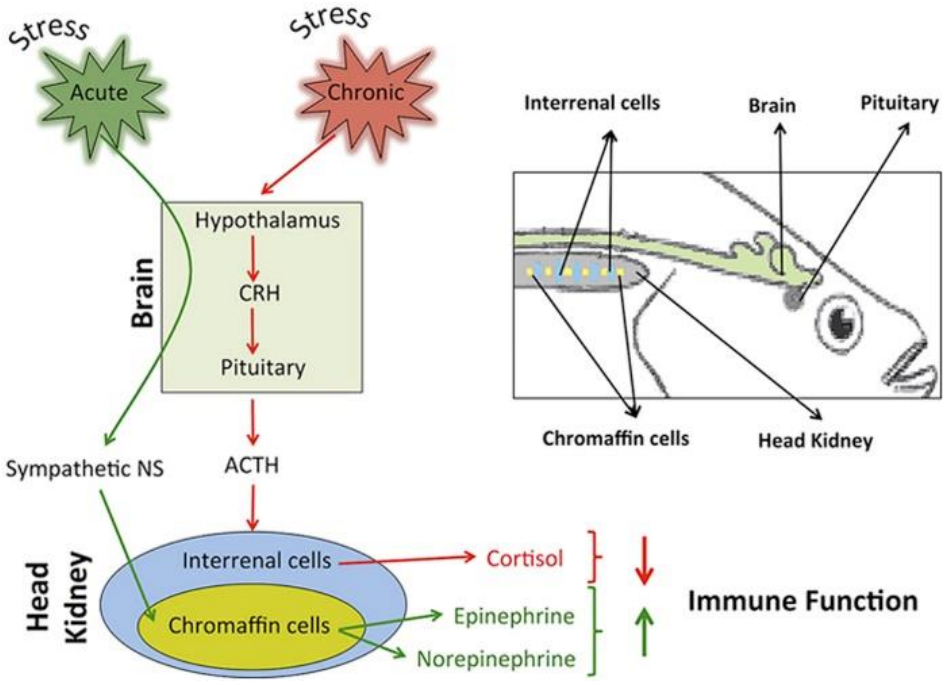

Figure 3.3. Endocrine pathway of stress hormones in fish (Nardocci et al., 2014)

3.1.6 Stress effect on immune response

Stress affects the fish health in various ways, one of which is making them more susceptible to diseases. The correlation of stress and diseases has been well studied. Stressed fish shows more mortality than unstressed ones when infected by pathogens suggests the one cause of suppression of immune system is stress response (Barton & Iwama, 1991; Stickney, 2005). In lower vertebrates, cytokines and neuropeptides play some role for both immune and neuroendocrine system. Also, some of the messenger of these systems are on same molecular class. Due to the lack of adrenal glands, the head kidney plays a major role in stress response in fish which is also a central organ for immune system. Hence, when the body imposes the system and the messengers for stress response, its involvement in immune response gets reduced (Tort, 2011). Whereas acute stress does not seriously affect the immune system, persistent chronic stress require continuous supply of energy that makes the energy deficient for immune system to produce antibodies, complement, and other proteins (Tort, 2011). Depletion of circulating lymphocytes, one of the maladaptive responses of fish, is another major mechanism by which stress causes increased disease susceptibility (Barton & Iwama, 1991). When the allostatic load reduces the function of the immune defense, pathogen can act with greater efficiency (Tort, 2011).

For any industry, sudden spreading of diseases can be devastating and can result in complete loss. Diseases in aquaculture animals cost over US$3 billion annually to aquaculture industry worldwide (Stickney, 2005). To prevent or cure these diseases, farmers tend to use antibiotics and various other synthetic chemicals which are of partial success (Logambal et al., 2000). The risk of using these drugs repeatedly poses a huge risk for consumers' health and the environment.

During the last few decades, farmers used antibiotics for prevention of diseases as well as for growth improvement and gaining high feed conversion efficiency in farmed animals (Pandiyan et al., 2013). While using of drugs are highly regulated in some areas for food fish, there is a lot of regions which are not regulated at all. Regarding non-food fish, there is even less regulation. Some previously used antibiotics in aquaculture as chloramphenicol were discovered later that people can die if exposed to this drug due to allergic reaction. Some chemicals that are currently being used can also be harmful to either the culture animals or the culturists or both, formalin for example. If the amount of these drugs is not strictly maintained, it can cause serious problems like direct mortality (Stickney, 2005).

Not only the treatment but also the process of the treatment can also lead to harm to culture animals. For example, dip treatment requires the fish to be handled before dipping which adds up additional stress to fish which is already stressed due to the disease (Stickney, 2005).

Treating the culture animals directly or by treating water possess serious health risks and imbalance to the wild environment and its microbiota too, as it can develop antibiotic resistant bacteria when the water goes to the natural sources, especially in case of cage and net pen cultures (Stickney, 2005; Pandiyan et al., 2013).

Facing all the problems, scientists are being interested more and more to study the effects of nutraceuticals to lessen the maladaptive responses to stressors as well as to enhance the immunity.

3.1.7 Nutraceuticals

Nutraceuticals are food or food additives which have several health benefits. Adding nutraceuticals to everyday diet has become a leading trend in recent times to health-conscious customers as studies have shown a direct relationship between dietary habit and disease risk (Espín et al., 2007). Using nutraceuticals also can lessen the uses chemical drugs and antibiotics. They are not harmful for consumers or the environment. For all these reasons, there has been a growing interest of researchers on health benefits of nutraceuticals focusing on their beneficial properties and their benefits against specific diseases (Bernal et al., 2011).

3.1.8 Holy basil as a nutraceutical

Recent studies showed a promising side of using different herbal compounds collected from medicinal plants as they show antimicrobial and immunostimulatory activities. Holy basil or *Ocimum sanctum* L. (Tulsi in Bengali and Hindi), belongs to family Lamiaceae, is a particular species of interest among them (Das et al., 2015). This indigenous plant of Indian subcontinent serves as a very common medicinal plant in Asia. People of Hindu religion treat the holy basil as a goddess of healing for its medicinal properties (Miller & Miller, 2003).

Classification of Holy Basil is given below.

Kingdom: Plantae
Phylum: Angiosperms
Class: Magnoliopsida

Order: Lamiales
Family: Lamiaceae
Genus: *Ocimum*
Species: *O. sanctum* L. (or *O. tenuiflorum*)

Several antioxidant and cyclooxygenase inhibitory phenolic compounds have been isolated from Holy basils.

Over the last decade, more than one hundred research have been conducted about various properties and effects of basil- both *in vitro* and *in vivo* using animal models (Jamshidi & Cohen, 2017). Some of the studies on effects of Holy basil on animal model or *in vitro* cell culture are given in table 3.2.

Table 3.2. Some studies on effects of Holy basil on animal model or *in vitro* cell culture

Experimental animals/ cells	**Effects**
Swiss albino male mice	Hypoglycaemic and stress modulator (Gholap & Kar, 2004)
Rats	Hypoglycaemic/antihyperglycaemic (Grover et al., 2002)
Mouse Lewis lung carcinoma cells	Antioxidant and anti-metastatic (Kim et al., 2010)
Rats	Ameliorative (Muthuraman et al., 2008)
Mice	Anticancer (Joseph & Nair, 2013)
Albino rats	Hepatoprotective (Lahon & Das, 2011)
Human fibro sarcoma cells and mice	Anticancer (Karthikeyan et al., 1999)
Rats	Anti-inflammatory (Singh et al., 1996)
Rats	Anti-arthritic and anti-edema (Singh & Majumdar, 1996)
Albino mice	Anticonvulsant (Jaggi et al., 2003)

The anti-inflammatory effects of basil are comparable to Ibuprofen, Naproxen, Aspirin (Cohen, 2014). For the wide range of medicinal properties, Holy basil has been selected for this study to observe its effects on Nile tilapia.

3.1.9 Nile tilapia

In this study, Nile tilapia has been chosen as the species of interest considering their importance in aquaculture. Tilapia is the common name for over 70 species among which Nile tilapia is the most produced species and accepts a higher percentage of plant protein (FAO, 2018). Classification of tilapia is given below.

Phylum: Chordata
Subphylum: Vertebrata
Superclass: Actinopterygii
Class: Teleostei
Superorder: Acanthopterygii
Order: Perciformes
Family: Cichlidae
Genus: *Oreochromis*
Species: *Oreochromis niloticus* L.

Farming of tilapias is believed to be started 4000 years ago from now in Egypt (Gupta & Acosta, 2004). Tilapia farming was first recorded in Kenya in 1924, since then it spread exponentially (Gupta & Acosta, 2004) and the production grew 12% annually (Cai et al., 2017). In 1990s, the production was nearly a half million tonnes which became more than 5 million tonnes in mid-2010s (Cai et al., 2017). Today, tilapia is among the most popular species to fish farmers and it is cultured in more than 120 countries (Cai et al., 2018). Among the tilapias, Nile tilapia is most used species in aquaculture and of most economic importance (Gupta & Acosta, 2004). Although the primary worldwide distribution was of *Oreochromis mossambicus, Orechromis niloticus* became more desirable to farmers and consumers after its distribution started during 1960s. The sex reversal technique and other research on tilapia nutrition and farming led to rapid expansion of tilapia culture industry (Rakocy, 2005).

3.1.10 Research Objectives

Based on the review of previous researches, the objectives of this study were the followings:

1. Produce quality protein: The main purpose of the study was to produce good quality tilapia, by making them healthy and immunocompetent even if they were raised in a stressed condition by providing them basil supplemented feed.
2. Use resource appropriately: By farming in aquaponics.

3.2 Materials and Methods

For this study, fish were reared in aquaponics system in greenhouse. There were four groups of fish -each was fed with different feed.

3.2.1 Experimental design

Aquaponics Setup

The study was conducted at the recirculating/coupled aquaponics system in the greenhouse at science building of Purdue University Fort Wayne was used. There were four fish tanks below two water beds and a gravel bed. The whole system was connected such a way that the water from the fish tanks went through a pipe to the gravel bed. From the gravel bed the water entered the water beds and then fell through separate pipes in every fish tank (Figure 3.4). Plants were transferred in both the gravel bed and water beds.

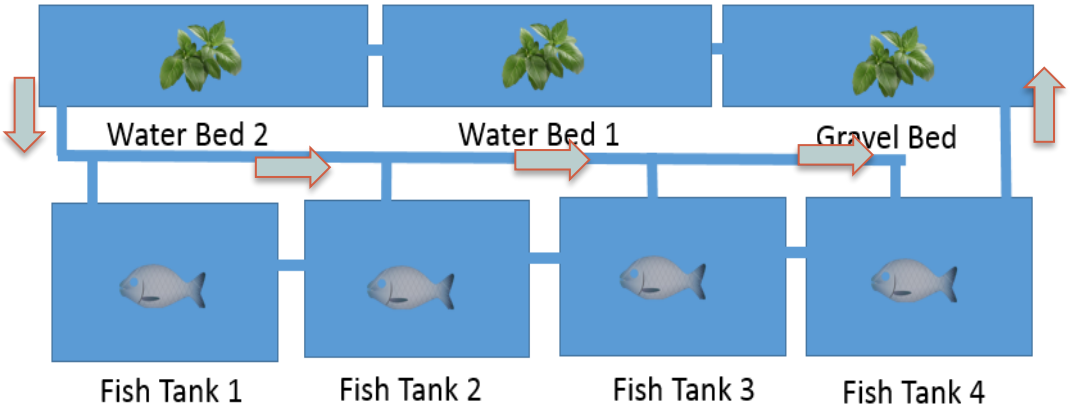

Figure 3.4. Aquaponics system setup

Fish acquisition and management

168 disease free tilapia fingerlings were obtained from Troyer Fish Farms, Geneva, Indiana. Upon arrival, fish were acclimated using optimum water conditions (Table 3.3) in a recirculating system for one week. After the initial acclimation period, fish were transferred to aquaponics system and were divided into four experimental groups of 42 fish in each group (each group had two replicates). There, fish were again acclimated for two weeks before first sampling (Kutty, 1972) and maintained following the animal care approved protocol thereafter (Table 3.3). The water quality was maintained according to Ostrander (2000).

