MICROBIOLOGICAL, IMMUNOLOGICAL, AND PHYSIOLOGICAL EFFECTS OF LONGEVITY SPINACH (*GYNURA PROCUMBENS*): *IN-VITRO* AND *IN-VIVO*

by

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I would like to dedicate this thesis to my parents, who provided everything to ensure I would have the opportunity of an education. Their efforts and struggles have allowed me to have a key to unlock the mysteries of our world and beyond.

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ABSTRACT

Longevity spinach, *Gynura procumbens*, has been traditionally used to treat a variety of diseases such as eruptive fevers, rash, kidney disease, migraines, constipation, hypertension, diabetes, and cancer. Recently, several papers have reported that this plant also has anti–Herpes simplex virus, anti–hyperglycemic, anti–hyperlipidemic, anti–inflammatory, analgesic, and reduced blood hypertension properties. However, to the best of our knowledge, nothing has been done to modulate stress in aquatic animals using *G. procumbens*. Therefore, we have investigated the potential of *Gynura* to modulate stress and immune responses in fish, tilapia.

I examined the effects of *Gynura* by well and disc-diffusion methods against nine selected bacteria. In the well diffusion method, *Gynura* in ethanol extract showed significant sensitivity against *E. feacalis* ($p<0.05$) and *P. aeruginosa* ($p<0.05$) with the presence of zone of inhibition. In the disc diffusion method, *Gynura* in ethanol extract showed significant sensitivity against *B. subtilis* ($p<0.05$), *S. aureus* ($p<0.05$), and *S. epidermidis* ($p<0.05$). Similarly, *Gynura* in methanol extract also showed significant sensitivity against *B. subtilis* ($p<0.05$) and *S. aureus* ($p<0.05$).

I used different concentrations of ethanol and aqueous extracts of *Gynura* to investigate its effects on the inhibition of B16 murine melanoma cells and proliferation of spleen cells collected from BALB/c mice. In these experiments, 4g/mL and 0.8g/mL of ethanolic extract significantly inhibited the melanoma cell growth in-vitro ($p<0.05$). However, there was neither significant proliferation nor inhibition of spleen cells ethanol or aqueous extracts of *Gynura* with different concentration ($p<0.05$).

I used *Gynura*-supplemented feed to prevent stress and to treat stressed fish for both short and long propagation periods. In these experiments, *Gynura* significantly prevented the stress hormone, cortisol, from increasing in the prevention group ($p<0.05$) and significantly reduced cortisol in the treatment group ($p<0.05$). The blood glucose level was decreased in prevention and treatment group compared to control. Because of their correlated effects, improvement of overall immunity was observed in the immunological responses, especially in serum lysozyme activity ($p<0.05$).

Gynura procumbens has the potential to be used as the nutraceuticals to modulate stress and immune responses in vertebrates.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Introduction

Plants are the most significant source of traditional medicines used to treat a wide range of illnesses [1]. Despite the fact that the importance of medicinal plant use had previously been placed on treatment of a disease rather than prevention, research work on the use of medicinal plants and their constituents in disease prevention has been raised recently.

Longevity spinach, *Gynura procumbens*, from the family Astereceae, is one such medicinal plant, mainly found in the Southeast Asia. In Malaysia, *G. procumbens* is called as Sambung nyawa, meaning "prolongation of life" in English [1]. This plant has traditionally been used to treat a variety of diseases including fevers, rash, kidney disease, migraines, constipation, hypertension, diabetes, and cancer [2]. Several studies have recently reported that this plant has anti–Herpes simplex virus, anti–hyperglycemic, anti–hyperlipidemic, anti–inflammatory, analgesic, and blood hypertension-lowering properties [3][4][5]. However, to the best of knowledge, nothing has been done to modulate stress in aquatic animals using *G. procumbens*. Therefore, I wanted to carry out experiments to investigate the potentials of *G. procumbens* to modulate stress and immune response there-after, in fish namely, tilapia.

To accomplish my research objectives, I first, investigated the antimicrobial effects of *G. procumbens* on selected gram-positive and gram-negative bacteria. Then I investigated immunological effects of *Gynura.* Specifically, I investigated its ability to inhibit melanoma cell growth in the murine cell line as well as its ability to modulate specific immune cells- both B and T, *in-vitro*. Finally, I investigated its physiological and immunological effects *in-vivo* using vertebrate animal model. In these *in-vivo* experiments, we used Nile tilapia. I stressed tilapia using hydrocortisone and studied their physiological and immunological responses in both acute and chronic conditions against *Gynura.* In the acute stress experiment, I investigated three different concentrations of *G. procumbens* on the modulation of physiological and immunological effects and picked the best concentration to be used in the chronic stress experiment. In the chronic experiment, I investigated the effects of *G. procumbens* on prevention of stress in animals as well as treatment of stressed animals in order to find the best solutions for researchers and farmers for future use.

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CHAPTER 2. ANTIBACTERIAL ACTIVITIES OF *GYNURA PROCUMBENS* AGAINST SELECTED GRAM-POSITIVE AND GRAM-NEGATIVE PATHOGENIC BACTERIA, *IN VITRO*

2.1 Abstract

Antibiotic resistance is a global concern and the development of alternative therapeutics is, therefore, an urgent need. Experiments were conducted to evaluate antimicrobial effects of different *Gynura procumbens* extracts against selected gram positive and gram-negative pathogens. Three different *Gynura* leaf extracts were prepared with the concentration of 0.25 ml/g in water, 25% ethanol, and 100% methanol solutions. The antimicrobial effects of the extractions were examined by well diffusion and disc diffusion methods against nine selected pathogenic bacteria. In the well diffusion method, *Gynura* in ethanol extract has shown significant sensitivity against *E. feacalis* (p<0.05) and *P. aeruginosa* (p<0.05) with the presence of zone of inhibition. In the disc diffusion method, *Gynura* in ethanol extract has shown significant sensitivity against *B. subtilis*, *S. aureus* (p<0.05), and *S. epidermidis* (p<0.05). Similarly, *Gynura* in methanol extract has also shown significant sensitivity against *B. subtilis* (p<0.05) and *S. aureus* (p<0.05). *Gynura* has a preferential effect on gram positive bacteria over gram negative bacteria. The current investigation demonstrates that *Gynura* leaf extracts have some antibacterial activity against pathogenic bacteria. Future investigations should be undertaken to isolate the components accountable for the efficacy.

2.2 Introduction

The ubiquitous use of antibacterial medicines for preventive and therapeutic purposes has resulted in a rise in antibiotic resistance. Pathogenic bacteria frequently acquire resistance when exposed long-term to conventional antibacterial medicines, making infections more challenging to control [1, 2]. The introduction of novel antimicrobials or antimicrobial substances is one solution to combat the rise of the antibiotic resistance concern. Plant extracts are being investigated as a possible alternative to presently deployed antimicrobials, and several plant extracts have been shown to exhibit significant antimicrobial properties over pathogenic microorganisms.

Herbal treatments are highly acknowledged to address a wide range of health conditions. Herbs are a reliable, less hazardous, cost-effective, safe, and consistent natural

supply of therapeutics all over the world [3]. The use of herbal medicine among tribal locals and medicinal healers is an important aspect of Asian history, and it is still extensively practiced today [3]. In traditional Chinese medicine, *Gynura procumbens* (*Gynura*), also known as sambungnyawa, is used to treat skin rashes, infections, rheumatism, inflammation, renal illness, headache, constipation, high blood pressure, diabetes, and malignancies [4]. It is rich in phenolic and flavonoids, natural products, saponins, tannins, terpenoids, and sterol glycosides. The presence of phenolic secondary metabolites like as flavonoids and glycosides in *Gynura* has been linked to its positive qualities. Astragalin, kaempferol-3-Orutinoside, kaempferol, rutin and other phytochemicals are also abundant in *Gynura* [5]. *Gynura* leaves or leaf extracts, besides its antimicrobial effects, have been shown to be efficacious against hyperglycemia and hyperlipidemia. *Gynura* has also shown to have anti-inflammatory, anticarcinogenic, antihypertensive, antiproliferative, antioxidative, and antiulcerogenic qualities [6–11]. Kaewseejan's phytochemical research indicated that the components of *Gynura* extract in ethanol have chlorophyll-a and -b, carotenoids, alkaloids, and volatile oils but they didn't find any antibacterial effect against the bacteria they tested. Haron and Jusoh discovered that an acidic extract of *Gynura* exhibits a favorable response to *E. coli*, *S. aureus*, and *C. albicans* [12, 13].

In this experiment, I wanted to investigate the antibacterial activity of *G. procumbens* leaf extracts prepared in ethanol, methanol, and water against selected pathogenic gram-positive and gram-negative bacteria. I found that ethanolic *Gynura* extract has some antibiotic activity against primarily gram-positive species. I hypothesize that our findings will be helpful for further development of nutraceuticals and therapeutics.

2.3 Material and methods

2.3.1 Preparation of *Gynura procumbens* extract

Fresh leaves of *Gynura procumbens* (*Gynura*) were collected from plants grown without any fertilizer and external treatment in a greenhouse at range from 24 to 32°C and around 80% humidity. Leaves were washed with water and finely mashed with mortar and pestle. *Gynura* paste was prepared with 0.25 g/mL in 25% ethanol, 100% methanol and water. Prepared *Gynura* paste was filtered with a double layer of cheese cloth. Filtered *Gynura* extract (GPE) was mixed for 20 minutes on a rotating shaker mixture. After that, GPE was centrifuged at 10°C at 10000 rpm for 10 minutes [14]. Finally, the prepared extract was stored at 4°C.

2.3.2 Pathogenic bacterial strain and growth conditions

Four gram-positive pathogenic bacteria; *Enterococcus faecalis*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus epidermidis,* and five gram-negative *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa,* and *Salmonella typhimurium* were used in this study. These pathogens were subcultured on trypticase soy agar media plates at 37°C for 24 hours. To keep the bacteria viable and to prevent contamination from other microorganisms, subcultures were kept at 4°C and used for further repetitions.

2.3.3 Preparation of bacterial suspension

Freshly cultured colonies from the stock were diluted with 0.85% saline to approximate the density of 0.5 McFarland standard, which represented an estimated concentration of $1.5x10^8$ colony forming units (CFU/ml). Commercial 0.5 McFarland standard was used to prepare standard bacterial suspension.

2.3.4 Well diffusion method

After incubating fresh subculture plates for 24 hours, they were placed in the laminar chamber and used to make standard bacterial suspensions with autoclaved sterile saline. Following that, an autoclaved sterilized cotton swab was used to perform lawn culture on sterile large plates containing 45 ml Mueller Hinton Agar (MHA) to ensure uniform growth of bacteria. A well was prepared in the plates with a cork-borer No. 2. Fifty microliters of each experimental solution were placed on the surface of the inoculated agar well. The plates were incubated for 18-24 hours at 37°C. The zone of inhibition surrounding the well plates, indicating the presence of antibacterial activity, was measured and recorded. The antibiotic discs were used as positive controls, and 25% ethanol and 100% methanol were used as negative controls against each specific pathogen [15], [16].

2.3.5 Disc diffusion method

After incubating fresh subculture plates for 24 hours, they were placed in the laminar chamber and used to make standard bacterial suspensions with autoclaved sterile saline. Following that, an autoclaved cotton swab was used to perform lawn culture on sterile large plates containing 45 ml Mueller Hinton Agar (MHA) to ensure uniform growth of bacteria. The blank discs (6 mm in diameter) were immersed with different solution of *G. procumbens*

extracts, namely *Gynura* water extract (GW), *Gynura* ethanolic extract (GE), *Gynura* methanolic extract (GM) and extraction solvents: Ethanol (ET) (25% without *Gynura*) and methanol (ME) (100% without *Gynura*). They were air dried. After the discs were completely dried, they were placed over bacterial lawn of MHA plate. The plates were incubated at 32°C for 24h. The diameter of inhibition zone around each disc were then measured using a millimeter ruler. The antibiotic discs were used as positive controls, and 25% ethanol and 100% methanol were used as negative controls against each specific pathogen [17].

2.3.6 Statistical analysis

All assays were performed in triplicate (n = 3) and the data is expressed as mean and standard Deviation (SD). Comparisons were made between the negative controls and different extracts of *G. procumbens* using Students' t-test with Bonferroni adjustments. Statistical significance was considered at 95% confidence intervals ($P<0.05$). Diameters of zones of inhibition ≥ 10 mm and ≤ 6 mm were considered active and inactive, respectively. Zones of inhibitions between 10 and 6 mm were considered mild [18].

2.4 Results

Results of the antimicrobial activities of *Gynura* water extract (GW), *Gynura* ethanol extract (GE), *Gynura* methanol extract (GM), Ethanol (ET), methanol (ME), and antibiotics has been summarized using well diffusion and disc diffusion methods in Table 2.1 and Table 2.2, respectively.

In well diffusion method, pure ethanol and pure methanol without *Gynura*, showed only mild effects against *B. subtilis. S. aereus, S. epidermidis,* and *S. typhimurium* with high deviations from the means, and no effect against *E. faecalis, P. mirabilis, P. aeruginosa, E. coli,* and *K. pneumoniae*. In contrast, *Gynura* ethanol (GE) extract was significantly effective ($P<0.05$) against *E. feacalis* and *Pseudomonas aeruginosa*. *Gynura* methanol (GM) extract has showed only mild effects against *B. subtilis. Gynura* water extract (GW) did not show any antimicrobial activity against any bacteria (Table 2.1).

Table 2.1 Antibacterial activity of *Gynura procumbens* extract on pathogenic bacteria using well diffusion method. Values are recorded as the mean diameter of inhibition zones (mm) ± standard deviation (n=3). A diameter more than 10 mm was considered active and less than 6 mm was considered as inactive. GW: *Gynura* water extract; GE: *Gynura* ethanol extract; GM: Gynura methanol extract. "-" indicates no activity. Antibiotics; GM (Gentamicin), P (Penicillin), SXT (Sulfamethoxazole-Trimethoprim) & E (Erythromycin). * means significantly active against the bacteria (p<0.05).

Name of pathogens	Antibiotics	Diameter of inhibition zone (mm)					
		Positive control	Ethanol (25%)	Methanol (100%)	GW	GE (Mean ± SD)	GM (Mean ± SD)
Enterococcus feacalis	GM	19.33±0.882	-	-	-	11.00±0.57*	7.33±7.33
Bacillus subtilis	P	33.66±0.333	7.66±3.93	7.21±4.16	-	12.66±0.33	8.66±4.72
Staphylococcus aureus	SXT	32.33±0.882	6.66±3.33	5.77±3.33	-	12.00±1.00	11.33±0.667
Streptococcus epidermidis	E	34.66±0.333	7.00±3.51	6.35±3.66	-	15.00±0.57	8.33±4.25
Escherichia coli	GM	25.33±0.333	6.66±3.33	-	-	-	-
Klebsiella pneumoniae	GM	25.33±0.577	4.00±4.00	-	-	-	-
Proteus mirabilis	GM	25.00±0.577	-	-	-	-	-
Pseudomonas aeruginosa	GM	24.66±0.882	-	-	-	9.33±0.33*	-
Salmonella typhimurium	GM	24.33±0.667	3.66±3.66	6.35±3.66	-	-	3.66±6.35

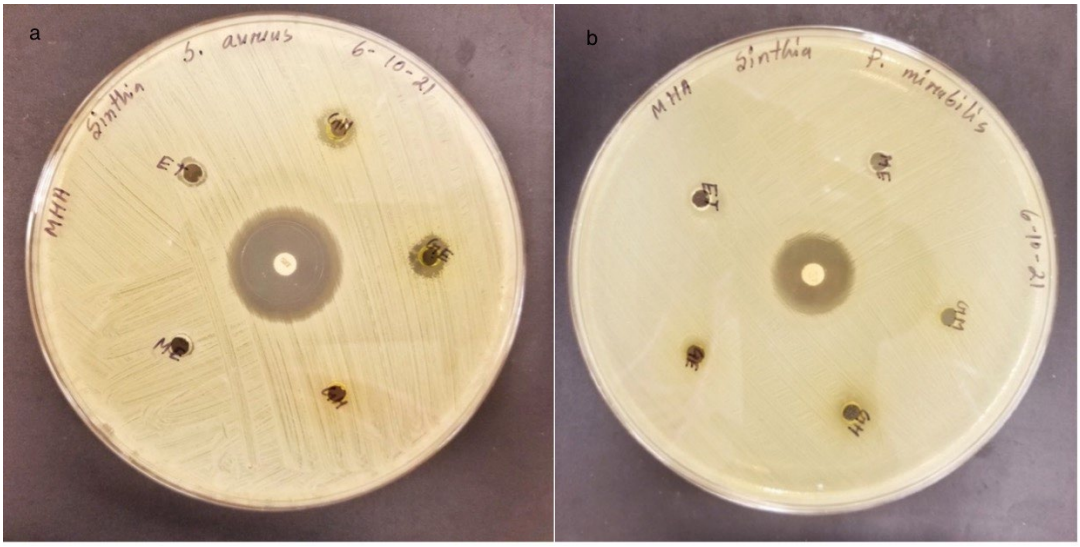

Figure 2.1 Antimicrobial effect of *Gynura procumbens* against *Staphylococcus aureus* and *Proteus mirabilis*; a. Sensitivity effect of *Gynura* ethanolic and methanolic extract on *S. aureus* by agar well diffusion method along with positive antibiotic control and solvents; b. Resistance effect of *Gynura* ethanolic and methanolic extract on *P. mirabilis* by agar well diffusion method along with positive antibiotic control. GE= *Gynura* ethanolic extract, GM= *Gynura* methanolic extract, GH= *Gynura* aqueous extract, ME= Methanol & ET= Ethanol.