Table 3.3. Water quality parameters maintained on the study

Parameter	**Reference Range**
Temperature	70-80$_0$F
Dissolved Oxygen	>4.7 ppm
pH	7-7.6
Nitrate	<40 ppm
Nitrite	< 5 ppm
Ammonia	<0.05 mg/L

Fish experimental groups: Group 1: Control (unstressed) fish were fed with commercial feed. Group 2: Stressed fish were fed with commercial feed supplemented with oral hydrocortisone. Group 3: Control (unstressed) fish fed with basil-supplemented commercial feed. Group 4: Stressed fish fed with basil and oral hydrocortisone-supplemented commercial feed. Fish were fed with oral cortisol (hydrocortisone) to confirm the stress condition. The details of the feed component are described on 3.2.2.

3.2.2 Fish diet preparation

All fish were fed with Purina® Aquamax® Fingerling Starter 300. This feed is 100% nutritionally complete and suitable for omnivorous fish as tilapia (Table 3.4). This feed was used throughout the experiment and modified according to different fish experimental group.

Control Diet: Control diet were prepared by mixing 1kg of commercial feed with 500mL of pure ethanol (obtained from the Biology Department stock supply at Purdue University Fort Wayne) by spraying it equally to make it similar with other types of supplemented feed. The feed was dried in laminar hood overnight.

Cortisol supplemented diet: As tilapia are adaptive to most natural stressors, stronger stress was induced in them by adding cortisol in feed at 0.01% in laboratory setup. Cortisol was obtained as 98% hydrocortisone (cortisol) powder (Acros Organics, New Jersey, USA). 100 mg of hydrocortisone powder was mixed with 500mL of pure ethanol (Biology Department stock supply, Purdue University Fort Wayne) and sprayed on the commercial feed. This concentration of cortisol in fish feed has been reported to induce stress previously (Barton et al., 1987).

Basil supplemented diet: Basil powder was obtained from Alka Ayurvedic Pharmacy, Ahmedabad, India (GMP certified). 30g of basil powder was dissolved in 200 mL of distilled water. The extract was collected using sterile vacuum filter (Nalgene, Thermo Fisher Scientific, New York, USA) with 0.20 µm pore size. The weight of basil in the extract was calculated as 15% of the crude powder after measuring the dry weight of extract. The extract was mixed with the feed in such way that the dry weight of basil will be 0.2% of the feed. This concentration has shown positive effect on fish health previously (Das et al., 2015). The extract was mixed with a portion of control diet and cortisol supplemented diet to prepare basil control feed and basil stress feed respectively.

Table 3.4. Contents of fish feed

	Amount (%)			
Content	Control Diet	Stress Diet	Control Basil Diet	Stress Basil Diet
Crude Protein (min)	50.00	50.00	50.00	50.00
Crude Fat (min)	16.00	16.00	16.00	16.00
Crude Fiber (max)	3.00	3.00	3.00	3.00
Calcium (Ca) (min)	2.35	2.35	2.35	2.35
Calcium (Ca) (max)	2.85	2.85	2.85	2.85
Phosphorus (P) (min)	1.30	1.30	1.30	1.30
Sodium (Na) (max)	0.60	0.60	0.60	0.60
Hydrocortisone	0	0.01	0	0.01
Holy Basil Extract	0	0	0.2	0.2

The feeds were again dried at room temperature (25°C) overnight and stored in fresh container in refrigerator at 4°C.

Fish was fed with experimental feed (four different groups) of 3% of body weight twice a day (1.5% each time). Before feeding each time, the water circulation was stopped and began again after 5 minutes of feeding to prevent possible transferring of feed from one tank to another. The fish were weighed every week to adjust the feed amount to the body weight.

3.2.3 Sampling

Three fish from each replicate were sampled on each sampling day. So, the total number of sampled fish was 24 fish (6 fish per treatment x 4 treatments) on subsequent sampling days x 4 sampling days = 96 fish.

Fish were euthanized by MS 222 (250 mg/L) (Tricaine-S, Western Chemical, Inc, Washington, USA) within 2 minutes of catching. This dose is high enough to cause rapid immobilization (Leary et al., 2013) and does not let cortisol level to go up due to handling stress.

Then they were kept in MS 222 for about 10 minutes until opercular movement stopped before collecting samples and analyses.

To record the chronic stress response and modulation of stress, samplings were conducted at week 0 and week 6. For recording cortisol levels, samplings were conducted at week 0, 2, 4 and 6.

3.2.4 Sample collection

Total length (from snout to tip of the tail) and weight (wet) of each fish were recorded using regular ruler and weighing scale. After that, blood was drawn from caudal vein using heparinized syringe (BD syringe, New Jersey, USA), (1000 iu/mL dilution of Heparin sodium powder, 150 iu/mg, Acros, New Jersey, USA) was used for heparinization. After collection, the syringe containing blood was immediately transferred to ice. This blood was used to measure physiological and immunological parameters.

3.2.5 Physiological parameters

Serum Cortisol

1mL blood was transferred to sterile Eppendorf tubes, then were centrifuged for ten minutes at 5000 RPM to get the serum (Stahl et al., 1992). After centrifugation, the supernatant (serum) was transferred to another sterile Eppendorf tube and kept at -80ºC. All samples were analyzed using a Cortisol ELISA Kit (Cayman Chemical, Michigan, USA) based on the instruction provided with the kit without any modification.

Packed Cell Volume

Few drops of blood from the syringe were taken into the capillary tube until it was 2/3rd full and then sealed by critocap on one side. They were centrifuged at 10,000 RPMs for five minutes in micro-hematocrit centrifuge. After the centrifugation was done, the blood on the tubes got separated into blood cells and plasma. The tubes were then placed on Micro-Hematocrit Capillary Tube Reader (Leica Biosystems) and the cell percentage was measured according to the scale. This method was adopted based on Wedemyer et al. (1990).

Total Plasma Protein

After the PCV reading was done, the plasma was collected from capillary tube and placed on a standard protein refractometer (VEE GEE Scientific, Kirkland, Washington). Before using, the refractometer was calibrated using distilled water. The reading was taken according to the scale to the nearest mg/dL. This method was adopted based on Riche (2007).

Hepatosomatic Index

After getting length and weight data and collecting blood, fish were dissected, and the livers were collected. The wet weight of liver was weighted immediately using AG204 Delta Range analytical scale (Mettler-Toledo, LLC, Columbus, Ohio). The weight of the liver was recorded to the ten-thousandths of a gram, and the weight was used to calculate hepatosomatic index (HSI) of the corresponding fish. This method was adopted based on Yue & Zhou (2008). The formula used to calculate HSI is given below.

$$HSI = \frac{liver\ weight\ (g)}{body\ weight\ (g)} * 100$$

Spleen-somatic Index

During dissection, spleen was also collected and weighted with same procedure as liver. This method was adopted based on Hadidi et al. (2008). The formula used to calculate spleen-somatic index (SSI) is given below.

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

Lysozyme Activity

After centrifuging the blood, the serum was collected and placed in a sterile Eppendorf tube. A suspension of 0.2mg of *Micrococcus lysodeikticus* in 0.05M (pH = 6.2) 1 mL of sodium phosphate buffer solution was made for the lysozyme assay. It is a gram-positive bacterium which was obtained freeze dried from Sigma, St. Louis, MO. This suspension was vortexed. Then 1 mL

of the suspension was taken into a sterile Eppendorf tube. Next, 50 µL of serum was added to the Eppendorf tube, then vortexed. Then 1 mL of this solution was put in a cuvette, and the absorbance at 540 nm was measured using Spectronic 601 spectrophotometer (Milton Roy Company). Before using, the spectrophotometer was calibrated using uninoculated sodium phosphate buffer (0.05M, pH - 6.2). The absorbance was recorded at 1 minute and at 5 minutes from mixing the serum. These readings were used to calculate the lysozyme activity (LA) using the following equation:

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

Total elapsed time was four minutes in this study. This method was adopted based on Parry et al. (1965).

Macrophage Phagocytic Capacity

For macrophage collection, during dissection, head kidney was removed aseptically by scrapping as the head kidney in fish has the interrenal cells which produce macrophages. Collected kidney was placed in centrifuge tube with 2mL L-15 Leibovitz medium with glutamine (Sigma, At. Louis, MO, USA) and the tube was placed on ice to store if needed. Then the kidney was macerated through sterile metal sieve (80 mesh/190µm) using sterile plunger. Meshes were changed between samples. The cells were collected again in centrifuge tube in L-15 then centrifuged at 1000 rpm for ten minutes. Cell free supernatant was discarded. Then 2mL of L-15 was added to the tube with the cell in form of pellet and vortexed to mix it. This centrifugation process was repeated three times. After third centrifugation, cells (pellet) were resuspended in 1 mL L-15.

For macrophage phagocytic assay, 50 µL aliquot of macrophage with L-15 was placed on to 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 paper wet by PBS. PBS was added to papers as needed. After incubation, 50 µL of heat killed bacteria (*Basillus megabacterium*) was added on the wells of the slides. Slides were kept at 25°C for two hours keeping cells moist. Next, slides were gently washed with PBS. Then the slides were dipped into absolute methanol for 1 minute to fix the smears. Then the slides were dipped in Wright-Giemsa stain solution for 1 minute. Then the smears were rinsed in PBS and dried in air.

Then cells were randomly counted from both wells of the slides. At least 50 cells were counted per well. 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.

Growth

From length and weight data, following parameters were measured- weight, length and Fulton's condition factor.

Fulton's condition factor (K) was measured by the following formula according to Ricker (1975).

$$K = \frac{Weight\ (g) * 100}{(Length\ (cm))^3}$$

3.2.6 Statistical Analysis

All data obtained in this study were analyzed using SigmaPlot 14.0 Scientific Graphing & Statistical Analysis Software (Systat Software, Inc., San Jose, California). One-way analysis of variance (ANOVA) followed by Tukey's test was performed for significance ($P<0.05$). All graphs were prepared using SigmaPlot 14, and additional labels to show significant differences were added using Microsoft PowerPoint. All graphs and data are presented as the means ± standard error of the means (SEM).

Figure 3.5. Centrifuge machine used for measuring PCV

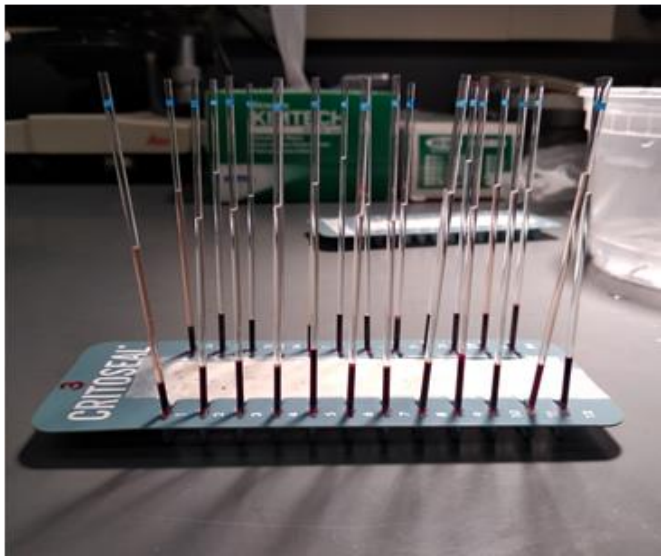

Figure 3.6. Microcapillary tubes after centrifugation showing plasma cells and plasma separately

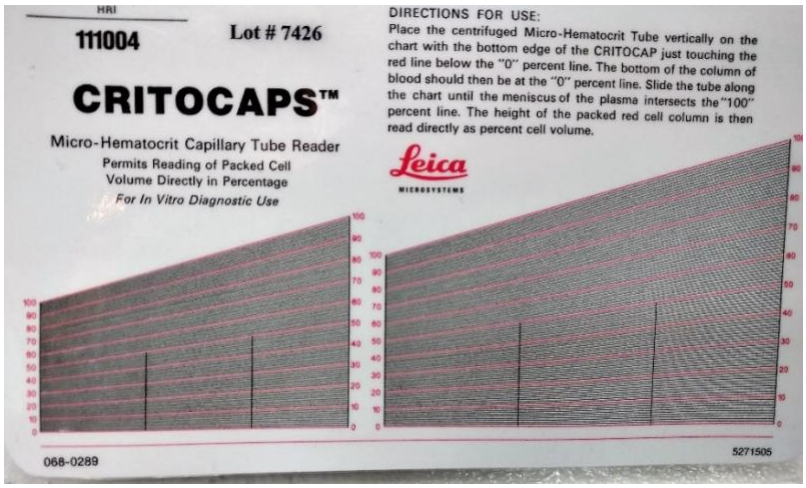

Figure 3.7. Capillary tube reader

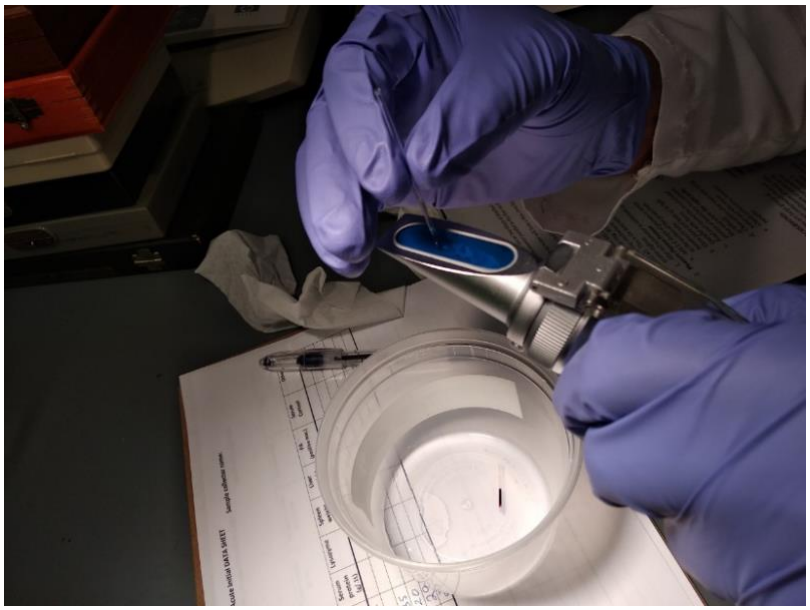

Figure 3.8. Measuring plasma protein using refractometer

Figure 3.9. Dissected fish showing the internal organs

Figure 3.10. Centrifuge machine used for differentiating serum from blood.

Figure 3.11. Fish from different groups (top- Control group, Bottom-Stress group)

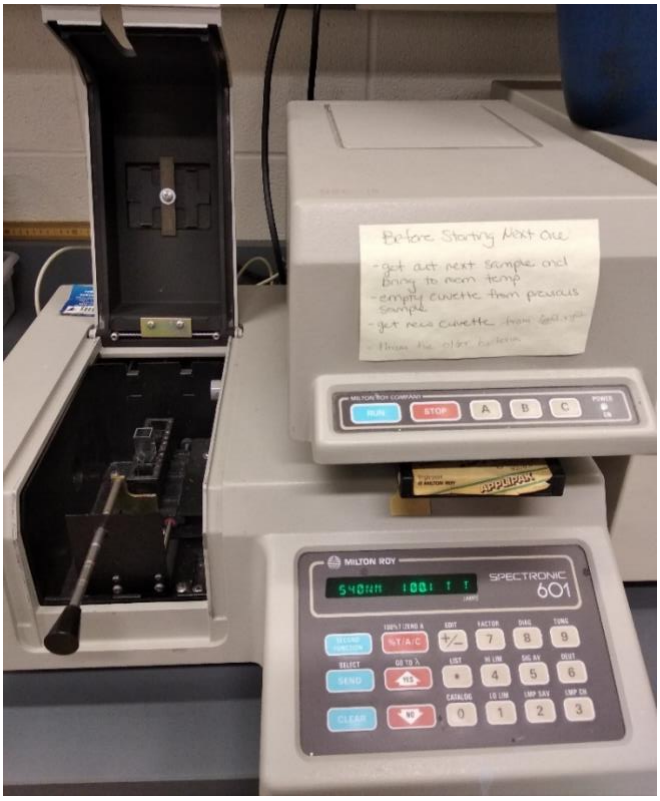

Figure 3.12. Spectrophotometer used to read the absorbance to measure lysozyme activity

Figure 3.13. Mesh used to extract macrophages from head kidney

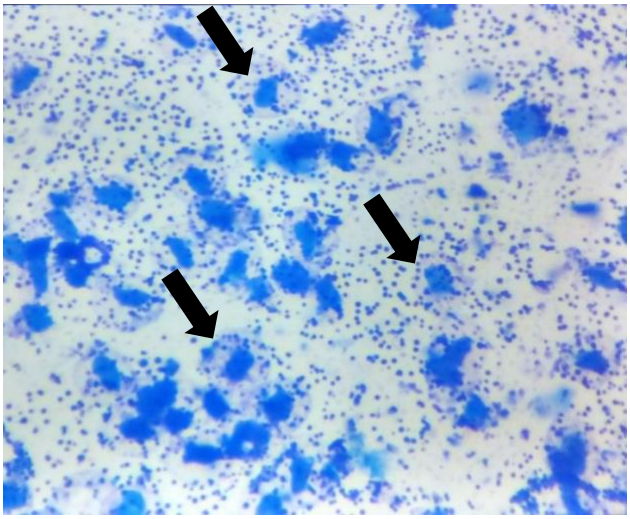

Figure 3.14. Photograph showing macrophages (black arrows) with phagocytized bacterial spore inside them (1000X)

3.3 Results and Discussion

3.3.1 Serum Cortisol

Secreting cortisol is an important primary response or hormonal response of fish to stress (Mazeaud et al., 1977). It is an indicator of both acute and chronic stress and is easier to measure. Also, cortisol releases a few minutes after the fish face the stress, so the plasma cortisol remains same to allow enough time to sample the fish. It is more stable than catecholamines during freezing storage (Schreck & Moyle, 1990). For these reasons, researchers mostly use cortisol as a measurement of stress (Barton & Iwama, 1991).

In fish, stressor stimulates the hypothalamic-pituitary-interrenal (HPI) axis and stimulates cortisol secretion from interrenal cells of the head kidney. These cells are equivalent to the adrenal cortex of higher vertebrates which is stimulated by adrenocorticotrophic hormone (ACTH) (Barton & Iwama, 1991). This release of cortisol is regulated by negative feedback of cortisol itself on the HPI axis (Fryer & Peter, 1977). It has been found that the negative feedback sites of corticosteroids are nucleus lateral tuberis (NLT) and preoptic-telencephalon regions which suppress the secretion of ACTH (Fryer & Peter, 1977). Plasma cortisol level comes down to the basal level (prestress level) within 24 hours after acute stress in fish (Laberge et al., 2019) due to increased metabolism of cortisol in liver.

After getting into the liver, it conjugates with other metabolites and is reduced to mainly tetrahydrocortisol, 20 β cortolone, tetrahydrocortisol, and 5 β dihydrocortisone, and cortisone (Truscott, 1979) which diminish its physiologic activity (McKay and Cidlowski, 2003). 80-90% of the conjugated cortisol then reabsorbed in the distal tubule of the kidney and the remaining is excreted through kidney (McKay and Cidlowski, 2003). These findings justify the methods of this study.

Serum cortisol results are presented graphically in Figure 3.15. The mean ± SEM baseline (week 0) serum cortisol (pg/mL) value was 3749.49±414.29. The mean ± SEM serum cortisol (pg/mL) values for the Control, Stress, Control Basil, and Stress Basil groups for week 2 were 4890.82±986.173, 2443.61±1246.96, 5190.80±1019.854, and 2764.667±1179.951, respectively; for week 4 were 4345.669±624.44, 207.36±73.67, 4040.76±360.62, 1880.05±543.296 respectively; and for week 6 were 2860.56±383.97, 1359.659±411.98, 2865.896±660.445, and 920.63±518.581, respectively.

Statistically significant differences were found between the baseline (week 0) serum cortisol values and both Stress groups at week 4 ($P<0.05$) and week 6 ($P<0.05$). Differences ($P<0.05$) were also observed between Control groups and Stress groups at week 4 ($P<0.05$) and week 6 ($P<0.05$). Present study did not demonstrate any effect of basil on serum cortisol level among the experimental groups, but it shows statistically significant difference between unstressed groups and stressed groups ($P<0.05$). The stress group fed with hydrocortisone mixed feed showed lower serum cortisol level at 4^{th} and 6^{th} week of the experiment which was also evident in cortisol fed Catfish (Davis et al., 1985), *in vitro* tissue culture in Coho salmon (Bradford et al., 1992), in tilapia in high density condition (Khan, 2016), and in tilapia fed with cortisol (Furnas, 2019; Saillant, 2019).

The results found in the present study can be explained by the negative feedback mechanism of cortisol. As fish were fed with cortisol added feed, it was absorbed in the blood which gave negative feedback to HPI axis and suppressed the secretion of ACTH and cortisol itself. As the fish were kept starved 24 hours before the sampling, the plasma cleared the cortisol by that time and measured serum cortisol in cortisol fed stressed groups was less than that of the control groups.

3.3.2 Packed Cell Volume (PCV)

Packed cell volume is a hematological indicator of secondary stress response (Barton & Iwama, 1991). It is the percentage of volume of blood cells to plasma.