Table 2.2 Antibacterial activity of *Gynura procumbens* extract on pathogenic bacteria using disc diffusion method. Values are recorded as the mean diameter of inhibition zones (mm) ± standard deviation (n=3). A diameter more than 10 mm was considered active and less than 6 mm was considered as inactive. GW: *Gynura* water extract; GE: *Gynura* ethanol extract; GM: *Gynura* methanol extract. "-" indicates no activity. Antibiotics; GM (Gentamicin), P (Penicillin), SXT (Sulfamethoxazole-Trimethoprim) & E (Erythromycin). * means significantly active against the bacteria (p<0.05).

Name of pathogens	Antibiotics	Diameter of inhibition zone (mm)					
		Positive control	Ethanol (25%)	Methanol (100%)	GW	GE (Mean ± SD)	GM (Mean ± SD)
Enterococcus feacalis	GM	19.33±0.882	-	-	-	-	-
Bacillus subtilis	P	34.33±0.882	-	-	-	13.00±1.53*	16.67±0.67*
Staphylococcus aureus	SXT	32.66±0.882	-	-	-	10.67±0.33*	12.33±0.33*
Streptococcus epidermidis	E	34.66±0.333	-	-	-	11.00±0.58*	9.67±4.84
Escherichia coli	GM	25.33±0.333	-	-	-	4.33±4.33	-
Klebsiella pneumoniae	GM	23.33±0.333	-	-	-	-	-
Proteus mirabilis	GM	25.00±0.577	10.33±0.57	-	-	-	-
Pseudomonas aeruginosa	GM	24.66±0.882	-	-	-	-	-
Salmonella typhimurium	GM	24.00±0.577	-	-	-	-	-

In disc diffusion method, pure ethanol showed effects against *P. mirabilis* only while pure methanol did not show any effect against any bacteria. *Gynura* ethanol extract (GE) was the most efficacious (p<0.05), with antibacterial activity against *S. aureus* and *S. epidermidis*. Similarly, *Gynura* methanol extract (GM) has significantly effective (p<0.05) against *B. subtilis* and *S. aureus*. *Gynura* waters extract (GW) did not show any antimicrobial activity against any bacteria (Table 2.2).

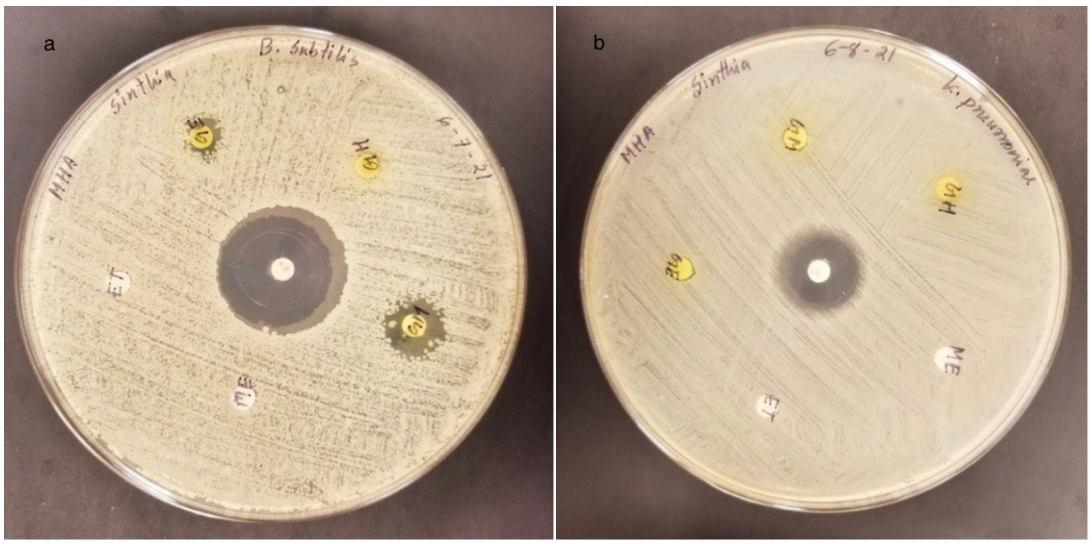

Figure 2.2 Antimicrobial effect of *Gynura procumbens* against *Bacillus subtilis* and *Klebsiella pneumoniae*; Sensitivity effect of *Gynura* ethanolic and methanolic extract on *B. subtilis* by disc diffusion method along with positive antibiotic control and solvents; b. Resistance effect of *Gynura* ethanolic and methanolic extract on *K. pneumoniae* by agar disc diffusion method along with positive antibiotic control. GE= *Gynura* ethanolic extract, GM= *Gynura* methanolic extract, GH= *Gynura* aqueous extract, ME= Methanol & ET= Ethanol.

2.5 Discussion and conclusions

The emergence of antibiotic resistance organisms is rapidly growing, and it has undoubtedly become a global challenge for scientists. The necessity for antibiotic replacements is growing in response to an increase in the number of antibiotic-resistant bacteria. New antimicrobial agents must be developed or established antibiotics must be altered such that they have wide range of effectiveness. In our experiment we focus on the impact of different extracts of *G. procumbens* against both gram-positive and gram-negative pathogenic bacteria to investigate if there are any antibacterial properties in *Gynura* plant. I found that the extract solvent performs a crucial role in demonstrating microbiological activities.

In the agar well diffusion method I found that ethanolic extract is more sensitive than methanolic extract against pathogenic bacteria with higher standard deviation which makes the results less reliable. In the well diffusion method GE showed significant efficacy against *E. feacalis*, and *P. aeruginosa.* On the other hand, GM did not show any significant antibacterial effect against any bacteria I used in our experiment. The disk diffusion method showed more consistent results in compared to well diffusion method. As I used 100% methanol for methanol extraction process that might evaporated before diffused into the agar when I used well diffusion method. In contrast, while performing disc diffusion method prepared disc were soaked into the solution and put them on the agar plate and showed more reliable results than the well diffusion method. Considering all thing together, from now on I will focus on disc diffusion results primarily.

Kaewseejan *et al*. reported that content of chlorophyll-a and -b, and carotenoids were present in ethanolic *Gynura* extract along with alkaloids and volatile oils [12]. They performed agar well diffusion method to determine antimicrobial activity against 5 different pathogens (*S. aureus*, *S. typhi*, *B. cereus*, *P. aeruginosa,* and *E. coli*) and found out that ethanolic extract did not show any effect against these bacteria [12]. In contrast, in my investigation, I found that ethanolic *Gynura* extract has significant activity against *E. feacalis* (n=3; p=0.0004), and *P. aeruginosa* (n=3; p<0.001) and moderate activity (statistically nonsignificant) against *B. subtilis*, *S. aureus*, and *S. epidermidis* (n=3; p=0.08), and in the well diffusion method. In the disc diffusion method from our experiments, ethanolic *Gynura* extract showed around 35% of inhibition against *B. subtilis*, *S. aureus* and *S. epidermidis* compared to the active antibiotics. This accounts for five out of nine gram-positive and gram-negative pathogenic bacteria. This could in part be due to the fact that their extraction process differed from ours. They utilized dried powder form of *Gynura* mixed with ethanol, while I prepared the extract using freshly harvested *Gynura* leaves immersed in ethanol @ 0.25g/mL. The process of drying and making a powder form of the plant might have affected the active substances in the plant those were responsible for inhibiting the pathogens. Another study found that *G. procumbens* extracts have antioxidant capacity and reductive ability. It was discovered that a 100 mg/mL methanol extract of *G. procumbens* suppressed *S. aureus* growth by 42%, which was 6-times higher than the resistant strain of *S. aureus* [19]. Similarly, methanolic extract exhibited significant efficacy against *B. subtilis* (n=3; p<0.001) and *S. aureus* (n=3; p<0.001) compared to the negative control.

Rahman *et al*. in their experiment determined antioxidant, antibacterial, and antifungal properties of *Gynura procumbens* leaf extracts in n-hexane (HX), dichloromethane (DCM), methanol (ME), and ethyl acetate (EA) extracts [20]. DCM and EA extracts had moderate antibacterial activity against most of the bacteria and fungus they used in their experiment, but HX and ME extracts did not. They used only disc diffusion method to determine the antimicrobial activity. In my experiment, the GE extract exhibited significant inhibition against *B. subtilis* (n=3; p=0.001), *S. aureus* (n=3; p<0.001) and *S. epidermidis* (n=3; p<0.001). GM also showed moderate inhibition against *S. aureus* and *B. subtilis*. Although this was not statistically significant (p>0.05) however GE and GM showed inhibitory effect against *B. subtilis* and *S. epidermidis,* respectively in disc diffusion method. The different extraction process could be a reason behind these contradictory results as Rahman *et al.* used sun dry and oven dry process with 40°C to prepare the extract that might degrade or intensify specific substance responsible for exhibiting their results. However, in my experiment, the utilization of centrifuge method might have ruptured plant cell walls and resulted in releasing of compounds and molecules. I did not apply any thermal treatment, which helps protect the functional particles and substance which may have ability to inhibit the bacteria. These differences may provide some explanation for the non-identical results between these experiments.

Nawi *et al*. expressed in their experiment that while using the disc diffusion technique, only methanol extracts of *G. procumbens* demonstrated antibacterial action, whereas hexane extracts exhibited no activity. *S. aureus* was the most susceptible to *G. procumbens* extract of the four bacteria investigated in the study [21]. On the other hand, *K. pneumoniae* and *P. aeruginosa* showed resistance towards *G. procumbens* extract [18]. Highest antimicrobial activities were recorded against *S. aureus* at 400 mg/mL concentrations with 10.5 mm of inhibition zone. I subsequently used disc diffusion method to determine the antibacterial activity of various *G. procumbens* extractions against selected pathogenic microorganisms. The disc diffusion results of ethanolic *Gynura* extract resembled the well diffusion results, while methanolic *Gynura* extract demonstrated activity against priorly mentioned three bacteria specifically in the disc diffusion method. Because we used 100% methanol to prepare the extract, it is possible that the methanol extract evaporated before it completely diffused into the agar during the well diffusion method, whereas the disc diffusion method soaked and placed the disc on the agar and had better diffusion than the well diffusion method.

The aforementioned study discovered that the various extracts of *G. procumbens* had antibacterial properties, lending credence to the herb's traditional usage in the treatment of bacterial illnesses. The potent antibacterial properties of this plant's ethanol and methanol extracts can be investigated for their potential values in decreasing illnesses or disorders caused by pathogenic microorganisms. My study also found that *G. procumbens* seems to have higher antibacterial properties against gram-positive species than gram-negative species. These results may provide guidance for future studies investigating its effectiveness against specific species. In addition, in this experiment, I observed that disc diffusion method illustrated more reliable and consistent results when compared to the well diffusion method. This conclusion came to after 100% methanol and 25% ethanol did not exhibit any visible results when disc diffusion method was used instead of well diffusion method. Further research with higher concentrations of *G. procumbens* may also illustrate more visible results.

In the future, a comprehensive chemical research and separation of molecules responsible for antibacterial and cytotoxic activities on animals may be required. Specific research could also be conducted to find out if *G. procumbens* have some impacts on certain bacterial infections and diseases. Further exploration into secondary plant metabolites will lead to significant advances in pharmacology and will greatly aid in the development of contemporary pharmacotherapeutics.

2.6 References

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CHAPTER 3. IMMUNOLOGICAL EFFECTS OF *GYNURA PROCUMBENS* EXTRACT ON B16 MURINE CELLS *IN-VITRO*

3.1 Abstract

Although *Gynura procumbens* extract (GPE) has been broadly reported to have an inhibitory effect on different cancer cell, studies specifically investigating its effects on melanoma cancer are lacking. Therefore, the purpose of this study was to see if the different concentration of ethanol and aqueous extracts of *G. procumbens* were capable of inhibiting B16 murine melanoma cells and proliferating spleen cells collected from BALB/c mice in cell proliferation assays. The final concentration 4g/mL and 0.8g/mL of ethanolic extracts significantly inhibited the melanoma cell growth *in vitro*. Spleen cell proliferation assay was conducted in the presence of concanavalin A (ConA) and lipopolysaccharide (LPS) mitogen for BALB/c mice. ConA and LPS acted as mitogen, enhance the mitosis cell division. The result indicates that there was neither significant proliferation nor inhibition in spleen cell proliferation assays with 25% ethanol or aqueous extract of *Gynura* with different concentrations. The effective concentration for melanoma cell inhibition did not interfere with the T cell or B cell proliferation in presence of mitogen *in vitro*. Taken together, the findings of this study suggest that GPE may be an effective melanoma cancer inhibitor and has no adverse effect on immune cell stimulation.

3.2 Introduction

Melanoma is one of the most aggressive skin cancer types, accounting for the fifth most commonly diagnosed cancer type in men and the sixth most common cancer type in women. It is associated with sun exposure and has seen an increase in occurrence in recent years [1], [2]. Melanoma is a cancer that arises when melanocytes, which naturally contain melanin, become mutated [3]. The consequence of melanin pigmentation on the malignant behavior of melanoma, with results so far being ambiguous [4]. Melanoma's aggressiveness is caused by somatic mutations and overexpression of genes encoding proteins implicated in signal transduction pathways, among other molecular mechanisms. Mutated B-Raf proto-oncogene (BRAF) is one of the most significant regulators of melanoma progression, and it has been found in more than 40% of patients with this cancer [5]. Melanoma tumors resemble black or brown moles in appearance. Some cancers, on the contrary, may appear pink, skin-colored, purple, red, white, or blue. Melanoma tumors come in a variety of shapes and sizes. Tumors

that penetrate deeper into the extracellular pathways are considered early stages, while tumors that penetrate deeper into the intracellular routes are considered later stages [6]. Its prevalence has increased over the years, but the treatment is only improved for localized melanoma after surgery [7]. Melanoma sometimes metastasizes, and the prognosis remains poor, with a low survival rate [8]. As a result, developing novel therapeutic approaches would be beneficial in the battle against this cancer.

The spleen is one of the most essential organs for blood filtration in the human body. The T and B cell responses to these antigenic targets in the blood are controlled by antigen-presenting cells (APCs) particular to the spleen. One of the spleen's main functions is to filter bacteria and abnormal cells from the blood and to promote low-probability encounters between APCs and lymphocytes [9]. Macrophages associate with T cells to stimulate them in target organs, and they are themselves triggered by T cell-produced cytokines [10]. T cells are the most essential elements in regulating cell-mediated responses. T cells are used in a specific immune response to neutralize cells associated with certain microbial species. Cytotoxic T cells are one of three groups of T cells responsible for destroying virus-infected cells during the cell-mediated immune response, whereas helper T cells are involved in both the antibody and cell-mediated immune responses. As suppressor T cells are activated, they deactivate T cells and B cells, stopping the safe reaction from being too strong [11]. To maintain an adequate defense while preventing autoimmunity, T cells must be closely controlled [12].