PCV values are presented graphically in Figure 3.16. There were no significant differences found for the PCV (%) among experimental groups within sampling period (week 6). There were statistically significant differences between the PCV of baseline (week 0) and week 6 Control Basil group, and PCV of Baseline and Stress Basil group. The mean ± SEM baseline PCV on week 0 was 26.333±1.229 (not shown in the graph). The mean ± SEM PCV values for the Control, Stress, Control Basil, and Stress Basil groups at week 6 were 32±1.789, 32.667±0.494, 36±2.898, and 34.833±2.587, respectively. When the value of PCV of Control group was adjusted to 100%, the values of other groups changed to 102%, 112% and 106% respectively.

In this study, stress group did not show any significant difference of PCV than control group. Hrubec et al. (2000) reported PCV (%) in hybrid tilapia as 27-37 which is similar to all groups in the current study. PCV has been reported to get reduced in fish following acute

(Akinrotimi et al., 2009) and chronic stress (Ishibashi et al., 1992 ; Gensic et al., 2004). Striped Bass, *Morone saxatilis* showed reduced PCV after simulated transport (Lebelo et al., 2001). Significant decrease of PCV were also recorded in carps exposed to toxicity of *Moringa oleifera* seed extract (Kavitha et al., 2012).

On the contrary, increase of PCV was reported as a result of hypoxia where fish release red blood cell to compensate the insufficient oxygen supply (Hughes & Kikuchi, 1984), also in diseased fish where it indicates the increase of white blood cells as a defense mechanism (Lebelo et al., 2001). This can be a reason why all the fish groups failed to show any significant increases from normal level as they were neither hypoxic nor diseased.

Another possible explanation of this result can be that, the release of epinephrine and norepinephrine during stress results in the release of additional erythrocytes and swelling of those cells (Wendelaar, 1997). Because fish were stress with cortisol not epinephrine or norepinephrine, and cortisol gives negative feedback to release of these two hormones, the PCV did not increase significantly.

Basil fed groups showed apparently higher PCV than fish groups fed with non-supplemented diet which is similar to the effects found in other studies in farmed fish fed with *Cucurma longa*, (Sahu et al., 2008), mixture of *Brussica nigra*, *Chelidonium majus*, *Echinacea purpurea*, *Inula helenium* and *Tussilago farfara* extracts (Abasali & Mohamad, 2010), a mixture of *Cinnamomum zeylanicum*, *Juglans regia*, *Mentha piperita* and *O. basilicum* extracts (Abasali & Mohamad, 2010), *Aegle marmelos* (Pratheepa et al., 2010). Higher PCV (within limit) in fish leads to an improvement in health and immune competence (Bulfon et al., 2015).

3.3.3 Plasma Protein

Plasma contains more than 100 protein as albumins, globulins (includes immunoglobulins), hormones, tissue derived enzymes, lipoproteins, transport proteins, proteinase etc.; many of which are of great physiological and immunological importance (Putnam, 2012). Increase in plasma protein can be an indicator of stress (Ellis et al., 2012) or immune response to infections (Dawood et al., 2017).

Plasma protein values obtained from fish groups in this study are presented graphically in Figure 3.17. There were no significant differences found among the experimental groups at week 6. The mean ± SEM baseline (week 0) plasma protein (g/dL) was 5.267±0.345. The mean ± SEM

plasma protein (g/dL) for the Control, Stress, Control Basil, and Stress Basil groups from week 6 were 4.683±0.164, 5.150±0.141, 4.683±0.138, and 4.767±0.148, respectively. When the value of Control group was adjusted to 100%, other groups' values were changed to 109%, 100%, and 101% respectively. All of these were within the range 3-7.7 g/dL described by other researchers (Hrubec et al., 2000; Mauel et al., 2007). Healthy tilapia in recirculating system showed less than 5 g/dL (Chen et al., 2003). All groups' plasma protein values were less than 5 g/dL except for that of the Stress group, which showed increased plasma protein. Significant increase in plasma protein was previously found in tilapia reared in high-density system (Hrubec et al., 2000), in tilapia under thermal stress (Zaragoza et al., 2008), and in carp under pollution stress (Gopal et al., 1997).

It has been found that, in fish, the rate of cortisol binding to a plasma protein is relatively high (30-55%) which makes the cortisol biologically inactive and it helps the fish to tolerate high levels of cortisol released in their blood when stressed (Idler & Freeman, 1968). This gives one explanation why there is an increase of plasma protein in blood in stress groups- it is an adaptive feature of fish to tolerate stress. Stress also elicit stress proteins (commonly known as heat shock proteins) which increases total plasma protein value (Iwama et al., 2015). Increase in plasma protein value also can be a result of organ damage and dysfunction which is a result of alteration of enzyme activity under stress condition (Kavitha et al., 2012).

3.3.4 Hepatosomatic Index

Hepatosomatic Index or HSI is the ratio of weights of liver to whole body which indicates liver health. Liver is important for metabolism and absorption of carbohydrate and secretion of enzymes in fish (Ighwela et al., 2014).

HSI values are presented graphically in Figure 3.18. There were no significant differences observed for HSI among experimental groups within the sampling period (week 6). The mean ± SEM baseline HSI on week 0 was 2.134±0.431 (not shown in the graph). The mean ± SEM HSI for the Control, Stress, Control Basil, and Stress Basil groups from week 6 were 2.476±0.114, 2.886±0.166, 2.809±0.138, and 2.660±0.263, respectively. When the HSI value of Control group was adjusted to 100%, other groups' values were changed to 116%, 113%, and 107% respectively.

HSI normally decreases in stressed fish (Barton et al., 2002) but it depends on a various factors. Some species of fish as well as mammals show increase in liver size, weight and storage of glycogen in response to cortisol treatment. Eel, *Anguilla japonica* showed increased glycogen

store when injected with cortisol (Chan & Woo, 1978). On the other hand, cortisol fed goldfish, *Carassius auratus*, showed no significant difference on glycogen store as well as HSI (Storer, 1967) which is similar to the result found in present study.

It has been also found that HSI increases in Tilapia (Al-Ghais, 2013), Grey Mullet, *Mugil cephalus* and Grass Goby, *Zosterisessor ophiocephalus* (Corsi et al., 2003) and African Sharptooth Catfish, *Clarias gariepinus* (Mdegela et al., 2010) living in polluted water compared to fish living in fresh water. This suggests that HSI also can increase due to continuous metabolism of pollutants in liver as it is the major detoxification organ and pollutants can alter morphology and activity of it (Sadekarpawar & Parikh, 2013). Increased HSI is possible in stressed fish as they have to metabolize the added exogenous cortisol in plasma. It can make the liver hypertrophic and hyperplastic (Vosylienė & Kazlauskienė, 1999). Thornfish, *Terapon jarbua* which showed high HSI under thermal stress which was remedied by Vitamin C supplemented feed (Chien & Hwang, 2001). However, HSI is a poor indicator of fish energy status as it depends on various factors (Chellappa et al., 1995). Higher HSI in stress group does not necessarily indicate high energy status in fish of that group.

3.3.5 Spleen-Somatic Index

Spleen-somatic Index or SSI is the ratio of weights of spleen to whole body and it is an indicator of spleen health. Spleen is an important part of immune system and a healthy spleen in necessary for an active immunity against pathogens. It also has a erythrocyte reservoir which supplies additional erythrocyte to compensate the oxygen demand in certain circumstances as stress (Pearson & 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.

SSI values are presented graphically in Figure 3.19. The mean ± SEM of baseline SSI on week 0 was 3.373±0.0718 (not shown in the graph). The mean ± SEM SSI for the Control, Stress, Control Basil, and Stress Basil groups from week 6 were 0.461±0.0.0551, 0.303±0205, 0.460±0.0616, and 0.299 ±0.0417, respectively. When the control SSI value was adjusted as 100%, the other values were 66%, 100%, and 65%, respectively.

Stress and Stress Basil groups showed significantly lower value than Control and Control Basil group. There were no significant differences between Stress and Stress Basil groups or between Control and Control Basil groups.

Both stressed groups (Stress and Stress Basil) showed lower SSI than the control group suggesting negative effect of stress which was also evident in other stressed fish as Dab, *Limanda limanda* (Pulsford et al., 1994), Rainbow Trout, *Oncorhynchus mykiss* (Pearson & Stevens, 1991; Vosylienė & Kazlauskienė, 1999; Lai et al., 2006), Amur sturgeon, *Acipenser schrenckii* (Ni et al., 2014). On the other hand, Basil groups did not show any changes in SSI to their corresponding control groups which suggests Holy basil could not restore the altered SSI to normal values.

3.3.6 Lysozyme Activity

Measuring innate immunity in fish is a powerful tool to understand overall fish health and environmental impact on fish population- specially impact of toxicants (Bols et al., 2001). As fish does not have 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 glycoside bond of cell wall of bacteria (Fearon & Locksley, 1996). Fish has lysozyme in gill, skin, serum, brain, kidney and liver (Saurabh & Sahoo, 2008) and Nile tilapia (*O. niloticus*) has the highest lysozyme activity in serum (Panase et al., 2017).

Lysozyme activity results are presented graphically in Figure 3.20. There were no statistically significant differences found for the serum lysozyme activity values among the experimental groups within the sampling period (week 6). Significant differences have been observed between the baseline values at week 0 and the values of all groups at week 6 ($P<0.001$). The mean ± SEM baseline serum lysozyme activity (Absorbance/minute) value on week 0 was 0.0120±0.000815 (not shown in graph). The mean ± SEM serum lysozyme activity (Absorbance/minute) values for the Control, Stress, Control Basil, and Stress Basil groups from week 6 were 0.00650±0.000616, 0.00608±0.000441, 0.00795±0.000759, and 0.00660±0.000691, respectively. When the control lysozyme activity value was adjusted as 100%, the other values were 93%, 122%, and 101%, respectively. Control Basil group showed relatively higher activities compared to stress group.

Effect of stress on lysozyme activity depends on its intensity and duration (Yildiz, 2006). Following chronic stress, tilapia showed lower lysozyme activity (Caruso & Lazard, 1999). Rainbow trout (*Oncorhynchus mykiss*) showed significantly lower level of lysozyme and decreased lysozyme activity following acute stress and it took two weeks to return to normal stage (Möck & Peters, 1990). It was expected that the lysozyme activity would be lower in stressed groups than the unstressed group. But the differences among groups of this study were statistically non-significant. Basil previously showed enhanced lysozyme activity when mixed with peppermint and English Walnut in commmon carp (Abasali & Mohamad, 2010) and in rohu (Das et al., 2015). *Astragalus radix* and *Scutellaria radix* fed tilapia showed enhanced lysozyme activity (Yin et al., 2006). Tilapia also showed increased lysozyme activity when fed with bovine lactoferrin (Welker et al., 2007), *Solanum trilobatum* (Divyagnaneswari et al., 2007) and *Eclipta alba* (Christybapita et al., 2007) and they showed enhanced protection against different pathogens. Enhanced lysozyme activity was also found in different fish species fed with glucan (Saurabh & Sahoo, 2008) and vitamin C (Waagbø et al., 1993; Roberts et al., 1995). On the other hand, no significant differences were found in lysozyme activity in tilapia fed with propolis and Aloe extract (Dotta et al., 2014), and probiotic bacterium (*Enterococcus faecium)* (Wang et al., 2008).

3.3.7 Macrophage Phagocytic Capacity

The capacity of macrophages to kill the pathogens or antigens is another important immune mechanism in fish. As mentioned before, fish relies on its innate immune response to fight most of the pathogens and macrophage is an important part of it (Das et al., 2015). Macrophages kill the pathogens in fish by metabolic activation, degranulation and releasing reactive oxygen and nitrogen species (Neumann et al., 2001). Acute stress stimulates the activity of macrophages (Pulsford et al., 1994) but chronic stress causes reduction of both number (Belo et al., 2005) and phagocytic capacity (Mustafa et al., 2000) of macrophages in fish which makes them susceptible to diseases.

Macrophage Phagocytic Capacity values are presented graphically in Figure 3.21. No significant differences were found for capacity among experimental groups within the sampling period (week 6). The mean ± SEM baseline Macrophage Phagocytic Capacity on week 0 was 63.165± 6.737 (not shown in graph). The mean ± SEM Macrophage Phagocytic Capacity for the Control, Stress, Control Basil, and Stress Basil groups from week 6 were 97.900± 2.100, 70.333±

12.354, 89.850± 6.037, and 92.400± 6.467, respectively. When the Control group's Macrophage Phagocytic Capacity was adjusted as 100%, the other values were 72%, 92%, and 94%, respectively.

Control group had highest mean capacity and the Stress group showed the lowest (72% of Control group). Basil fed Stress group showed relatively higher capacity than the Stress group. But as not statistically significant, Effect of stress or Basil is not evident on macrophage phagocytic capacity. Different other herbal extracts previously showed enhanced phagocytic capacity as garlic (Nya & Austin, 2009), laurel (Bilen & Bulut, 2010) and smoked tree (Bilen et al., 2011) in rainbow trout, Chinese mahogany (Wu et al., 2010), omega-3 fatty acid (Hough et al., 2016) and mampat (Rattanachaikunsopon & Phumkhachorn, 2010) in tilapia.

3.3.8 Growth

Growth of animals in aquaculture is a key factor for profit. Stress occur physical disturbance in fish which is associated with metabolic cost and this causes reduction in growth (Barton & Iwama, 1991). Cortisol is a component of stress response which results in reduced growth in fish (Barton & Iwama, 1991). Cortisol fed channel catfish showed reduction in weight (Davis et al., 1985) which is similar to studies on rainbow trout (Barton et al., 1987) subjected to chronic stress. Although, it has been reported that acute stress does not have an effect on growth (Pickering, 1990) which suggests impaired growth is a result of chronic stress. Results of growth parameter are given below.

Length

Length values are presented graphically in Figure 3.22. There were significant differences ($P<0.05$) found for the length (cm) between Control and Stress group and between Control and Stress Basil group. Control Basil group did not show any significant differences with any other groups, although its mean was higher than both Stress and Stress Basil group. The mean ± SEM baseline length (cm) on week 0 was 11.917± 0.214 (not shown in graph). The mean ± SEM length (cm) for the Control, Stress, Control Basil, and Stress Basil groups from week 6 were 34.973± 16.810, 28.319± 11.754, 34.616± 17.757, and 28.687± 12.523, respectively. When the Control group's length was adjusted as 100%, the other values were 91%, 93%, and 89% respectively.

Weight

Weight values are presented graphically in Figure 3.23. There were significant differences ($P<0.05$) found for the weights (g) between Control Basil group (82%) and Stress Basil group (60%), and between Control (100%) and Stress group (66%). The mean ± SEM baseline weight (g) on week 0 was 33.217± 2.291 (not shown in graph). The mean ± SEM length (cm) for the Control, Stress, Control Basil, and Stress Basil groups from week 6 were 118.333± 8.698 78.600± 3.280, 97.067± 10.691, and 71.333± 4.955, respectively. When the Control group's weight was adjusted as 100%, the other values were 66%, 82%, and 60%, respectively.

Condition Factor

Condition Factor values are presented graphically in Figure 3.24. There were statistically significant differences observed between Control and Stress groups, between Control and Stress Basil groups, between Control Basil and Stress Basil, and between Control Basil and Stress at week 6 ($P<0.001$). The mean ± SEM baseline Condition factor on week 0 was 1.946± 0.0580 (not shown in graph). The mean ± SEM Condition factor for the Control, Stress, Control Basil, and Stress Basil groups for week 6 were 1.953 ± 0.0393, 1.724 ± 0.0330, 1.973 ± 0.0328, and 1.674 ± 0.0245, respectively. When the Control group's condition factor was adjusted as 100%, the other values were 88%, 101%, and 86%, respectively.

All growth parameters showed persistent results in this study. Control and Control Basil groups showed significantly higher values than both Stress and Stress Basil groups. This shows the negative impact of stress in both groups fed with basil or not. Overall, basil did not show any significant impact on fish growth. Basils are commonly used as immunostimulatory compound, not as a source of protein, but there are studies showing effects of basil to enhanced growth of tilapia (Panprommin et al., 2016; Brum et al., 2017) and broilers (Sheoran et al., 2017) although it varies widely upon doses and mode of extraction.

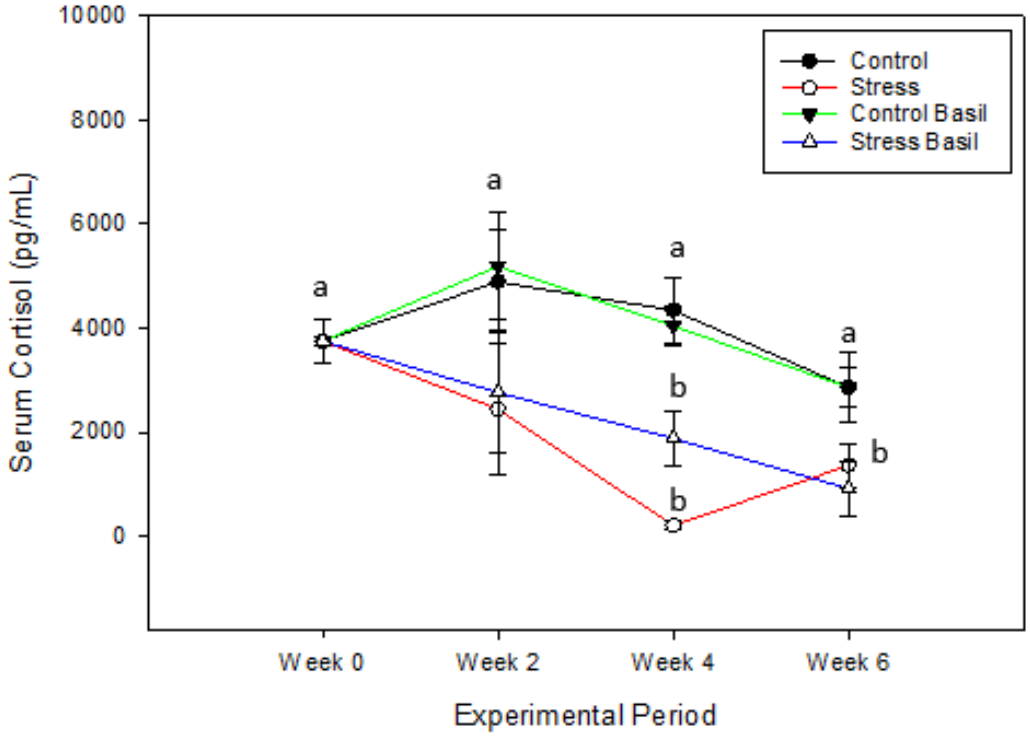

Figure 3.15. Serum cortisol levels throughout the experiment. Same alphabet means there is no statistically significant difference ($p>0.05$), different alphabet means there is/are statistically significant difference/s between/among groups ($p<0.05$).

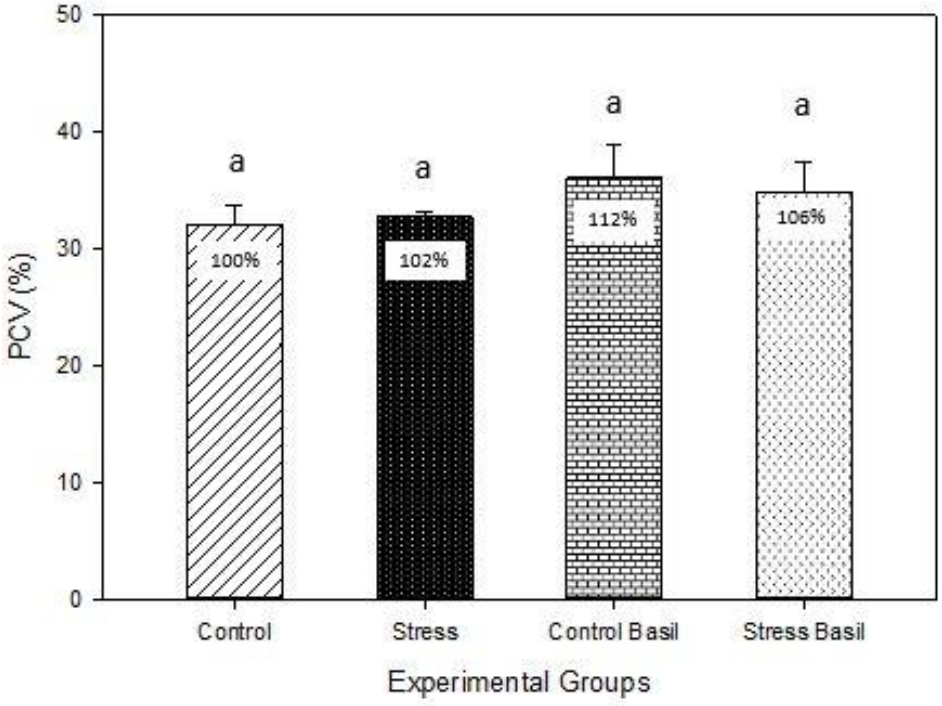

Figure 3.16. Packed Cell Volume (PCV) of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no significant difference ($p>0.05$).

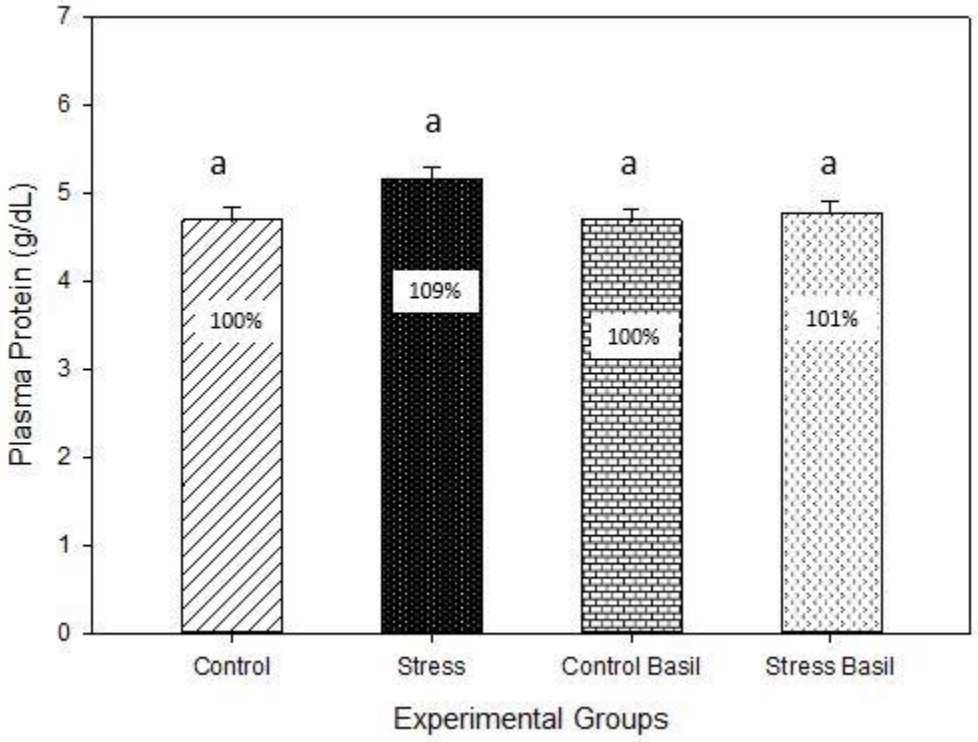

Figure 3.17. Plasma Protein of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no significant difference ($p>0.05$).