Gynura procumbens, a member of the Compositae family, is an herbaceous plant. In Southeast Asia, particularly Indonesia, Malaysia and Thailand, the plant is traditionally known for the treatment of several ailments including fevers, kidney disease, migraines, constipation, hypertension, diabetes mellitus and cancer [13]. Flavonoids, saponins, tannins, terpenoids, and sterol glycosides are among the active chemical constituents found in *Gynura*. The chemical constituents of *G. procumbens* can inhibit calcium influx in blood vessel muscles. Because of the lack of calcium, blood vessel muscles relax and blood pressure falls. Furthermore, the anti-diabetic and pro-fertility effect of *G. procumbens* on streptozotocin-induced male rats demonstrated that *G. procumbens* aqueous extract has a pro-fertility effect and anti-diabetic activity [14]. Previous studies had also reported that *Gynura* leaves extracts contained rutin, kaempferol and two potential antioxidant components which are kaempferol-3-O-rutinoside and astragalin. It can treat topical inflammation, rheumatism, and viral ailments. Kaempferol, another active phytoconstituent, was discovered to have an anti-inflammatory effect by inhibiting glycogen synthase kinase- 3β (GSK-3β) [15].

Gynura procumbens extract has previously been shown to stimulate apoptosis and prevent proliferation and metastasis in U2OS (osteosarcoma) cells by inhibiting nuclear translocation of NFB [16]. Recently, an ethanol extract of *G. procumbens* was shown to reduce azoxymethane-induced aberrant crypt foci in rats by approximately 80%, indicating potential for colon cancer prevention [17]. *Gynura procumbens* extracts inhibited the expression of matrix metalloproteinase (MMP)1 and MMP9 caused by UVB irradiation by inhibiting the release of proinflammatory cytokine mediators and the production of reactive oxygen species (ROS). MMPs are required for the degradation of the basement membrane, and MMP2 and MMP9 are the most strongly associated with tumor invasion and metastasis [16]. *G. procumbens* has also been shown to effectively suppress the proliferation of breast cancer and mammary gland epithelial cells. Furthermore, research has shown that the treatment of *G. procumbens* will decrease the occurrence of tumors in the animals studied [18].

Throughout the years, intensive research done on *G. procumbens* has provided extensive scientific evidence of its therapeutic potential. Based on prior carcinoma research I was expecting that *Gynura procumbens* might have an inhibiting effect on melanoma cell growth. In this present experiment I observed the effect of different dilutions of ethanolic and aqueous extracts of *Gynura* on growth of melanoma cell line. I also wanted to explore immune stimulating potential of *Gynura procumbens* in the presence of specific mitogen.

3.3 Materials and methods

3.3.1 Experimental animal

For this study I chose 1-year BALB/c mice as it is widely used in immunological research and is a great model organism for the human immune system. The mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were taken care of following Purdue University Animal Care and Usage Committee (PACUC) approved protocols.

3.3.2 *Gynura* extract preparation

Fresh leaves of *Gynura Procumbens* were obtained from plants and grown in the aquaponics system of Purdue University Fort Wayne Biology greenhouse. The leaves were washed with water and finely mashed with mortar and pestle. To prepare the *G. Procumbens* ethanolic and aqueous extract prepared the *Gynua* paste contained 1g of *Gynura* with 0.25 ml of 25% ethanol and 0.25 ml of Deionized (DI) water, respectively. The prepared paste was

filtered with double layer of cheese cloth. The filtered GPE was shaken for 20 minutes with a rotating shaker mixture. After that, the GPE was centrifuged at 10°C temperature with 10000 rpm for 10 minutes. The supernatant was filtered with filter paper and sterilized by passing through a sterile syringe filter (mesh size: 0.2 micron). Finally, the extract was stored at 4°C for further experiment.

3.3.3 Melanoma cell line preparation

The culture flask containing the tumor cells was rinsed two times with 10 mL of Roswell Park Memorial Institute (RPMI) media without Fetal Bovine Serum (FBS). Then, 1.5 mL of Trypsin was added into the flask and the flask was shook until the cells detached. The flask was then placed under the microscope to observe the detachment. When most cells were released, 10mL of 10% FBS and RPMI media was added into the flask. Again, the flask was flipped up and down 3 times to further release the cells. The volume of cells and media in the flask was transferred into a 15 mL test tube for centrifuging at 1000 ppm for 5 minutes. After the completion of centrifugation, the supernatant was poured off and 1ml of 10% FBS and RPMI media was added to the tube. To a separate tube, 5uL of this media and cell mixture, 395uL of isotone and 100uL of trypan blue was added. From this mixture, 10uL was transferred to a hemocytometer to count the cells under the microscope. Cells in the upper left and lower right 18 squares were counted and averaged. Finally, the averaged amount was multiplied with 1000000 to determine the amount per milliliters.

3.3.4 Spleen cell collection and cell culture

To perform spleen cell proliferation, mouse spleen cells were first collected after mice were sacrificed by cervical dislocation and dissected. The spleen was separated aseptically, and a single-cell spleen cell suspension was prepared in sterile media using a screen wire mesh. The spleen was set on top of the mesh and tapped through the mesh with a sterile plunger until the suspension was ready. A syringe was used to extract the cells, which were suspended in 1 mL of RPMI 1640 (Mediatech Inc, Herndon, VA). To extract red blood cells, an aliquot of the cell suspension (5µL) was applied to a solution containing isotonic buffer (PBS 295µL), Trypan Blue (100µL), and RBC Lysis Buffer (100µL), and 10µL of this solution was put on a hemocytometer, and cells were counted under a microscope. After counting, the cells were diluted to $10X10^6$ cells/mL in cell media (RPMI + FBS solution). At a final concentration of $1X10^6$ cells per well, 100µL of this solution was transferred to each of the wells of the plate.

Every well received 100µL of RPMI 1640 (Mediatech Inc, Herndon, VA) media with 10% Fetal Bovine Serum (FBS) (Sigma, St. Louis, MO) [19].

3.3.5 Cell proliferation assay

Ninety-six well plates were used to conduct the experiment immunological efficiency of *Gynura procumbens.* For melanoma cell inhibition each plate had control (cultured melanoma cell), two different GPE (ethanol and aqueous) with 5 different dilution factors 1:1, 1:5, 1:25, 1:125 and 1:625 or the final concentrations of 4 g/mL, 0.8 g/mL, 0.16 g/mL, 0.032 g/mL and 0.062 g/mL in each well. Each experimental well contained 100 uL of prepared melanoma cells, 10 uL of factors, and 100 uL of 10% FBS and RPMI media; wells of control only contained 100 uL of prepared melanoma cell and 100 uL of 10% FBS and RPMI media. The same dilution factors used for the cellular and humoral immune response in mouse model. For cellular (T cell) and humoral (B cell) proliferation we used mitogen along with mouse spleen cell. In the case of T cells, Con A (Concanavalin A from *Canavalia ensiformis*, Type IV, lyophilized powder from Sigma) was used as a mitogen [20]. LPS (Lipopolysaccharides from *Escherichia coli* O26:B6) was used in the case of spleen B cells [21]. We used collected spleen cell with and without appropriate mitogen for control, again with mitogen in combination with 25% ethanol for control and factor dilution were tested with mitogen. 7.5 µL of 100 µg/mL Con A (final concentration of 3.45 µg/mL per well) and 7.5 µL of 80 µg/mL LPS (final concentration of 2.76 µg/mL per well) were added to each well except the control without mitogen (ConA/ LPS) [19][22]. To observe the effect of different concentrations and extract of *Gynura procumbens* were added (10µL in each well except the control). Each sample was run in triplicates and three repeats.

Plates were incubated for 48 hours in a CO_2 incubator at 37°C and 95% humidity. After incubation, 10µL (stock concentration: 0.0375 µCi/µL) of radioactive isotope (H3 Thymidine) (Moravek Biochemicals, Brea, CA) were added to each well. After that, the plates were incubated for another 24 hours under the same conditions. When spleen cells divide, thymidine is taken up as a pyrimidine nucleotide by the cells. As a result, the amount of radioactive thymidine inserted into the cells after incubation would be proportional to cell proliferation.

3.3.6 Cell harvesting

The cells were harvested onto filter paper strips (WhatmanTM) using a cell harvester (Brandel Cell Harvester, model M 24) by washing the wells twenty times with PBS. The strips

were then treated with 10% trichloroacetic acid (TCA) solution five times to rupture the cells. Then ethanol was passed through the filter five times to wash out the unincorporated H3 Thymidine. Filter paper with cell were air-dried behind a radioactive shield for 3 hours. Dried discs of filter paper were placed in separate scintillation vials. 3 mL of scintillation cocktail (EcoLume TM scintillation cocktail, MP Biomedicals, Irvine, CA) was added to each vial and then placed in a scintillation counter (Beckman CoulterTM, LS 6500 Multipurpose Scintillation Counter) that counts radioactivity in the vials as count per minute (CPM) proportional to the number of cell divisions of cellular and humoral cell [19].

3.3.7 Statistical analysis

The data obtained from these experiments were analyzed by one-way analysis of variance (ANOVA) using SigmaPlot® 14.5, (Systat Software Inc) to see if there were significant difference among the treatment groups. For all data that was statistically significant ($p<0.05$), a Tukey's HSD test (post ANOVA comparison of multiple means) was performed to determine any differences between treatments. The data are presented as means ± SEM.

3.4 Results and Discussion

Gynua extract has previously been shown to have anticancer properties *in vitro* in a variety of human cancers, including colon cancer, osteosarcoma, glioblastoma, and breast cancer [23], [24]. *Gynura*'s anticancer activities have been studied in relation to a variety of carcinogenic phenotypes, including cell proliferation, metastasis, and angiogenesis. GPE's anticancer effect against melanoma, on the other hand, has not been studied. Thus, in this study, we look at the effect of different concentrations of ethanolic and aqueous GPE on melanoma cells. We also investigated if GPE has any effect on immune cells.

Several studies have reported the *in vitro* and *in vivo* anti-cancer activities of crude extract of GP in human cell lines and animal models. The GPE at IC_{50} 90 µg/ml could increase the effectiveness of doxorubicin on T47D human breast cancer cell [25]. Furthermore, oral administration of GPE at 300 mg/kg significantly inhibited the carcinogenesis of dimethylbenz (a) antracene (DMBA) on male rat liver and female rat mammary glands induced by DMBA with no adverse effects [26]. In addition, the acute toxicity studies showed that the high dose of GPE at concentration 5 g/kg did not cause any toxicity in Sprague-Dawley rats after 14 days of oral administration [27]. *G. procumbens* ethanol extract is effective in preventing and

treating liver cancer by interfering with the inflammatory microenvironment during oncogenesis induced by nanoDEN [28]. In my experiment, more than 60% melanoma cells were inhibited with ethanolic extract of *Gynura* compared to the control (Figure 3.1). I found that concentrations of 4 g/mL and 0.8 g/mL significantly inhibited melanoma cell growth compared to control cell growth (n=14; F=5.794; p= 0.003) and (n=14; F=5.794; p= 0.036) respectively. Another concentration of ethanolic GPE (0.16 g/mL) inhibited cell growth around 68% compared to the control, but it was not statistically significant (p>0.05). The solvent used to prepare the extract, 25% ethanol, was also tested to ensure that it had no effect on the melanoma cell itself. In this experiment 25% of ethanol did not inhibit the melanoma cell *in vitro*. The two lowest ethanolic GPE concentrations, 0.032 g/mL and 0.062 g/mL, had no effect on the melanoma cancer cell.

The inhibitory effect of the aqueous extract was not comparable to that of the ethanolic extract (Figure 3.2). Despite the fact that the extraction process was similar, I did not find any inhibitory activity of *Gynura* aqueous extract against melanoma cells. Based on these findings, I can conclude that 25% ethanol might have been able to extract the bioactive compounds of *Gynura*, which may have an inhibitory effect on the cell (Figure 3.2). Previous studies have identifed peroxidase, catalase, glutathione transferase and harpin proteins in ethanol extract of *G. procumbens* [23]. The anti-cancer properties of *Gynura procumbens* leaves extract were investigated in two canine mammary cancer cell lines. The results indicate that this herbal extract inhibits the cell proliferation and migration, and induces apoptosis of both cancer cell lines via the inhibition of EGFR signaling pathway [29]. The protein fraction extracted from *G. Procumbens*, SN-F11/12, of *G. procumbens* was found to inhibit the growth of a breast cancer cell line. The down regulated expression of invasion markers, CCL2 in the SN-F11/12 treated the cancer cells reveals the possible route of cytotoxic mechanism of SN-F11/12 [23]. Another study found that GPE could significantly inhibit osteosarcoma cell proliferation and metastasis in vitro, and that inhibition of NF-B nuclear translocation appeared to be a potential molecular mechanism [24]. I used crude extract in our experiment, but protein fraction extraction could provide a better explanation of which compound is responsible for inhibiting melanoma cell growth. I could use that bioactive compound to develop a therapeutic product for the treatment of melanoma.

In the immune cell stimulation experiment, my observation differed from previous experiments performed by other researchers with *Gynura* Dwijayanti & Rifa, (2015), found that the extract of *G. procumbens* may increase proliferation of CD4+CD62L- T cell, T CD4+CD25+, and B220+ compared to the control [30]. In this experiment, with ethanolic

extract of *Gynura* lower concentrations 0.16 g/mL (n=6; F=6.553; p= 0.037), 0.032 g/mL (n=6; F=6.553; p= 0.001) and 0.062 g/mL (n=6; F=6.553; p= 0.002) were responsible to inhibit the T cell proliferation (Figure 3.3). However, the higher concentrations showed inhibitory effect on melanoma cell but did not affect the T cell of B cell proliferation in presence of mitogen. For spleen T cells (Figure 3.4), I found that all the concentrations (4 g/mL, 0.8 g/mL, 0.16 g/mL, 0.032 g/mL and 0.062 g/mL) of the aqueous extraction of *Gynura* had an inhibitory effect compared to control (p<0.001). All the concentration of aqueous extract of GP inhibited T cell 50- 80% compared to the control (p<0.001). However, all things considered these results exhibited that ethanolic extract (Figure 3.3) of GP (dilution 1:1 and 1:5) did not have any negative or inhibitory effects on normal immune cells as it did in the case of melanoma cells. Therefore, 25% ethanolic extract of *Gynura* specific concentration inhibited melanoma cell growth. *G. procumbens* can act as an immunostimulant as well as an immunosuppressant. Previous study found that immunosuppressants and immunostimulants function in different directions and they appear to block cytokine transcription, as well as allowing cytokines to play a part in cell activation [31].

Finally, in the case of spleen B cells (Figure 3.5 and 3.6), I see that there was no significant difference between control and experimental treatment for both ethanolic and aqueous extracts. I could see that the specific concentration (4 g/mL and 0.8 g/mL) that had inhibitory effect on melanoma did not interfere with the B cell proliferation. However, all the concentration with aqueous extraction of GP (Figure 3.6) was responsible to reduce the B cell count around 50% compared to the control. Going from 4 g/mL, 0.8 g/mL, 0.16 g/mL, 0.032 g/mL and 0.062 g/mL, we see a decrease in % of control, however it was not statistically significant (n=6; F= 1.858, p=0.116). It seems to be the aqueous extract of GP that acts to inhibit the B cell proliferation.

T cells, as a key component of adaptive immunity, are critical in protecting the host from pathogenic organisms. Unwanted T cell mediated immune responses, on the other hand, can occur in a variety of settings, including transplant rejection, asthma, and a variety of other immune diseases. As a result, it is critical to investigate effective methods of preventing T-cell activation and proliferation [32]. My study (Figure 3.4) exhibited that all the dilutions of aqueous extracts significantly inhibited the T cell proliferation in presence of mitogen compared to the control group (n=6; F=14.659; p<0.001). Three lower dilution factors of ethanol extract (1:25, 1;125, and 1:625) acted as T-cell inhibitors that helps orchestrate the complexities of adaptive immunity. According to our findings, the dilution concentration is critical for the immunological action they exhibit. As I used crude extract for this study, I could

not define which specific substance was responsible for the inhibitory response. Future research using this plant's chemical composition analysis will provide a better understanding of the specific elements contained by *Gynura procumbens* that are able to account for its inhibitory capacity.

The results of the research indicate that an ethanolic extract of GP at concentrations of 4 g/mL and 0.8 g/mL could be a potential anti-cancer agent, though more research is needed to confirm this. However, because GPE is made up of proteins, taking it orally will not result in the desired bioactivity because it will be absorbed by the digestive system. As a result, a good drug delivery system must be developed for its potential use as a chemotherapy drug.

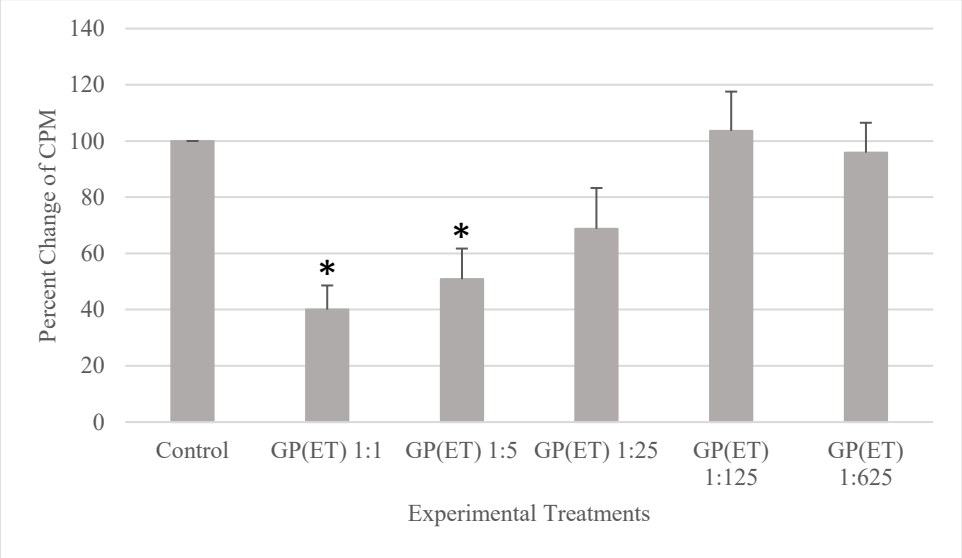

Figure 3.1 Melanoma cell counts (radioactive isotope counts in CPM) across the different concentrations of *Gynura procumbens* ethanolic extract applied. Results are presented as means± SEM. * means significantly different from the control (p<0.05).

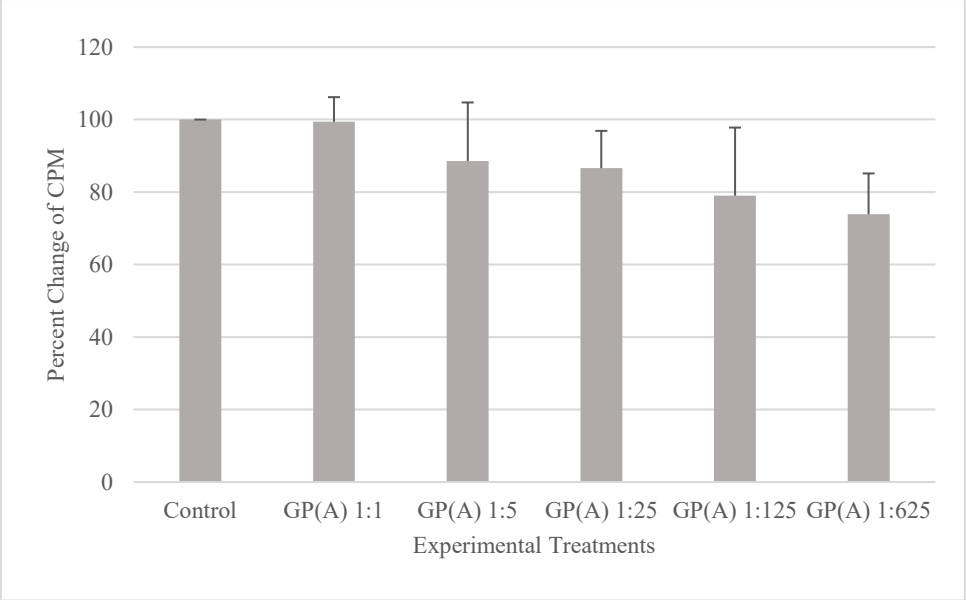

Figure 3.2 Melanoma cell counts (radioactive isotope counts in CPM) across the different concentrations of *Gynura procumbens* aqueous extract applied. Results are presented as means± SEM. Treatment Groups are not significantly different from control (p>0.05).

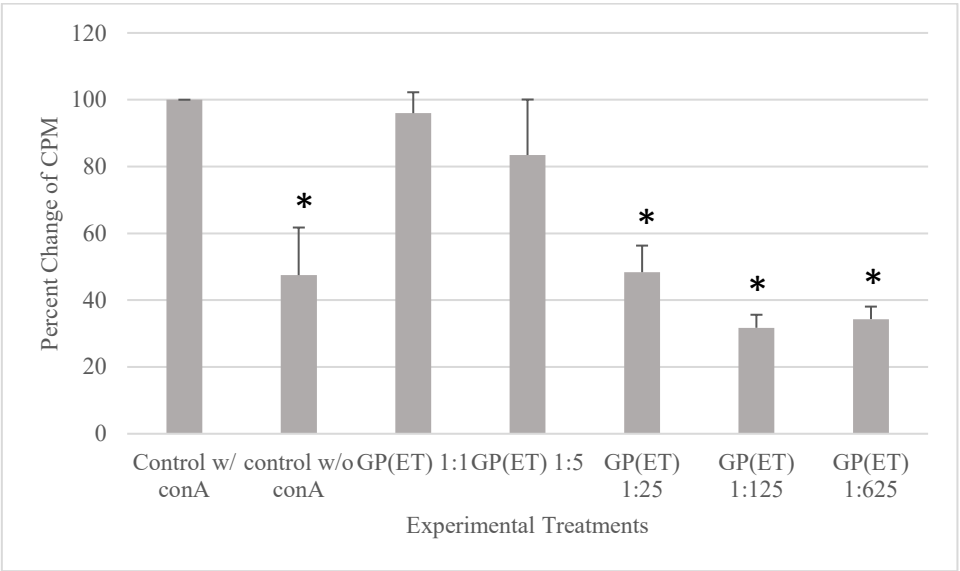

Figure 3.3 Proliferation of spleen cell (T cell) with different dilutions of ethanolic extract of *Gynura procumbens* in presence of ConA (Percentage of CPM). Results are presented as means± SEM. * means significantly different from the control w/ ConA (p< 0.05).

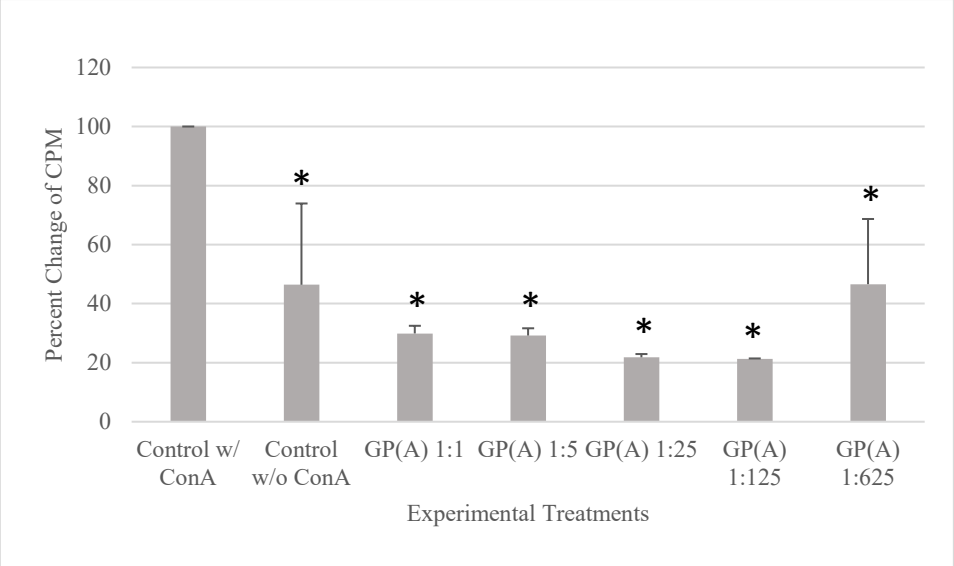

Figure 3.4 Proliferation of spleen cell (T cell) with different dilutions of aqueous extract of *Gynura procumbens* in presence of ConA (Percentage of CPM). Results are presented as means± SEM. * means significantly different from the control w/ ConA ($p< 0.05$).

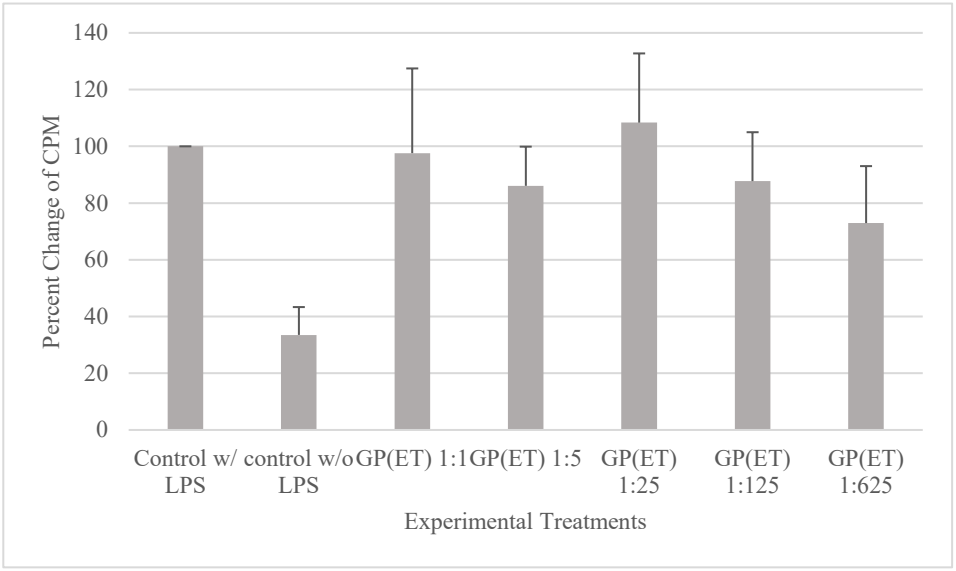

Figure 3.5 Proliferation of spleen cell (B cell) with different dilutions of ethanolic extract of *Gynura procumbens* in presence of LPS (Percentage of CPM). Results are presented as means± SEM. Treatment Groups are not significantly different from control ($p>0.05$).

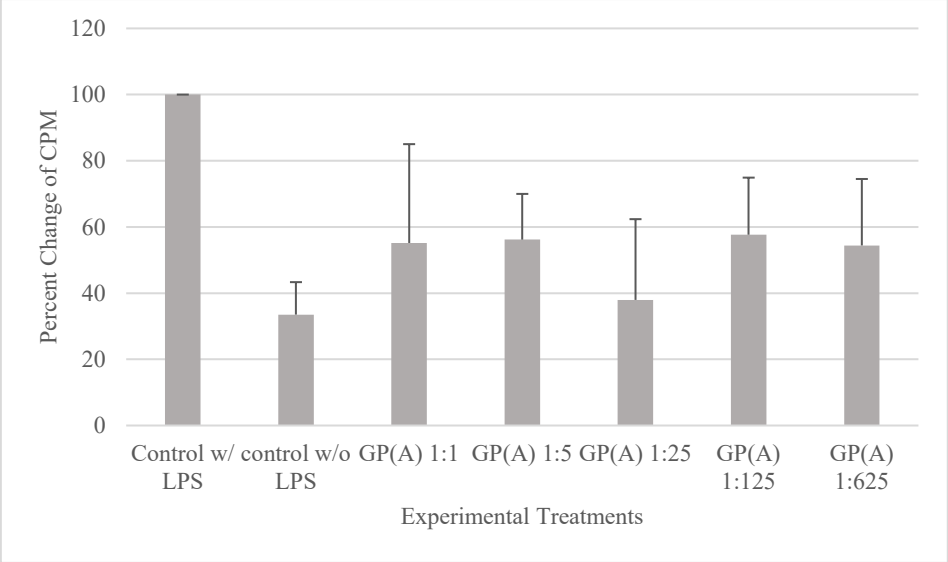

Figure 3.6 Proliferation of spleen cell (B cell) with different dilutions of aqueous extract of Gynura procumbens in presence of LPS (Percentage of CPM). Results are presented as means± SEM. Treatment Groups are not significantly different from control (p>0.05).

3.5 Conclusion

To the best of my knowledge, this is the first study to look at the anti-melanoma effect of an ethanolic extract of *Gynura* in a B16 Murine Melanoma cell line. According to the findings, this herbal extract significantly reduces melanoma cell proliferation. These efficacy data for GPE indicate that it has the potential to be developed as a single agent or in combination with other chemotherapy in the treatment of melanoma cancer. However, more research is needed to investigate the mechanism and interactions of combined treatment for melanoma cancer. Following those further studies with purified bioactive GPE compounds is needed to gain a better understanding of the effect of GPE on signaling pathways altered by oncogene expression in melanoma. Taken together, GPE's potent anti-cancer properties allow it to be used as a chemotherapeutic agent in the treatment of melanoma cancer.

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CHAPTER 4. MITIGATION OF ACUTE STRESS IN TILAPIA, *OREOCHROMIS NILOTICUS,* USING LONGEVITY SPINACH, (*GYNURA PROCUMBENS*), AS NUTRACEUTICALS

4.1 Abstract

Even though aquaculture may meet the world's demand for a significant amount of protein supply, it is not without shortcomings, the most serious of which is stress. Mitigating stress is therefore an important goal for aqua-culturists. In the present study, we investigated the effects of different concentrations of longevity spinach, *Gynura procumbens,* as a nutraceutical, on the hematological parameters of acutely stressed Nile tilapia, *Oreochromis niloticus,* and determined the best concentration from experiments conducted in a recirculating aquaculture system. We fed stressed tilapia commercial feed, supplemented with *Gynura procumbens* extracts (0% (stress control), 0.5g/kg, 1.0g/kg and 1.5g/kg of feed weight) in combination with hydrocortisone, a stress hormone (0.01% of fish body weight). In this experiment, we evaluated blood glucose, lysozyme activity, phagocytic capacity, hematocrit, spleen somatic index and hepatosomatic index. During the acute stress period, *G. procumbens* has shown to decrease the levels of blood glucose and bring the levels of serum lysozyme activity and phagocytic capacity in stressed fish to the levels of controlled fish. These findings are promising for the development of new nutraceuticals for aquaculture industry.

4.2 Introduction

To promote global nutrition and food sustainability, we must have a sufficient food supply available to sustain life. Aquaculture can yield an affordable source of food for the expanding human population [1]. Analyses of global fish intake have clearly demonstrated a rising trend in uptake as part of a balanced diet, demonstrating the relevance of aquatic food in the human diet [2]. It is essential to ensure nutritional value and aquatic food standards since there is not enough food accessible which is also inexpensive [3]. Since fish is becoming a diet staple, it's high time to give emphasis to fish safety. Fish safety has a substantial influence on the fish's stress response, health, and disease resistance, all of which have implications for the industry's long-term viability [4]. Dietary habits have a major impact on stress tolerance, thus fish must be given appropriate amounts of diets that fulfill all of their dietary requirements and keep them stress- and disease- free for proper growth and in order to produce a healthy proteins [5]. Nile tilapia (*Oreochromis niloticus*) is a common fishery and aquaculture species with

great ecological and economic value. Due to its growth, and adaptation to diverse production techniques, this species is one of the most significant candidates for farming world-wide [6].