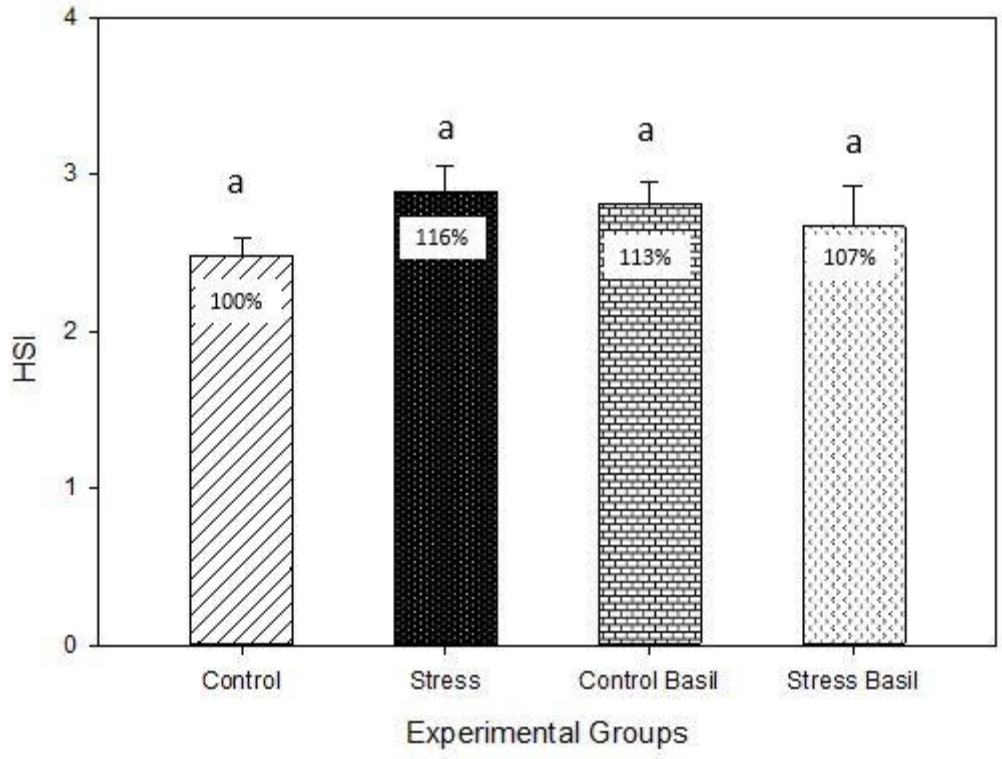

Figure 3.18. Hepatosomatic Index (HSI) of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no significant difference ($p>0.05$).

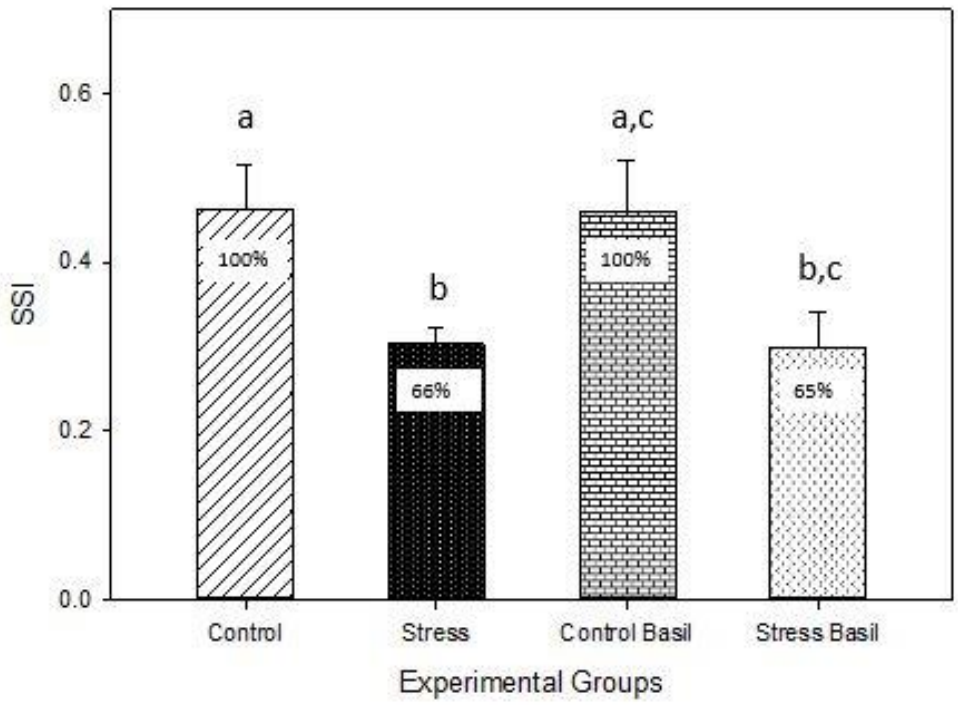

Figure 3.19. Spleen-somatic Index (SSI) of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no significant difference ($p>0.05$), different alphabet means there is/are statistically significant difference/s between/among groups ($p<0.05$).

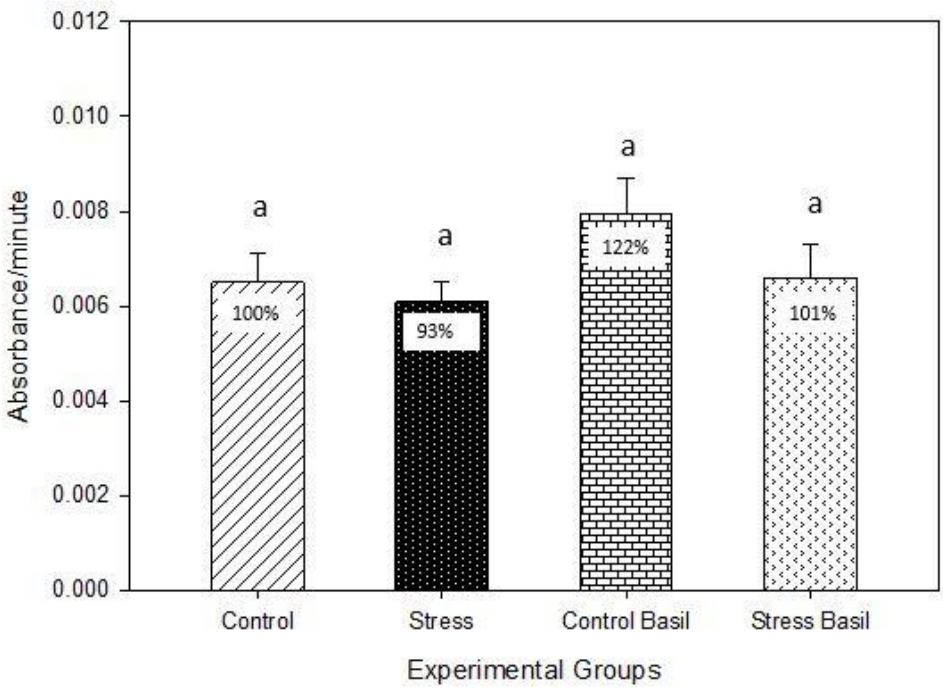

Figure 3.20. Lysozyme activity (absorbance/minute) of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no significant difference ($p>0.05$).

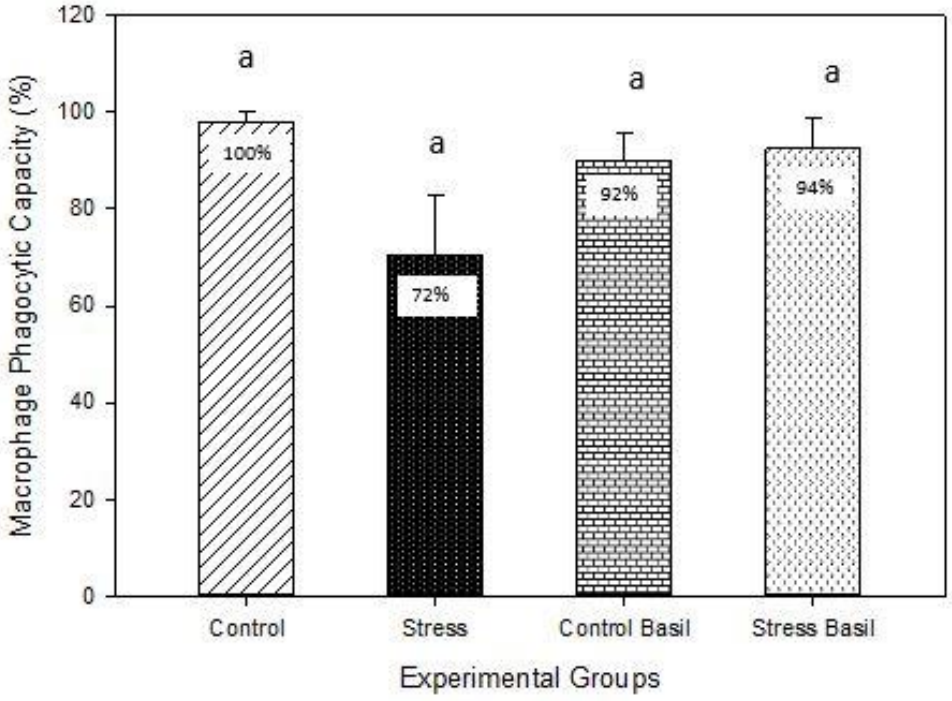

Figure 3.21. Macrophage Phagocytic Capacity of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no significant difference ($p>0.05$).