Fish are susceptible to a wide range of stresses. Capture, handling, transportation, overcrowding, fluctuations in water temperature, oxygen, and salinity, and the adverse consequences of pollution are all typical stressors for confined fish [7]. Stress is a physiological reaction that aims to keep an organism's baseline homeostatic levels of physiological parameter stable [8]. These physiological reactions in vertebrates have been classified as primary, secondary, and tertiary responses. In vertebrates, primary stress reactions involve the production of neuroendocrine messengers such as catecholamines, as well as other hormones [9, 10]. Secondary stress reactions are characterized by the activities induced by these hormones, which include alterations in energy resources and immune function [11]. Chronic stress results in the tertiary reactions. This is when the animal's energy reserves deplete, immune system is weakened, behavior and reproduction are impaired, and ultimately, the death of the animal [9]. In both natural and man-made environments, fish are always exposed to a number of stresses [12] and face these consequences.

In fish, the primary catecholamine of the humoral adrenergic system is epinephrine. It is critical for the rapid mobilization of energy resources during times of stress. Glycogenolysis, gluconeogenesis, and lipolysis can all be increased, leading to hyperglycemia and free fatty acid redistribution to provide energy to manage the stressful event [38, 39]. Post-stress, glucocorticoid levels can reveal information about specific stimuli. For example, fish can be stressed due to various capturing and handling circumstances, and severe environmental exposures. Increased glucose levels are one of the most commonly measured secondary stress markers [10]. The intensity of hyperglycemia varies based on the species, kind of stressor, length, and magnitude of the stress; nevertheless, glucose levels have been found to increase 30 minutes after a stressful event and can last for up to 24 hours [14]. The other stress hormone-corticosteroids such as cortisol, cortisone, 11-deoxycortisol, and corticosterone are the most common in fish. Cortisol is one of the most often utilized physiological stress markers in fish [10]. Stress, in whatever manner, has an adverse impact on all aspects of fish physiology, including growth, breeding, immunity, and disease resistance [15].

The research of stress in fish has exploded in recent years, and the notion of functional, helpful food has emerged as a novel way to promote overall health and control stress [16]. Several studies on fish farming have used the addition of particular biologically active compounds to commercial fish feed to regulate or reduce the stress reaction and, as a result, increase wellbeing [17-19]. Pharmaceuticals accumulating in aquatic habitats may have

unintended consequences for fish and other species [20]. Immunostimulants have been shown in fish studies to counteract the negative effects of stress and to enhance different defense mechanisms [21]. Because of issues such as the rise of antibiotic-resistant bacteria, environmental pollution, and the accumulation of antibiotic residues in fish, the use of conventional medications and antibiotics in fish production has been prohibited. As a result, various nutraceuticals have been proposed as an effective preventative strategy for decreasing distress and preserving farmed fish [22, 23]. Nutraceutical feeding proves to be a promising method for reducing stress and protecting fish from illnesses by improving their non-specific defense mechanism.

Gynura procumbens (GP), often known as 'longevity spinach', is a medicinal plant that contains phenolic chemicals that serve as natural antioxidants. Since antiquity, Southeast Asians have utilized this herb to treat a variety of illnesses [24], especially in reducing blood sugar levels and its consequences thereafter. Because of the qualities of these spinach plants, planting lifespan spinach is also quite straightforward. Despite its numerous uses, fresh longevity spinach is still hard to come by in today's market because of the necessity for low temperature preservation to maintain quality and potency [25]. The focus of this research is to assess acute stress responses in Nile tilapia after treatment with *Gynura procumbens* to determine if it can reduce blood sugar and eventually the other effects of stress in fish. The ultimate goal is to identify an appropriate additive and feeding concentration that will prevent fish from suffering, particularly in stress-related fish farming practices. The findings of this investigation might assist to improve the aquaculture of these species, and to the best of my knowledge, this is the first project to explore whether *G. procumbens* can contribute to how Nile tilapia deal with acute stress.

4.3 Materials and methods

4.3.1 Plant extract preparation

Fresh GP leaves were obtained from plants cultivated without fertilizer and external treatment at Purdue University Fort Wayne. The leaves were cleaned and mashed finely using a mortar and pestle. GP paste was, then, prepared with 0.25g/mL of 25% ethanol solution [26]. Prepared paste was filtered with double layered cheese cloth and mixed for 20 minutes on a rotating shaker mixture. After mixing, the GP extract (GPE) was washed at 10000 rpm at 10◦C temperature for 10 minutes [27]. Finally, the prepared extract was stored at 4◦C for future use.

4.3.2 Fish maintenance

Nile tilapia with an average weight of 462 ± 16 g and average length of 29 ± 0.34 cm was reared in the aquaponics system in the greenhouse at Purdue University Fort Wayne, Indiana. Fish were maintained in optimal conditions (pH: 6.0-7.0; ammonia: 0-3.0 mg/dL; temperature: 25-28 °C, dissolved oxygen: 5.00-7.00 mg/L; and photoperiod: 12 h:12 h, light: dark). Fish were fed a known amount of commercial feed Purina® AquaMax® Fingerling Starter 300 (Purina Mills, MO, USA) as well as experimental feed twice daily at 1.5% of body weight (a total of 3%/day). All fish were taken care of following the guidelines of an approved protocol of Purdue University Animal Care and Usage Committee (PACUC).

4.3.3 Feed preparation

The amount of feed required for each feeding group was determined by the fish body weight (3% body weight). The commercial feed was prepared into two groups: control feed and stress feed. For stress feed (to induce stress), hydrocortisone was added at 0.01% hydrocortisone per kilogram of fish body weight [28]. Stress feed was then divided into four groups: stress control without GP, 0.5g/kg, 1.0g/kg and 1.5g/kg of GP of feed weight. Proximate composition of control and experimental feed are presented in Table 4.1.

Table 4.1. The approximate content of feed components used to produce each of the five treatment feeding groups is represented by Control without Stress, Control with Stress, 0.5g/kg GP treatment with Stress, 1.0g/kg GP treatment with Stress, 1.5g/kg GP with Stress.

Ingredients (% feed weight)	**Control without Stress**	**Control with Stress**	**0.5g/kg GP treatment with Stress**	**1.0g/kg GP treatment with Stress**	**1.5g/kg GP with Stress**
Crude protein	50	50	50	50	50
Crude fat	16	16	16	16	16
Crude fiber	3	3	3	3	3
Calcium (Ca)	5.2	5.2	5.2	5.2	5.2
Phosphorus (P)	1.3	1.3	1.3	1.3	1.3
Sodium (Na)	0.6	0.6	0.6	0.6	0.6
Hydrocortisone*	-	0.01	0.01	0.01	0.01
GPE**(g)	-	-	0.5	1.0	1.5

*According to body weight

** According to feed weight

4.3.4 Experimental design

The fish in this investigation were separated into five treatment groups. Each group had two replicates. As previously stated, four of the five groups were stressed by supplementing commercial feed with hydrocortisone (0.01 % body weight) (stressed group) [28] and remaining groups were fed with regular commercial feed (non-stressed control group). The 3 stressed groups were fed with three different concentrations of GPE (0.5g/kg, 1.0g/kg and 1.5g/kg of feed weight) through feed supplementation to determine the best concentration to mitigate effects of stress on the hematological stress biomarkers. Specifically, Control group without stress was fed with control feed, without hydrocortisone and GPE; control group with stress was fed with control feed and hydrocortisone but without GPE; treatment groups with stress were fed commercial feed supplemented with hydrocortisone and GPE (0.5g/kg GP with stress, 1.0g/kg GP with stress, and 1.5g/kg GP with stress, respectively).

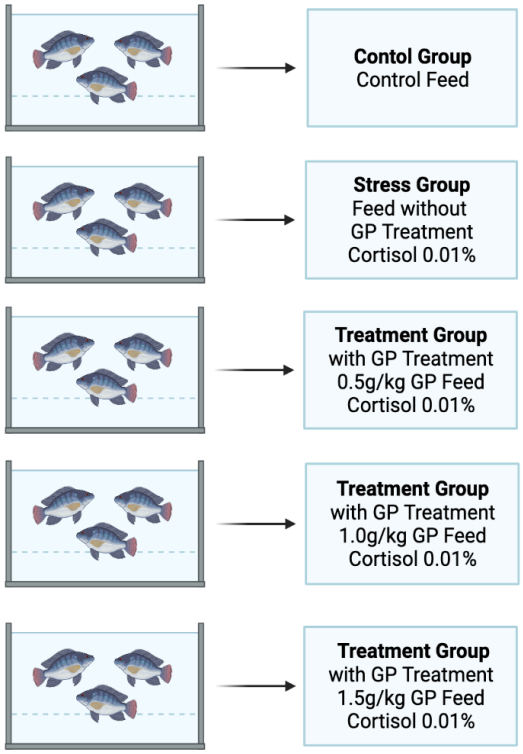


Figure 4.1 The picture represents experimental design for the acute study according to the groups and their treatment regime.

4.3.5 Fish sampling

To investigate the hematological stress parameters, the fish (3 X 2=6 fish/ group, 5 groups) were sampled at day 1, day 2 and day 3. For sampling at day 1 and 2, fish were

anesthetized with <100 mg/L tricaine methane sulfonate (MS-222; Western Chemicals, WA) and at day 3, fish were euthanized with >200 mg/L tricaine methane to reduce any stress caused by handling. Blood was drawn, within 2 minutes, from the caudal vein using heparinized syringes to avoid blood clotting. The blood was transferred to a 1.5 mL Eppendorf tube and placed on ice right away. The obtained blood was then used to measure blood glucose, packed cell volume, and lysozyme activity. Fish length, weight, spleen weight, liver weight, and phagocytic capacity were also measured from the day 3 samples.

4.3.6 Blood glucose

Blood glucose levels are one of the most vital stress indicators. The concentration of glucose in the blood is extremely sensitive to stress, allowing even minor changes in blood glucose concentration to be detected almost instantly, making it useful for detecting acute stress. Changes in blood glucose cause an increase in respiration, a decrease in metabolic activity, and a decrease in immunity, all of which are signs of stress. [29]. A glucometer (Freestyle, Abbott Laboratories, CA) was used to monitor the glucose levels in the blood [30].

4.3.7 Packed cell volume

The blood cell to plasma ratio is determined by packed cell volume (PCV) or hematocrit. It determines the proportion of red blood cells relative to all other cells found in blood [31]. A glass capillary tube was filled with blood (~75%) and then sealed on one side with a Crito-cap using capillary action. The capillary tubes were spun at 10,000 rpm for 10 minutes in a micro-hematocrit centrifuge. The percent PCV from the capillary tubes was read using a micro-hematocrit capillary tube reader.

4.3.8 Spleen somatic index

The spleen stores lymphocytes and blood cells, which are then deployed to regulate immunity. [32]. As a result, the spleen somatic index (SSI) is an excellent indication of a fish's overall health. The spleen somatic index measures the mass of a fish's spleen in relation to its overall mass. The SSI of fish was determined by weighing the harvested spleen and applying the following formula:

$$SSI = \frac{Spleen\ \ wight(g) * 100}{final\ weight\ (g)}$$

4.3.9 Hepatosomatic index

Glycogen is a type of energy that is stored in the muscles and liver of fish. Glycogen is mobilized in the presence of glucagon produced by the pancreas during growth and high stress situations. The liver's glycogen stores are broken down into glucose and released into the bloodstream, altering the liver's overall size and mass. As a result, the hepatosomatic index (HSI) is a reliable measure of fish nutrition, growth, and stress levels [33]. The hepatosomatic index measures the weight of a fish's liver in relation to its overall weight. The harvested liver from the fish was weighed, and the HSI of the fish was calculated using the formula below:

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

4.3.10 Lysozyme activity

Lysozyme is a bacterial cell-lysing enzyme, found in blood plasma that alters the opacity of a bacterial solution. Using a spectrophotometer, this experiment evaluates the capacity of endogenous lysozyme in the blood to remove the bacterial solution [34]. The more bacteria destroyed, the clearer the solution gets, resulting in a higher spectrophotometer transmittance (T) measurement. Higher transmittance means more light passes through the material. Thus, in this case less bacteria occlude the lights. The plasma was obtained from the acquired blood samples by centrifuging them for 10 minutes at 5000 RPM. Each sample's supernatant was collected and placed in Eppendorf tubes before being set aside. Then a suspension of *Micrococcus lysodeikticus* was made at a concentration of 0.2 mg/ mL in 0.05 M (pH = 6.2) sodium phosphate buffer. Then 1 mL of the suspension was added to an Eppendorf tube. 50 µL of the plasma was then added to the Eppendorf tube and vortexed. 1 mL of this solution was put into a cuvette to measure its transmittance at 540 nm using a spectrophotometer (Spectronic 601 spectrophotometer, Milton Roy Company, PA). At the one-minute and five-minute marks, readings were obtained. This process was carried out on each of the fish serum obtained. An uninoculated sodium phosphate buffer was used to calibrate the spectrophotometer. The lysozyme activity assay (LLA) was calculated using the following formula:

$$LAA = \frac{(Final\ transmittance - Initial\ transmittance)}{Total\ elapsed\ time\ (minute)}$$

As a result, the lysozyme activity assay is a measure of the lysozyme clearing bacteria's rate of increase in transmittance per minute.

4.3.11 Phagocytic capacity

Phagocytosis is defined as the ingestion of particulates larger than 0.5 micron in diameter by immune system cells such as macrophages or monocytes [46]. To collect and isolation of the macrophage a head kidney was removed aseptically and placed in centrifuge tube with 2ml L-15 with 2% FBS; stored on ice. Kidneys were then macerated through sterile double metal sieve using sterile plunger (double sieve is 80 mesh/190µm with 100 mesh/140µm) and cell suspensions were collected in L-15 with 2% FBS. Collected cell suspension was centrifuged at 1000 rpm for 10 minutes and the cell free supernatant was discarded. The cell pellet was then resuspended in 2 ml of L-15 and the process was repeated. After the second wash, the cell pellet was finally re-suspended in 1 ml L-15 with 0.1% FBS. The isolated macrophage cells were, thereafter, used to determine phagocytic capacity. 50µl of prepared cell suspensions were placed on both wells of double-etched microscope slides and incubated for 2 hours at room temperature in moist conditions by using soaked paper towel. After 2 hours of incubation, 50 µl of formalin killed bacteria was added on to each well of the slides and incubated for another 2 hours at in the same condition. After the final incubation period, the slides were gently cleaned in Phosphate buffered saline before being fixed in 100% methanol for 1 minute. After fixing, slides were stained with Wright-Giemsa stain. The stained slides were washed with phosphate buffer and deionized water. Under a microscope, the dried slides were used to count positive macrophage cells. Macrophages were considered positive for phagocytic capacity when they had more than 5 bacteria phagocytized within them. This protocol was followed after Mustafa *et al*. [35].

4.3.12 Statistical analyses

The Shapiro-Wilk test was used to determine whether the data was normally distributed or not. If the data passed the normality test, the data were analyzed statistically using one-way ANOVA of variance with Tukey's honestly significant difference test in post hoc analysis on SigmaPlot (Systat Software, San Jose, CA). Data are expressed as means ± SEM. Differences were considered significant at $p<0.05$.

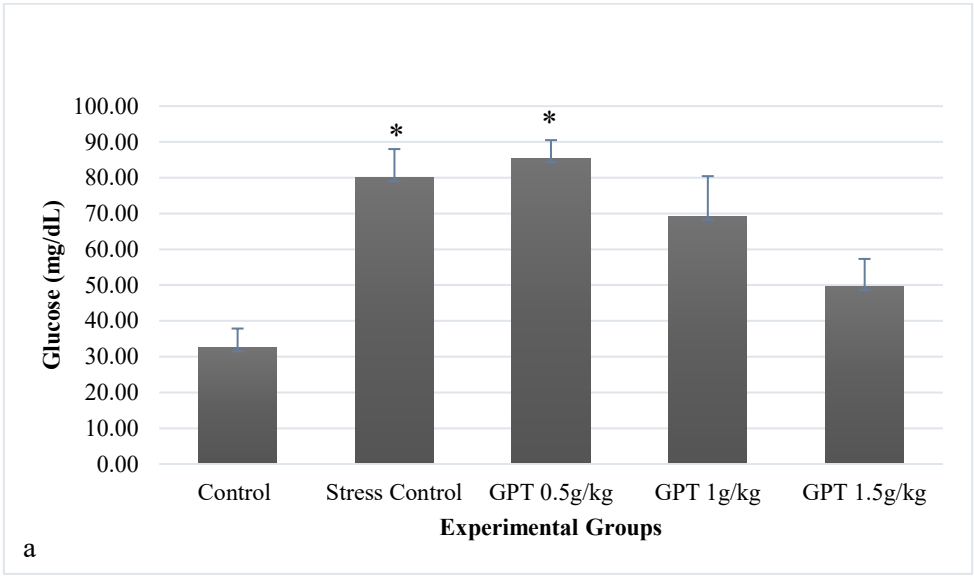
4.4 Results and discussion

To the best of my knowledge, this is the first experiment where longevity spinach, *Gynura procumbens,* has been used to mitigate the stress in Nile tilapia. Since there is a scarcity of research on the implications of *G. procumbens* on the acute stress response in fish, we have compared the effects of *G. procumbens* on other animal models for this study. I have used glucose, lysozyme activity, phagocytic capacity, hematocrit (packed cell volume), SSI (spleen somatic index) and HSI (hepatosomatic index) to investigate the effect of *G. procumbens* in this study.