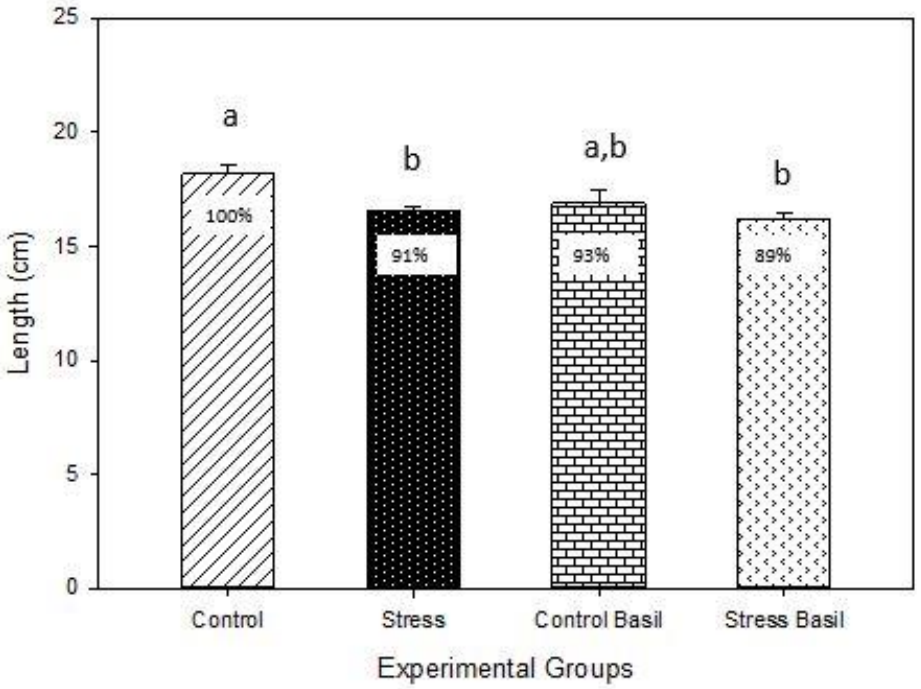

Figure 3.22. Length of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no significant difference ($p>0.05$), different alphabet means there is/are significant difference/s between/among groups ($p<0.05$).

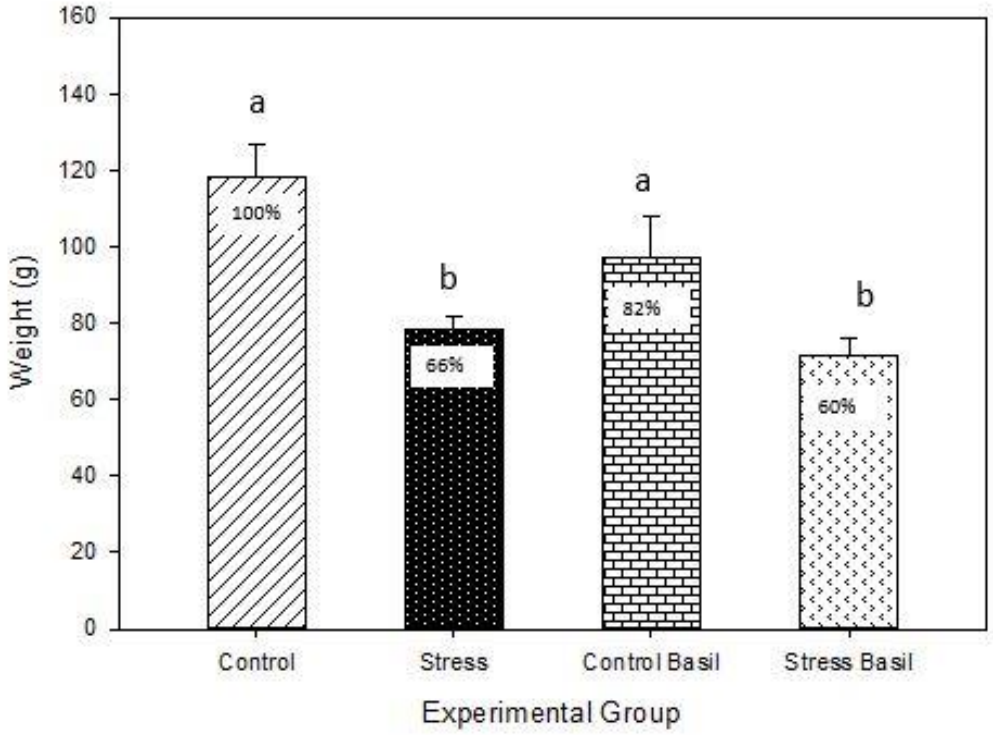

Figure 3.23. Weight of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no statistically significant difference ($p>0.05$), different alphabet means there is/are significant difference/s between/among groups ($p<0.05$).

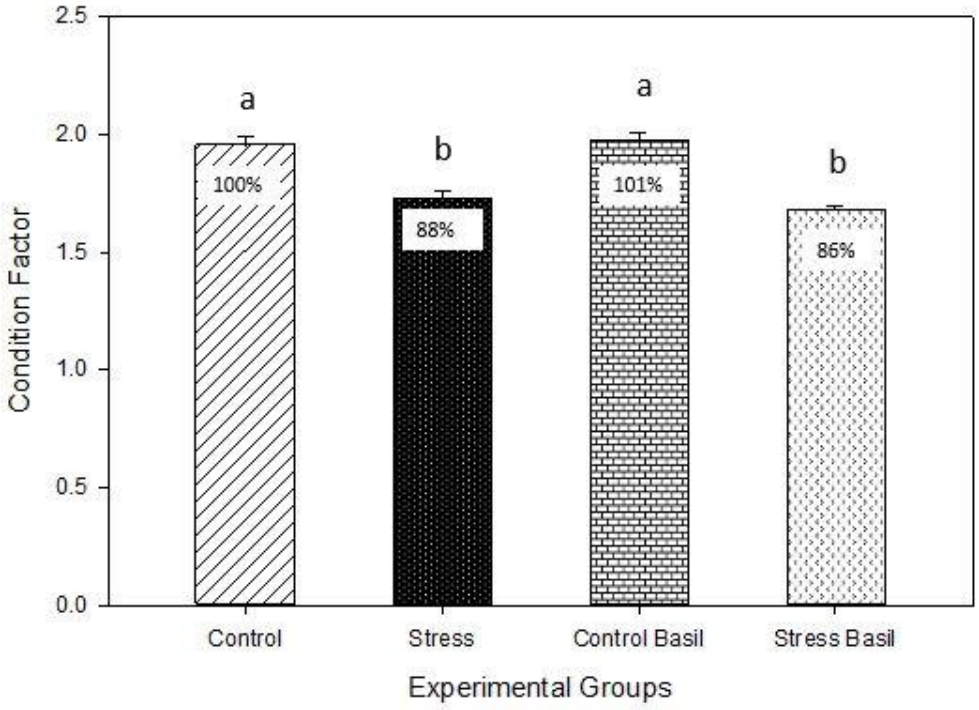

Figure 3.24. Condition Factor of four fish groups at week 6. For presenting the values in the box, the mean value of the control group was taken as 100% and the means of other groups were calculated based on that. Same alphabet means there is no statistically significant difference ($p>0.05$), different alphabet means there is/are significant difference/s between/among groups ($p<0.05$).

3.4 Conclusion

With continuing increase of world population and decrease of cultivable land, aquaculture is becoming a major dependable source of protein supply to meet the world demand. It is a healthier and more sustainable source of protein compared to others. Moreover, it reduces overfishing and depletion of natural habitat. But it is evident that the aquaculture practices induce both acute and chronic stress which impairs fish health and production which is also evident in this study. It also makes fish more susceptible to diseases that results in using antibiotics and other drugs in the water. This causes harm not only to the consumers' health-as the chemicals accumulates on fish meat, but also to the environment when it goes into the wild and raise the possibilities to introduce disease resisting microbiota and destroy beneficial ones.

While stress is unavoidable, scientists are focusing more and more to develop a way to modulate the stress to make the fish healthier and more productive. It also can reduce the use of drugs in the system. Nutraceuticals show a promising effect on this matter as they are not harmful to health, do not interfere with regular metabolism and do not have any negative impact on environment.

Holy basil is well studied plant as nutraceuticals. It has been widely used in different regions in Southeast Asia as herbal medicinal plant. Researchers conducted on various animal models suggest it improves immunity, increases survival rate when infected by lethal pathogens, reduces inflammation and increases growth. In the present study. Basil fed stressed group showed relatively higher lysozyme activity, relatively higher macrophage phagocytic capacity, relatively higher PCV and relatively lower plasma protein than the control stress group which suggests it can modulate stress at some extent. On the other hand, control group fed with regular feed and the control group fed with basil supplemented feed did not show any significant differences between them, which indicates that basil does not have any negative impact on healthy fish. Basil fed fish groups did not show any impact on increase of growth either, but the stress groups continued to show the negative effects on growth as expected.

There are several other studies showed different beneficial impacts of basils in different animal models which varies greatly on doses, study times, mode of application, medium of dilution etc. Hence, further investigations are needed to get the best concentration and biologically active components of this plant extract. It is possible that different dilution is capable of implicit different aspects of animal health and growth which can be determined by more extended research. Also,

the mode of modulation of stress also depends on type, extension and persistence of stress which may need different extraction and incorporation method of extracts to get the maximum result out of it. Mixing one nutraceutical with one or more other nutraceuticals also should be taken in consideration. There are some studies that showed beneficial effects of mixtures of two or more plant extracts on animals.

Once the maximum beneficial doses can be found for every health problem and the active compound/s responsible for that effect, nutraceuticals can replace the over usage of drug at some extent in aquaculture. That would be beneficial for the producers, consumers and the environment which is long expected.

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CHAPTER 4. PRODUCTION OF BASIL AND TILAPIA IN LABORATORY SCALE AQUAPONICS

4.1 Introduction

Aquaponics is getting attraction to various kind of farmers- hydroponic growers, fish farmers, and greenhouse growers as it only adds up the production and eases the maintenance of water quality (Diver & Rinehart, 2000). Considering all aspects, aquaponics system is very promising for future sustainable agriculture and fighting the pollution in both global and urban food production (Goddek et al., 2015). It has also been of vast interest in modern times for its ecological merits and extra economic advantage (Liang & Chien, 2013).

Aquaponics does not diminish essential non-renewable resources which is needed to continue the process (Lehman et al., 1993) and it resembles natural ecosystem (Francis et al., 2003). By the definition of sustainable agriculture, it can be said that aquaponics is a sustainable system of agricultural production (Goddek et al., 2015). In non- recirculating system, nutrients and water are needed from external sources which cost energy and fuels, also the waste generated here has chances for natural water pollution as it produces water considerably high in nutrients (Goddek et al., 2015). Considering this, aquaponics is a better system which not only reduces the chances of water pollution by reducing the waste but also reduces cost for secondary production (plant) by using the waste as nutrients. Comparing to beef production or fish production in semi-intensive/extensive aquaculture system, aquaponics requires almost 99.5% and 99.92% less water to produce 1 kg of meat (Goddek et al., 2015). This huge difference had been possible because aquaponics reuse almost 95-99% of the water (Goddek et al., 2015).

Both Holy and Thai basils are widely used in food as spice or taken as herbal medicine, mainly in Asia; and Nile tilapia is the most produced species among tilapia group and became very popular among farmers due to its adaptive capacity and low maintenance nature. For these reasons, basils and tilapia were chosen as the crops, and they were cultivated in the aquaponics system to investigate their productivity.

4.2 Materials and Methods

4.2.1 Aquaponics Setup

The research was conducted at the aquaponics facility in the greenhouse at science building of Purdue University Fort Wayne was used. This was a coupled system (Figure 4.1) with four fish tanks (aquaculture part) and two waterbeds and a gravel bed for plants (hydroponics part). The whole system was connected. Water from the fish tanks went to gravel bed. From the gravel bed the water entered the waterbeds and then fell through separate pipes in every tank. In both the gravel bed and water beds, plants were transferred at the same time as the fish.