In vertebrates, stress may affect nearly every aspect of physiology, including fertilization, immunity, hydromineral balance, and a variety of other biological activities. The sympathetic adrenergic and hypothalamic-pituitary-adrenal (HPA or HPI in fish) axis influence acute and chronic stress events, with catecholamines and glucocorticoids serving as key components of the endocrine response to stress [36]. Glucose mobilization is a vital component of an organism's adaptive response to stress. Catecholamines induce hyperglycemia primarily by stimulating glycogenolysis [13, 14]. Stress raises the energy requirements, which are needed to support the fight or flight response and establish homeostasis, resulting in a rise in blood glucose to meet the increased energy requirement. The pancreas produces glucagon, which stimulates the liver to break down glycogen into glucose. These two substances allow the body to keep up with the demand for blood glucose. Thus, increased blood glucose level is a strong indicator of stress [37]. In traditional medicine, *G. procumbens* is frequently used to treat diabetes, and in vivo investigations have shown that it has a hypoglycemic effect [38]. This plant extract has been demonstrated to produce a substantial reduction in fasting blood glucose levels and inhibition of increased glucose in diabetic rats [41, 42]. *G. procumbens* includes antidiabetic constituents, the majority of which are extracted in 25% ethanol. The active ingredients of this extract appear to work in a metformin-like manner. Oral acute and sub-chronic toxicity assessments in male and female Sprague Dawley rats showed the 25% ethanol extract of *G. procumbens* is safe to use [42, 43]. Sunarwidhi [42] discovered that combining *Azadirachta indica* and *G. procumbens* extracts had a greater hypoglycemic impact than using either of the extracts alone. Both extracts might be used to generate a blood glucose-lowering medication for diabetic animals [42]. In my experiment, treating the fish groups with cortisol showed that the level of control group glucose is significantly less than the stress control (n=6; F= 8.202; p=0.001) group which indicated that cortisol worked to stress the fish, therefore increasing the glucose level in the stressed fish group (Figure 4.2). After day 1, treating the fish

with decided *G. procumbens* (GP) concentrations, it has shown that the control group without any stress and *G. procumbens* treatment exhibited normal range of glucose. Among the stressed fish, fish treated with *G. procumbens* for 1 day,1.5g/kg of *G. procumbens* reduced the glucose level the most (Figure 4.2.a). While analyzing day 3 results we found a similar trend to day 1. Day 3 showed control group, which was not treated with cortisol or *G. procumbens,* had a lower glucose level and the stress control (without *G. procumbens* treatment) along with stress groups with *G. procumbens* treatment all had higher glucose levels. Although it was not statistically significant comparing to stress control 1.5g/kg *G. procumbens* showed lowest glucose level after 3 days of treatment (Figure 4.2.b). As we mentioned earlier, as this is the first experiment treating stressed fish with *G. procumbens* we do not have supporting documents to compare. In our future experiments we are planning to examine *G. procumbens* effects on the chronic stress responses.

Lysozyme and phagocytic activities are humoral aspects of the innate immune system that target the peptidoglycan layers of Gram-positive bacteria's cell wall. They are affected by a variety of stresses [43]. In hybrid tilapia exposed to cadmium, there was an increase in lysozyme activity and a decrease in other humoral immune components. Pollutants may enhance innate immunity while also reducing humoral mediated immunity, therefore enabling pathogen susceptibility in pollutant stressed fish [44]. Lysozyme is an important component of both freshwater and marine fish immune systems [45]. Stress causes an increase in lysozyme activity, phagocytosis, and cell-mediated cytotoxicity, as well as a reduction in antibody synthesis [46]. In this study, serum lysozyme activity in stress groups did not significantly ($n=6$; $F=0.782$; $p=0.549$) increase as compared to controls. It was not statistically significant, however, the stress control group showed increased lysozyme activity when compared to the stress group with 0.5g/kg *G. procumbens* treatment (Figure 4.3.a). These results were based on 3 days of treatment which may not be enough to determine the effect of *G. procumbens* on fish. Further investigations on immune responses of tilapia exposed to chronic stress with the treatment of *G. procumbens* may help understand its immunomodulatory effects. As mentioned before, another study revealed that in common carp, cortisol induced mRNA quantity increase the phagocytic activity [47]. Similarly, in our experiment it has been showed that the stress control without nutraceutical treatment had the highest phagocytic activity while the control group without stress and the stressed groups with nutraceutical treatments showed decreased phagocytic activity. According to prior interpretation it can be said that *G. procumbens* a trend to decrease the phagocytic activity which is an indicator that the stressed groups treated with *G. procumbens* were not as stressed as the stress control group (Figure 4.3.b).



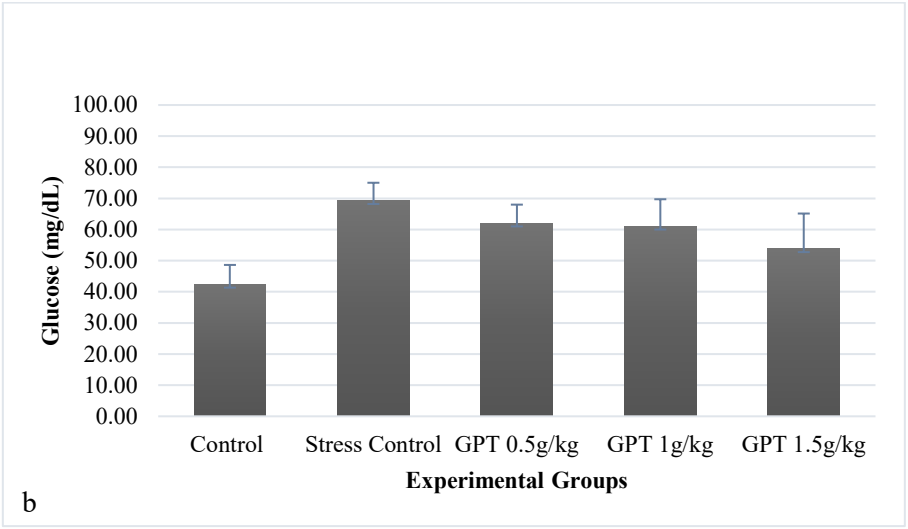


Figure 4.2 Blood glucose levels of Nile tilapia treated with different treatments, represented by control (control feed without stress and without *Gynura*), stress control (stressed group without *Gynura*) and stressed groups with *Gynura procumbens* treatments (GPT) (0.5,1.0 & 1.5 g/kg of feed). a) represents the glucose levels of Nile tilapia on Day 1 and b) represents the glucose levels of Nile tilapia on Day 3. Values are expressed as means ± SEM (n=6). Star means significantly different from control (p <0.05)

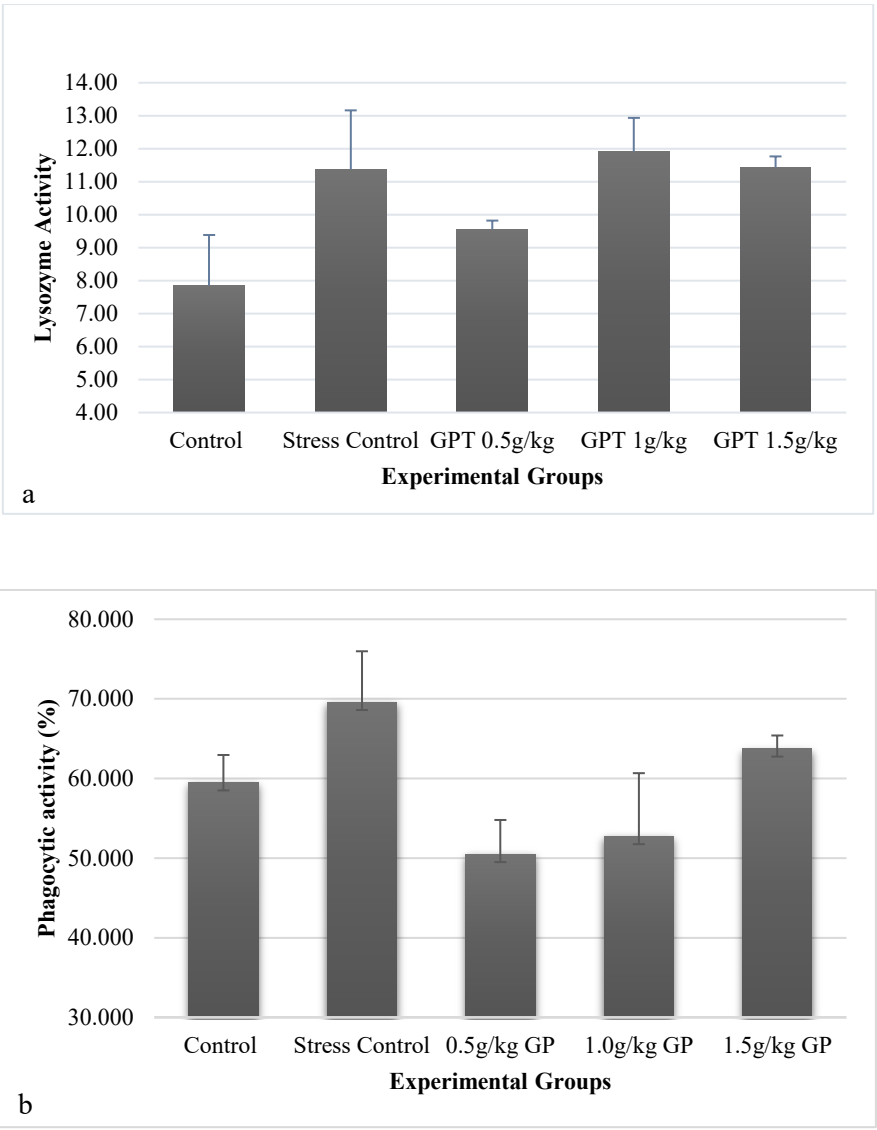


Figure 4.3 Lysozyme activity and phagocytic capacity of Nile tilapia after cortisol exposure and feed diets with or without *Gynura procumbens*. Nile tilapia treated with different treatments, represented by control (control feed without stress and without *Gynura*), stress control (stressed group without *Gynura*) and stressed groups with *Gynura procumbens* treatments (GPT) (0.5,1.0 & 1.5 g/kg of feed): a) represents the values lysozyme activity and b) represents the values phagocytic capacity of different groups of experimental treatment from Day 3 sample collection. Values are expressed as means ± SEM (n=6). There is no significant difference among the experimental groups (p>0.05)

Stress causes changes in blood composition, a slowdown in development reproduction rate, suppression of the immune system, electrolyte abnormalities, and a change in the quality of fish products, among other things. Hematological indicators are crucial for determining a fish's physiological condition and assessing stress [48]. Blood cells conduct key physiological activities in the body as well as immune modulation, therefore hematocrit (PCV) is a useful indication of physiological and immunological performance. Under stressful situations, there

will be more blood cells to adapt for the greater demand, resulting in a higher PCV percentage [49]. In our experiment, experimental treatment groups showed that the percentage of PCV in the stress control is higher than the pure control group, which is the ideal condition while assessing PCV (%) in stress condition (Figure 4.4.a). Interestingly, *G. procumbens* treatment was able to decrease the percentage of PCV in stressed fish compared to the fish in the stress control without, indicating that the nutraceutical treatment was able to minimize stress.

The role of the spleen in mammals has been extensively researched [50]; the cellular makeup, growth, and function of the fish spleen, on the other hand, are far less well understood [52-53]. The spleen is the biggest blood filter in animals, and it eliminates wounded or dormant cells that are unfit for ongoing circulation. It's also involved in iron recovery and absorption, and it's important for pathogen trapping and killing, as well as the development of adaptive immunity [51]. The spleen is a key source of antibody production in fish, and the spleen somatic index (SSI) reduced in fish exposed to significant hypoxia [52]. Results show that in our study there was a substantial decrease, but not significant in every stressed group with or without *G. procumbens* therapy compared to the pure control group (Figure 4.4.b). Gynura did not demonstrate any impact of maintaining a packed red blood cell volume under stressful conditions. This evaluation may need to be determined in the future as part of chronic stress research.

In fish, the liver is important for essential activities such as xenobiotic deposition and biostimulation [53]. The HSI is a significant biomarker that indicates how well a fish's liver functions and how healthy it is [54]. Hossain *et al*. demonstrated that high dose of insecticide in fish caused a spike in HSI, which is linked to numerous changes in the liver induced by insecticide (Sumithion) toxicity, such as fat accumulation in the liver during poisoning and glycogen transformation to glucose [55]. Our results did not reflect the previous ones since it was a short-term trial. Nevertheless, other experiment demonstrated that stress increases HSI in fish that had been exposed to the stressor for a long time (Figure 4.4.c). As a result of limited research, we are unable to draw any conclusions on HSI under stressful situations. If *G. procumbens* extract has any therapeutic capabilities to regulate the HSI, a chronic investigation may provide definitive results on HSI levels in stressful situations.

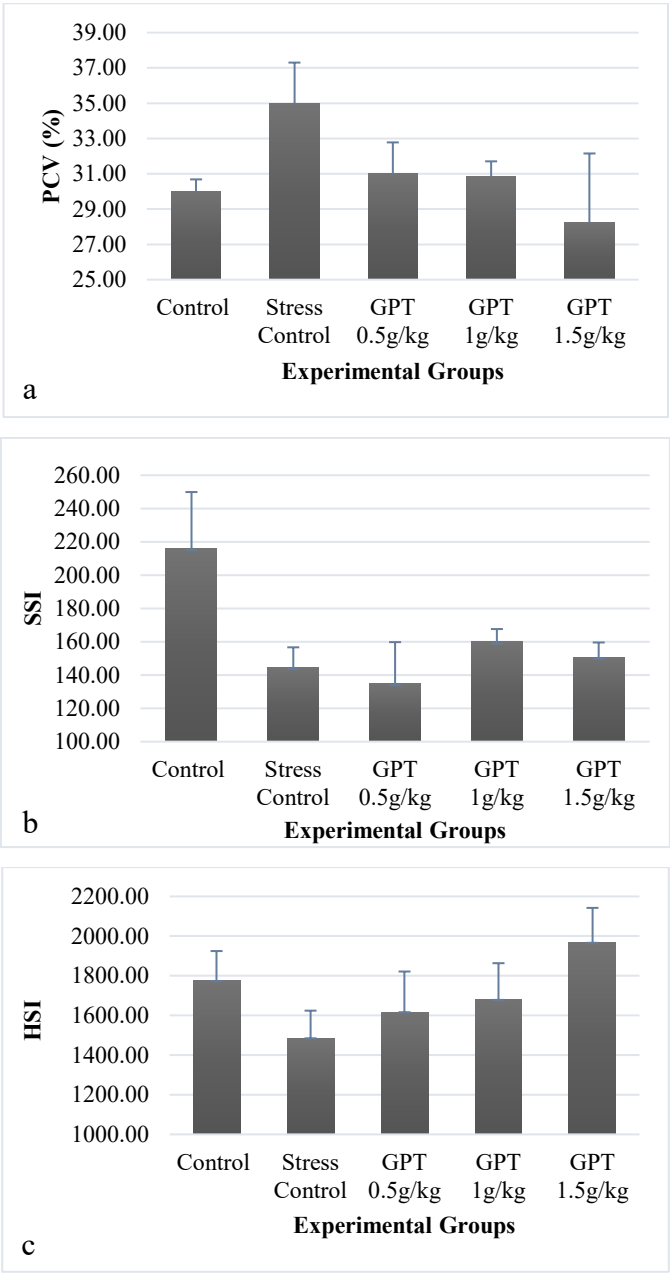


Figure 4.4 Represents Packed Cell Volume (PCV), Spleen Somatic Index (SSI), and Hepato-Somatic Index (HSI) of Nile tilapia after cortisol exposure and feed diets with or without *Gynura procumbens*. Nile tilapia treated with different treatments, represented by control (control feed without stress and without *Gynura*), stress control (stressed group without *Gynura*) and stressed groups with *Gynura procumbens* treatments (GPT) (0.5,1.0 & 1.5 g/kg of feed). a) represents the packed cell volumes (PCV %) values, b) represents the values spleen somatic index (SSI), and c) represents the values of hepato-somatic index (HSI) of different groups of experimental treatment from Day 3 specimen collection. Values are expressed as means ± SEM (n=6). There is no significant difference among the experimental groups (p>0.05)

4.5 Conclusion

It is essential to keep farmed fish healthy and stress-free in order to fulfill the rising population's protein needs. The purpose of utilizing functional feed additives (nutraceuticals) in aquafeeds is to increase fish performance by promoting intestinal health. Supplementing Nile tilapia with *G. procumbens* at 1.5g/kg of fish weight has the ability to regulate glucose levels under stressful conditions and enhance immunity. The oxidative state of Nile tilapia was similarly promoted in a specific way by *G. procumbens* consumption. *Gynura* supplementation provides an easy-to-use approach for enhancing the immunological state of Nile tilapia under chemical stress conditions with no discernible adverse effects. *Gynura* extract fed Nile tilapia have higher lysozyme activity than the stress control fishes. By decreasing glucose levels in blood and increasing the production of blood enzymes and proteins, boosting lysozyme activity, and activating bactericidal and phagocytic activities, this medicinal plant may have the ability to influence host immunity.