4.2.2 Plant acquisition and management

Two types of basil seeds were obtained from David's Garden Seeds, San Antonio, TX and were planted in the hydroponics system in the water beds: Holy basil and Thai basil. Seeds were planted in square rock wools held by hydroponic mesh pot basket. They were kept in closed plant germination room with optimal temperature (25°C) and photoperiod (12 hours). After germination (development of the root), the plants were transferred to the water and gravel beds. The baskets were placed on the Styrofoam floats on the plant beds in aquaponics system in such a way that the roots touched the water. There were two same sized water beds for plants, each of 1.47 sq. m (155*95cm); one for Holy basil and the other for Thai basil. There were nitrifying bacteria in the gravel bed to transform the fish toxic ammonia waste into non-toxic nitrate and nitrite. All the sections were joined together by a common water flow i.e., recirculating system.

Plant harvesting

The plants were harvested according to 'cut and come again' method. The weights of Holy basil and Thai basil were harvested separately. The plants were left for the leaves to come back. The leaves were harvested every month, started two months after the germination and every month thereafter for three more months. There were fish on the system throughout the study.

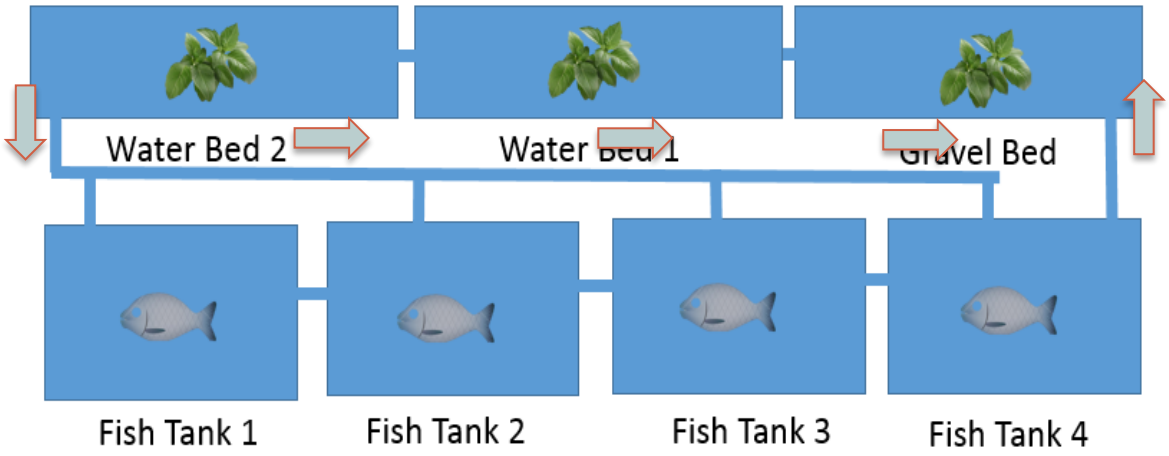

Figure 4.1. Aquaponics system

4.2.3 Fish acquisition and management

84 disease free tilapia fingerlings were obtained from Troyer Fish Farms, Geneva, Indiana. Upon arrival, fish were acclimated using optimum water conditions (Table 4.1) in a recirculating system for one week. After the initial acclimation period, fish were transferred to aquaponics system. There were two experimental groups, each had 42 fish in each group (each group had two replicates). There, fish were again acclimated for two weeks before first sampling (Kutty, 1972) and maintained following the animal care approved protocol thereafter (Table 4.1). The water quality was maintained according to Ostrander (2000).

Table 4.1. Water quality parameters maintained for farming of tilapia

Parameter	**Reference Range**
Temperature	70-80$_0$F
Dissolved Oxygen	>4.7 ppm
pH	7-7.6
Nitrate	<40 ppm
Nitrite	<5 ppm
Ammonia	<0.05 mg/L

Fish Diet

All fish were fed with Purina® Aquamax® Fingerling Starter 300. This feed is 100% nutritionally complete and suitable for omnivorous fish as tilapia (Table 4.2). This feed was used throughout the experiment and modified according to different fish experimental group.

Control Diet: Control diet were prepared by mixing 1kg of commercial feed with 500mL of pure ethanol (obtained from the Biology Department stock supply at Purdue University Fort Wayne) by spraying it equally to make it similar with other types of supplemented feed.

Cortisol supplemented diet: As tilapia do not respond to mild stress factors, stress was induced in them using cortisol in feed at 0.01%. Cortisol was obtained as 98% hydrocortisone (cortisol) powder (Acros Organics, New Jersey, USA). 100 mg of hydrocortisone powder was mixed with 500mL of pure ethanol (Biology Department stock supply, Purdue University Fort Wayne) and sprayed on the commercial feed. This concentration of cortisol in fish feed has been reported to induce stress previously (Barton et al., 1987).

The feeds were again dried at room temperature (25°C) in laminar hood overnight and stored in fresh container in refrigerator at 4°C.

Fish was fed with experimental feed (two different groups) of 3% of body weight twice a day (1.5% each time). Before feeding each time, the water circulation was stopped and began again after 5 minutes of feeding to prevent possible transferring of feed from one tank to another. The fish were weighed every week to adjust the feed amount to the body weight.

Fish sampling

For initial data, fish were sampled after acclimation (week 0) and for final data, they were sampled at week 6. For sampling, the fish were obtained from the tanks with nets, then anesthetized with MS 222 (15 mg/L) (Tricaine-S, Western Chemical, Inc, Washington, USA). This dose is high enough to cause sedation and does not let cortisol level to go up due to handling stress. The fish were measured for length and weight. Fulton's condition factor (K) was measured by the following formula according to Ricker (1975).

$$K = \frac{Weight\ (g) * 100}{(Length\ (cm))^3}$$

Table 4.2. Contents of fish feed

	Amount (%)			
Content	Control Diet	Stress Diet	Control Basil Diet	Stress Basil Diet
Crude Protein (min)	50.00	50.00	50.00	50.00
Crude Fat (min)	16.00	16.00	16.00	16.00
Crude Fiber (max)	3.00	3.00	3.00	3.00
Calcium (Ca) (min)	2.35	2.35	2.35	2.35
Calcium (Ca) (max)	2.85	2.85	2.85	2.85
Phosphorous (P) (min)	1.30	1.30	1.30	1.30
Sodium (Na) (max)	0.60	0.60	0.60	0.60
Hydrocortisone	0	0.01	0	0.01
Holy Basil Extract	0	0	0.2	0.2

4.3 Results

4.3.1 Fish Production

The fish production data are presented in table 4.3. The baseline average length and weight of the fish were 11.92 cm, 33.22 g respectively. After 6 weeks, Control group showed significantly higher length and weight, and better condition factor than those of the Stress group. Stress group showed 25.6% less length and 46.68% less weight than Control group.

Table 4.3. Growth data of fish (Different alphabet indicates significant differences between groups)

Parameter (Average)	**Control Group**	**Stress Group**
Initial Length cm (Week 0)	11.92	11.92
Final Length cm (Week 6)	18.17 a	16.57 b
Length Gain cm	6.25 (100%)	4.65 (74.4%)
Initial Weight g (Week 0)	33.22	33.22
Final Weight g (Week 6)	118.3333 a	78.6 b
Weight Gain g	85.11 (100%)	45.38 (53.32%)
Condition Factor	1.95a	1.72b

After six weeks, the net fish production from Control group was 4969.99 g (6 g/L) and from Stress group was 3301.2 g (3.98 g/L).

4.3.2 Plant Production

The plant production data are presented in table 4.4. After four harvests, a total of 9586g (6,521.1 g/sq. m) and 10613g (7219.73 g/sq. m) of leaves with stems (marketable size) were gained from Holy basil and Thai basil respectively. The average production from both species is 6,733 g/sq. m.

Table 4.4. Plant production

Species	**Total Leaf Harvest (With Stem) (g)**	**Total Leaf Harvest (g)**
Holy Basil	9586	4999
Thai Basil	10613	5333

4.4 Discussion

Both Control and Stress group of fish showed higher condition factor. Fish production was significantly higher in Control group than the Stress group. In conventional pond aquaculture system, condition factor in male tilapia has been recorded as 1.63 with a daily feeding rate of 5% of body weight (Dagne et al., 2013). Compared to that, we fed 2% less feed and still the fish's condition factor was higher. This suggests that, aquaponics is a promising and more productive sector of aquaculture that the traditional pond system and requires less feed. However, aquaponics requires good husbandry methods such as maintaining the water quality at optimum level.

Stress group of fish showed significantly lower values for all growth factors than the Control group. It is evident in this study that stress have negative impact on fish production. Even, it is better than that of the traditional aquaculture, lessening the stress on fish on aquaponics system can give even better production as well as profit.

The yield from Thai basil was more than Holy basil. From the observation, it is evident that Thai basil grows faster than Holy basil. But the total production was still compatible to traditional greenhouse basil production (Figure 4.2).

Figure 4.2. Plant growth (Top-1 month after germination, Middle- 2 months after germination, Bottom- 3 months after germination)

4.5 Conclusion

The scarcity of cultivable land and other natural resources are decreasing day by day. Hence, aquaponics is becoming more and more of scientists' and producers' interest. As it needs minimal space and can be placed in urban environment, its popularity is increasing (Love et al., 2015). It is currently used to produce food to supply food to urban local market with locally produced food. In addition, it utilizes the heat produced in urban industrial area (Lewandowski et al., 2018).

Our study showed good production of both fish and plant which is compatible to land based agriculture. Although, the stressed fish showed far lower production. So, it should be kept in mind not to stress the fish and keep the environment as ideal as possible when using aquaponics system. Also, it is important to note that, this experiment was done in a laboratory scale aquaponics system. At commercial production level, the stocking density of both crops is going to be higher that this for getting profit. So, it is important to find the threshold between the stocking density and production loss due to overcrowding stress. If we can successfully establish and utilize aquaponics system as an economically viable sector, this can be the primary resource of food production in future.

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CHAPTER 5. GENERAL CONCLUSION

The studies were conducted to observe the effects of Holy basil and Thai basil on spleen cell proliferation *in-vitro*, to observe the stress modulation of Holy basil in tilapia reared in aquaponics system *in-vivo*, and to get an estimation of aquaponics production of both plants and fish.

In the *in-vitro* study, basils did not show any statistically significant differences on spleen cell proliferation or spleen T cell proliferation. But overall, Holy basil showed higher proliferation than that of Thai basil in spleen cell proliferation.

In the *in-vivo* study, cortisol fed fish groups showed significantly lower serum cortisol level and condition factor which exhibits the inactivation of HSI due to negative feedback and reduced growth. Holy basil did not show any significant effects on these parameters. Holy basil showed to have some immunostimulatory effects on fish by modulating some immune responses. It is also evident that holy basil does not have any negative impact on fish health or growth.

Third study gives us the estimated production of Holy and Thai basils and tilapia in a laboratory scale aquaponics. The result shows that aquaponics production is competent with that of the land agriculture considering only the value of the products.

These studies are significant in various ways. Most of it was done to test the usefulness of using nutraceuticals as it is environment friendly and can be naturally obtained that makes it less expensive. This study can lead to future research to find the best method of using nutraceuticals. If the exact concentration and combination can be found to be effective to make fish (or to a broader extent, other animals including human) healthier whether under stress or not, it can lessen our dependency on chemical drugs and antibiotics; and if we can apply the method of incorporating nutraceuticals into fish feed which will lessen the negative impacts of stress in aquaponics, it can be a reliable agriculture in future which is environmentally sustainable and economically viable.