4.6 References

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CHAPTER 5. POTENTIALS OF *GYNURA PROCUMBENS* TO MODULATE STRESS AND IMMUNOLOGICAL RESPONSES IN NILE TILAPIA

5.1 Abstract

Discovering viable techniques to reduce stress in fish and increase their resistance to disease, as well as reducing farmers' reliance on conventional chemical treatments, is critical. *Gynura procumbens* (GP), also known as longevity spinach, is a popular medicinal plant in Southeast Asia for lowering blood cholesterol and glucose levels, controlling blood pressure, and even treating some types of cancer. In this experiment, we investigated the effects of 0.0015% *G. procumbens* extract on stress physiology and immunology of Nile tilapia for 12 weeks. In this experiment, four treatment groups were used: control, stress, prevention, and treatment. The stress hormone cortisol was measured for 12 weeks and the *Gynura* supplemented groups were found to control the hormone's release. When compared to the stressed groups, the most significant biomarker of stress- glucose levels, had decreased significantly over time ($p<0.05$). Other physiological parameters revealed that *Gynura* treatment can reduce stress by improving physiological functions in a stressful condition. All the physiological and immunological parameters considered; the prevention group outperformed the treatment group.

5.2 Introduction

Fish farming for human consumption is a thriving industry, and with increased interaction with captive fish populations, there is a growing interest in determining how to provide good welfare for the fish we farm [1]. Fish are subjected to handling and confinement in a variety of situations throughout their life cycle as farmed animals. Netting, weighing, sorting, vaccination, transport, and, finally, slaughter are all common events in farming [2]. Stress in fish is a significant issue in aquaculture due to the extensive husbandry techniques that contribute to reduced production efficiency [3]. The study of stress in fish has grown significantly in recent years, owing primarily to its close relationship to animal welfare. It is widely accepted that good fish welfare, like superior animal facilities, ensures a successful culture in fish farms. In this way, fish farmers are gradually recognizing it, because survival and growth, among other factors, have been shown to decrease in poor welfare conditions [4].

The severity and duration of the stressor influence the nature of the stress response. In almost all cases, the alarm phase consists of the activation of systems involved in flight, fight, and, most importantly, trying to cope [5]. During the resistance stage, the fish either (a) fully overcomes the stressor, allowing for the restoration of homeostatic standards, (b) overcomes the stressor sufficiently to allow it to nearly restore, or (c) begins a downward spiral leading to death. As we will see later, very low levels of stress (eustress) are adaptive, whereas higher levels of stress (distress, a difficult term to define) have both maladaptive and adaptive elements. We are looking at stress from the standpoint of distress here [6].

Fish physiological responses to environmental stressors have been broadly classified as primary and secondary (Figure 5.1). The release of catecholamines from chromaffin tissue is one of the primary responses, which involve the initial neuroendocrine responses [7], and stimulation of the hypothalamic-pituitary-interrenal (HPI) axis, which results in the release of corticosteroid into the bloodstream [6], [8], [9]. Essentially, the primary response to the perception of a stressor involves the activation of a neuroendocrine cascade response involving the secretion and synthesis of cortisol and related compounds, as well as catecholamines such as adrenaline and noradrenaline [6]. In lamprey, the main corticosteroid responding to stress is 11-deoxycortisol, whereas in elasmobranchs it is 1a-hydroxycorticosterone and cortisol, as well as other related steroids in chondrostean and teleosts [10], [11]. Cortisone, a cortisol metabolite, is also found in high concentrations in the blood of stressed fish [5].

Secondary responses include cardiovascular and respiratory responses, both of which increase the distribution of oxygen and energy substrates released into the circulation as a result of the stress response. Adrenaline alters gill blood flow patterns and permeability, both of which favor water flowing down its osmotic gradient, either in or out of the fish depending on the salinity of the environment. Cortisol's logical role in this regard would thus be to restore osmotic balance [12]. Changes in plasma and tissue ion and metabolite levels, hematological characteristics, and heat shock or stress proteins (HSPs) are all related to physiological changes such as metabolism, respiration, acid-base condition, hydromineral stability, immunity, and cellular responses [13], [9]. Another response to cortisol and possibly other control factors is immunosuppression. While hormones ultimately benefit the organism by directing energy to where it is needed during stressor exposure, many tertiary responses, or those at the whole animal level, are maladaptive. Health and disease resistance, reproduction, growth, learning, and other behaviors like predator avoidance are all hampered [14]. The stress response is unquestionably a necessary fish mechanism for overcoming severe challenges and, if possible, restoring homeostasis. Nonetheless, it is critical to recognize that under normal, non-stressful

conditions, a low circulating level of stress hormones is required for the maintenance of routine life functions such as growth, the immune system, and learning. That is, at low levels of stress, the effects of cortisol and catecholamines on routine life functions are positive, whereas the effects become negative at higher and more prolonged elevations in concentration. In other words, the individual components of the stress response are components of homeostasis [5].

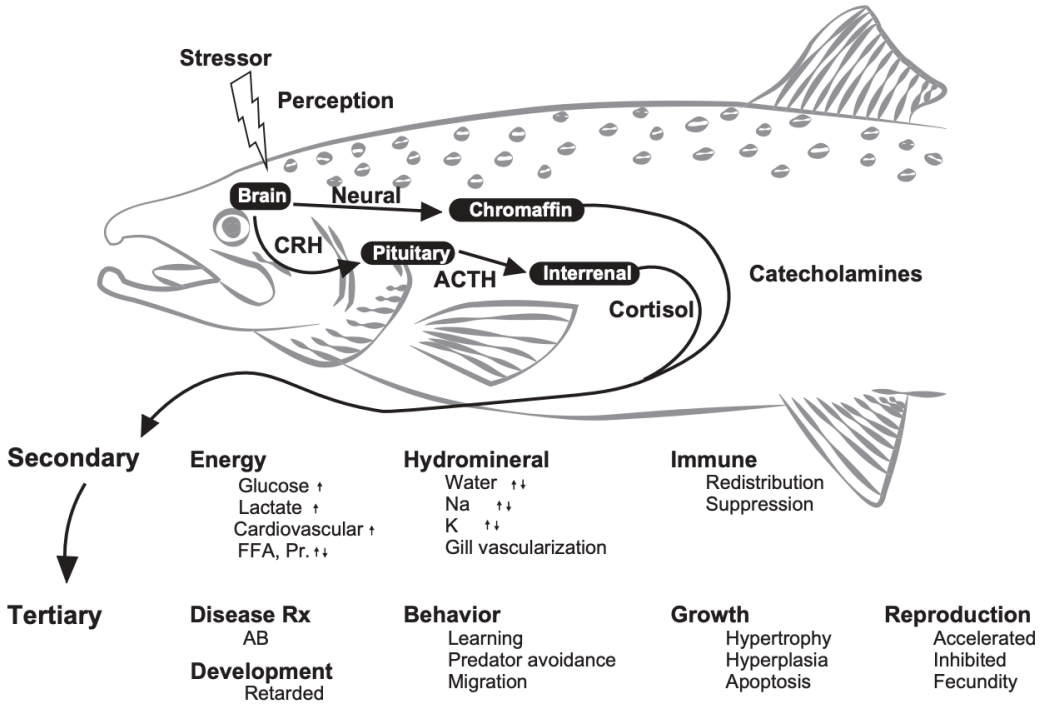

Figure 5.1 This picture demonstrates different phases of stress response in fish. The primary, secondary, and tertiary responses of fish during Stress. Corticotropin releasing hormone (CRH); adrenocorticotropic hormone (ACTH); free fatty acids (FFA); proteins (P); antibodies (AB) [5].

Several zootechnical systems and variables are adjusted in fish farming to achieve maximum animal welfare without compromising productive yield, though finding the right balance can be difficult at times. Aside from technological and infrastructure changes, implementing new feeding strategies is a straightforward and practical way to improve fish welfare. In this context, the concept of functional food (food with beneficial effects on the organism that are not nutritional in nature) has emerged as a new method to improve overall health, including welfare [15]. As a result, several works on fish farming are based on the addition of specific biologically active substances to conventional commercial fish feed in order to modulate or attenuate the stress response and, thus, succeeding [16]–[18].

Gynura procumbens, also known locally as "Sambung Nyawa," is mainly grown for medicinal purposes in Southeast Asia, particularly in Indonesia, Malaysia, and Thailand. It is a member of the Asteraceae family and has long been used as both a vegetable and a medicine. As a traditional medicinal plant, the leaves of this plant are commonly used to treat a variety of diseases caused by oxidative stress, including inflammation, cancer, diabetes, hypertension, and hyperlipidemia [19]. *G. procumbens* has been shown in prior pharmacological research to have anti-inflammatory, antihypertensive, antihyperlipidemic, antioxidative, and cardioprotective attributes [20]. Several organic compounds, including syringic acid, quercetin, N, N-dimethylanthranilic acid, dehydrovomifoliol, β-sitosterol 3-O-β-D-glucopyranoside, schottenol, and montanic acid, were isolated and their structures determined. The results of the tests revealed that the parts of *G. procumbens* were active as -glucosidase inhibitors, which could help with diabetes treatment [21]. Intensive research on *G. procumbens* has provided extensive scientific evidence of its therapeutic potential over the decades. Because they have the ability to mitigate diabetics and glucose levels are one of the major biomarkers of stress, we tested the effect of *Gynura* on mitigating the chronic stress response of Nile tilapia in this study. We measured plasma cortisol (primary stress biomarker), blood glucose levels, plasma protein levels, packed cell volume, liver and spleen somatic indices (secondary stress biomarkers), lysozyme activity, and macrophage capacity (tertiary stress biomarkers) in stressed and non-stressed tilapia, *O. niloticus*.

5.3 Materials and methods

5.3.1 Plant extract preparation

Fresh *Gynura* leaves were obtained from plants cultivated at Purdue University Fort Wayne. The leaves were cleaned and mashed finely using a mortar and pestle. *Gynura* paste was, then, prepared with 0.25g/mL of 25% ethanol solution [22]. Prepared paste was filtered with double layered cheese cloth and mixed for 24 hours on a shaker mixture at room temperature [23]–[25]. Finally, the prepared extract was used for preparing the fish feed according to the treatment groups.

5.3.2 Fish maintenance

Nile tilapia with an average weight of 38±2.45g and average length of 13±0.96 cm was reared in the aquaponics system in the Life Sciences Research Center, Purdue University Fort

Wayne, Indiana. Fish were maintained in optimal conditions (pH: 6.0-7.0; ammonia: 0-3.0 mg/dL; temperature: 25-28 °C, dissolved oxygen: 5.00-7.00 mg/L; and photoperiod: 12 h:12 h, light: dark). Fish were fed a known amount of commercial feed Purina® AquaMax® Fingerling Starter 300 (Purina Mills, MO, USA) as well as experimental feed, twice daily at 1% of their body weight (a total of 2%/day). All fish were taken care of following an approved protocol, approved by Purdue University Animal Care and Usage Committee (PACUC) following the guidelines of the US National Research Council's Guide for the Care and Use of Laboratory Animal and Purdue University Aquatic Animal Standard Operating Procedures.

5.3.3 Feed preparation

The amount of feed required for each feeding group was determined by the fish body weight (2% body weight). The commercial feed was prepared into two groups: control feed and stress feed. For stress feed (to induce stress), hydrocortisone was added at 0.01% hydrocortisone of fish body weight [26]. Stress feed was then divided into three groups: stress group (feed with hydrocortisone throughout the experiment), prevention group (1.5g/kg *Gynura* treatment with induced stress for the complete experiment) and treatment group (first 6 weeks induced stress with 0.01% hydrocortisone and after 6 weeks started 1.5g/kg *Gynura* treatment). Proximate composition of control and experimental feed are presented in Table 1.

5.3.4 Experimental design

The fish in this investigation were separated into four treatment groups. Each group had two replicates. As previously stated, three of the four groups were stressed by giving hydrocortisone (0.01 % body weight) supplemented commercial feed (stressed group) [26] and the other group were fed with regular commercial feed (non-stressed control group). The two stressed groups prevention group was fed *Gynura* with hydrocortisone for 12 weeks of experiment and treatment group fed hydrocortisone feed from the beginning to end of 12 weeks of experiment however after 6 weeks this group treated with *Gynura*. This different groups of feed supplementation were provided to determine the best regimen to mitigate effects of stress on the hematological stress biomarkers.

Table 5.1 The approximate content of feed components used to produce each of the five treatment feeding groups is represented by control, stress, prevention, and treatment.

Ingredients (% feed weight)	**Control**	**Stress**	**Prevention**	**Treatment**
Crude protein	50	50	50	50
Crude fat	16	16	16	16
Crude fiber	3	3	3	3
Calcium (Ca)	5.2	5.2	5.2	5.2
Phosphorus (P)	1.3	1.3	1.3	1.3
Sodium (Na)	0.6	0.6	0.6	0.6
Hydrocortisone*	-	0.01	0.01	0.01
Gynura procumbens extract*	-	-	1.5g/kg	1.5g/kg (after 6 weeks)

*According to body weight

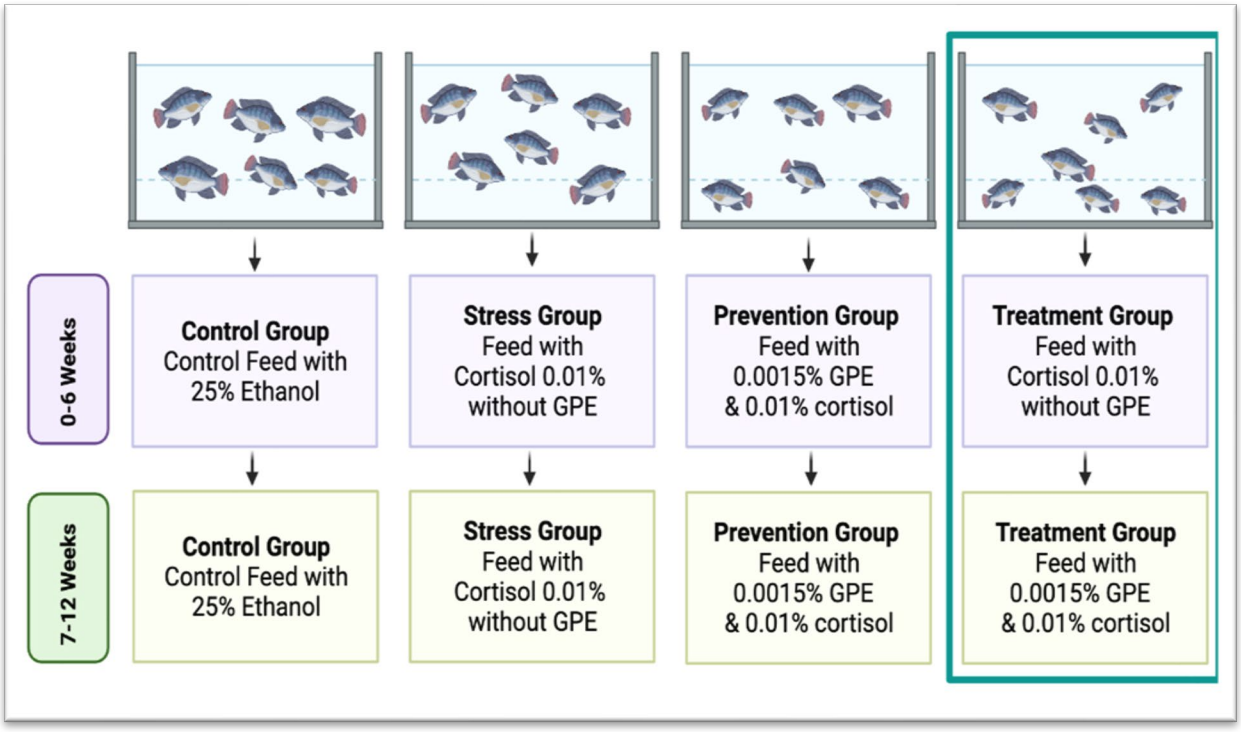

Figure 5.2 The picture represents experimental design for the chronic study according to the groups and their treatment regime.

5.3.5 Fish sampling

To investigate the hematological stress parameters, the fish (3 X 2= 6 fish/ group, 5 groups) were sampled at week 0 (baseline), week 2, week 4, week 6, week 8, week 10 and week 12. For sampling days fish were euthanized with >200 mg/L tricaine mesylate to reduce any stress caused by handling (all within two minutes of catching the fish). Blood was drawn from the caudal vein using heparinized syringes to avoid blood clotting. The blood was transferred to a 1.5 mL Eppendorf tube and placed on ice right away. The obtained blood was then used to measure blood glucose, packed cell volume, and lysozyme activity. The fish were then dissected using aseptic techniques to remove the liver and the spleen. Fish length, weight, spleen weight, liver weight, and phagocytic capacity were also measured.

5.3.6 Plasma cortisol

Cortisol is one of the major stress hormones that are released by the inter renal cells of the head kidney tissue of the fish and thus indicative of stress [26]. The blood was centrifuged at 5000 rpm for 10 minutes to collect the plasma. The supernatant plasma was collected and preserved at -80°C for future use. To measure the cortisol level in the plasma, Cayman Chemical Cortisol ELISA kit (Item No. 500360) (Ann Arbor, MI, USA) was used, following manufacturer's protocol.

5.3.7 Blood glucose

One of the most significant indicators for measuring stress is blood glucose. The concentration of glucose in the blood is extremely sensitive to stress, allowing even little variations in blood glucose to be noticed almost instantly, making it useful for detecting acute stress. An increase in respiration, a decrease in metabolic activity, and a decrease in immunity, all of which are signs of stress, are caused due to changes in blood glucose [27]. A glucometer (Freestyle, Abbott Laboratories, CA) was used to monitor the glucose levels in the blood [28].

5.3.8 Packed cell volume

The blood cell to plasma ratio is determined by packed cell volume (PCV) or hematocrit. It determines the proportion of red blood cells relative to all other cells found in blood [29]. Using capillary action, a glass capillary tube was filled with blood (~75%) and then sealed on one side with a Crito-cap. In a micro-hematocrit centrifuge, the capillary tubes were

spun at 10,000 rpm for 10 minutes. The percent PCV from the capillary tubes was read using a micro-hematocrit capillary tube reader.

5.3.9 Spleen somatic index

The spleen stores lymphocytes and blood cells, which are then deployed to regulate immunity. [30]. As a result, the spleen somatic index is an excellent indication of a fish's overall health. The spleen somatic index measures the weight of a fish's spleen in relation to its overall weight. The SSI of fish was determined by weighing the harvested spleen and applying the following formula:

$$SSI = \frac{Spleen\ \ wight(g) * 100}{final\ weight\ (g)}$$

5.3.10 Hepatosomatic index

Glycogen is a type of energy that is stored in the muscles and liver of fish. Glycogen is mobilized in the presence of glucagon produced by the pancreas during growth and high stress situations. The liver's glycogen stores are broken down into glucose and released into the bloodstream, altering the liver's overall size and mass. As a result, the hepatosomatic index (HSI) is a reliable measure of fish nutrition, growth, and stress levels [14]. The hepatosomatic index measures the weight of a fish's liver in relation to its overall weight. The harvested liver from the fish was weighed, and the HSI of the fish was calculated using the formula below:

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

5.3.11 Lysozyme activity

Lysozyme is a bacterial cell-lysing enzyme that alters the opacity of a bacterial solution. Using a spectrophotometer, this experiment evaluates the capacity of endogenous lysozyme in the blood to remove the color of the bacterial solution [31]. The more bacteria destroyed, the clearer the solution gets, resulting in a higher spectrophotometer transmittance (T) measurement. The ratio of light falling on a substance to light passing through it is known as transmittance. The term "higher transmittance" refers to the amount of light that passes through the material. The plasma was obtained from the acquired blood samples by centrifuging them

for 10 minutes at 10000 RPM. Each sample's supernatant was collected and placed in Eppendorf tubes before being set aside. Then a suspension of *Micrococcus lysodeikticus* was made at a concentration of 0.2 mg/ mL in 0.05 M (pH = 6.2) sodium phosphate buffer. Then 1 mL of the suspension was added to an Eppendorf tube. 50 µL of the plasma was then added to the Eppendorf tube and vortexed. 1 mL of this solution was put into a cuvette to measure its transmittance at 540 nm using a spectrophotometer (Spectronic 601 spectrophotometer, Milton Roy Company, PA). At the one-minute and five-minute marks, readings were obtained. This process was carried out on each one of the fish serums obtained. An uninoculated sodium phosphate buffer was used to calibrate the spectrophotometer. The lysozyme activity assay (LLA) was calculated using the following formula:

$$LAA = \frac{(Final\ transmittance - Initial\ transmittance)}{Total\ elapsed\ time\ (minute)}$$

As a result, the lysozyme activity assay is a measure of the lysozyme clearing bacteria's rate of increase in transmittance per minute.

5.3.12 Phagocytic capacity

To collect and isolation of the macrophage head kidney was removed aseptically and placed in centrifuge tube with 2ml L-15 with 2% FBS; stored on ice. Kidney was then macerated through sterile double metal sieve using sterile plunger (double sieve is 80 mesh/190µm with 100 mesh/140µm) and cell suspension was collected in L-15 with 2% FBS. Collected cell suspension was centrifuged at 1000 rpm for 10 minutes and the cell free supernatant was discarded. The cell pellet was then resuspended in 2 ml of L-15. After the second wash, the cell pellet was finally re-suspended in 1 ml L-15. The isolated macrophage cells were, thereafter, used to determine phagocytic capacity. 50µl of prepared cell suspension placed on to both wells of double-etched microscope slides and incubated for 2 hours at room temperature in moist condition. After 2 hours of incubation, 50 µl of formalin killed bacteria was added on to each well of the slides and incubated for another 2 hours at room temperature in moist condition. After the final incubation period, the slides were gently cleaned in PBS before being fixed in 100% methanol for 1 minute. After fixing, slides were stained with Wright-Giemsa stain. The stained slides were washed with phosphate buffer and deionized water. Under a microscope, the dried slides were used to count positive macrophage cells.

Macrophages were considered positive for phagocytic capacity when they had more than 5 bacteria phagocytized within them. This protocol was followed after Mustafa *et al*. [32].

5.3.13 Growth and metabolism

The Fulton formula is used to calculate the Fulton condition factor. In the early 1900s, Fulton proposed this formula: K= 100 × weight/length^3. The weight is given in grams, and the length is given in centimeters. As a result, the weight of the fish divided by its length cubed yields a number [33].

$$\text{Fulton Condition Factor (K)} = \frac{weight(g) * 100}{length(cm)^3}$$

5.3.14 Statistical analysis

The Shapiro-Wilk test was used to determine whether the data was normally distributed or not. If the data passed the normality test, the data were analyzed statistically using one-way ANOVA of variance with Tukey's honestly significant difference test in post hoc analysis on SigmaPlot 14.5 (Systat Software, San Jose, CA). Data are expressed as means ± SEM. Differences were considered significant at $p<0.05$.

5.4 Results and discussion

As of today's knowledge, the effects of *Gynura prcumbens* extract (GPE) on fish stress physiology are a novel area of research. We reviewed literature that tested the effects of GPE on mice models whenever fish model research was unavailable due to a lack of research on the effects of GPE on the stress physiology of fish. As a result, our study is one of the first to look into the potential use of GPE as a stress-relieving agent in fish, specifically *O. niloticus*. GPE on mouse models, on the other hand, has received a lot of attention in this field. We review the stress physiology of fish because it is a well-reviewed and well-known area of research when discussing the effects of stress on animal physiology.

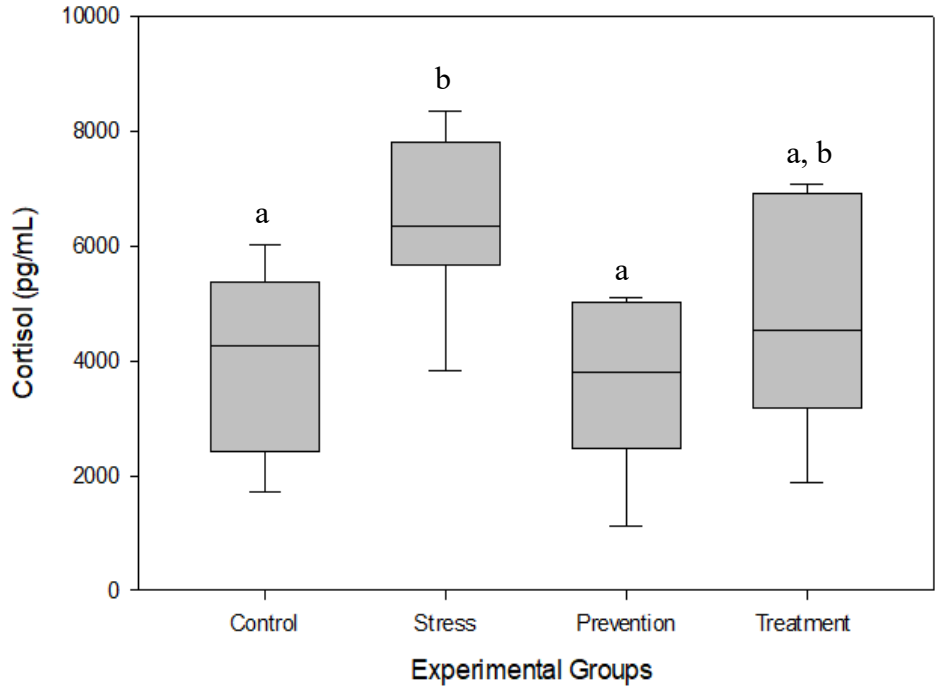

Figure 5.3 Cortisol concentration in different experimental groups over the 12 weeklong experimental period. Boxplot indicates the median (the midline in the box) and the 5th, 25th and 75th percentile from the bottom to top. Different alphabets indicate significant difference (p<0.05).

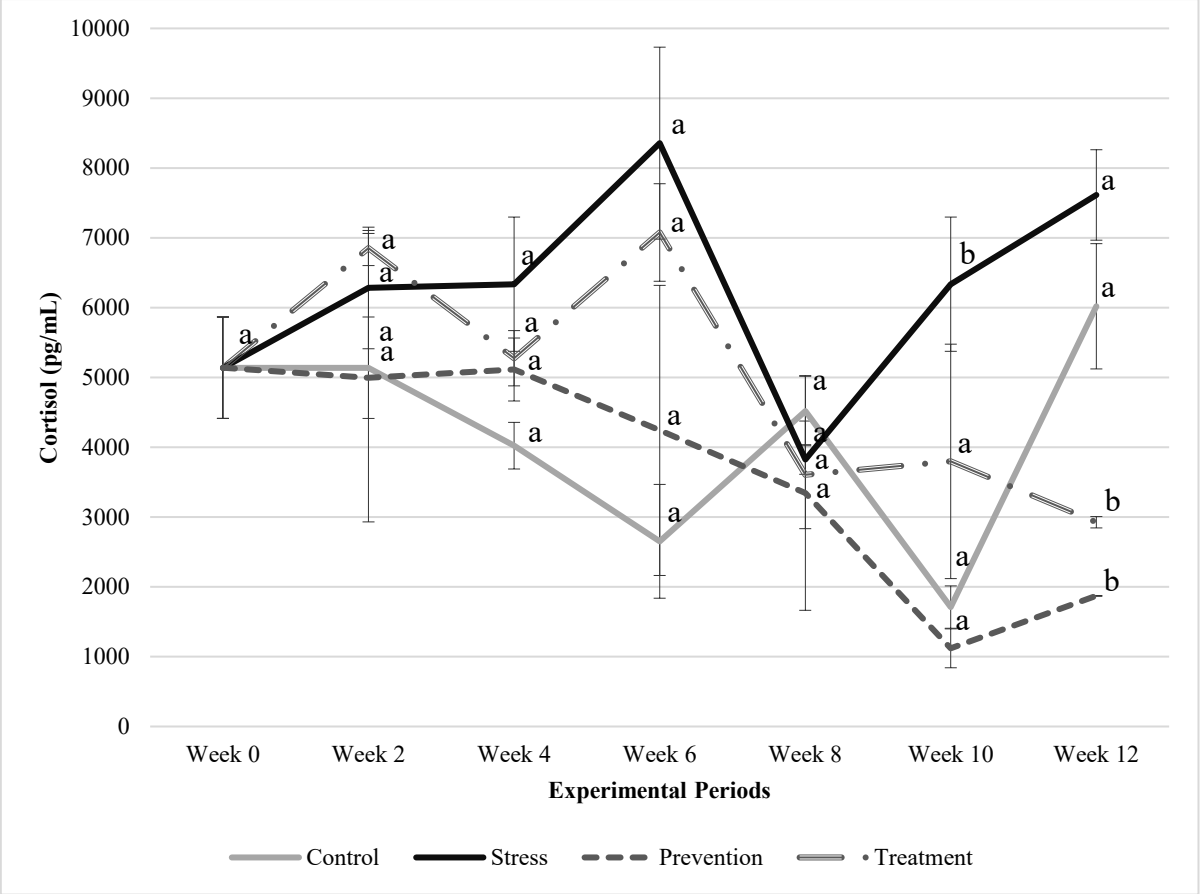

Figure 5.4 Plasma cortisol concentration in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

Firstly, cortisol ELISA assay was done to see whether the hydrocortisone fed to the fish was able to stress the fish effectively. To interpret the results, we must first understand how cortisol is generated in the body and how long it remains active. In fish, stressors stimulate the hypothalamic-pituitary-interrenal axis (HPI) which prompts the release of adrenocorticotrophic hormones (ACTH). This in turn prompts the interrenal cells of the head kidney tissue to generate cortisol in response. This is similar to the cortisol production in mammals who have hypothalamic-pituitary-adrenal cortex (HPA) and are also stimulated by the adrenocorticotrophic hormones (ACTH) [14]. The plasma cortisol levels are kept in check by regulating how much of it is produced endogenously. In fish, the plasma cortisol levels come down to the basal levels 24 hours after an acute stressor are perceived. Cortisol is rapidly

metabolized in the liver due to its action on it, and then filtered and excreted by the kidney [34]. In their experiment, Iskander *et al* demonstrated that the hexane and toluene fractions extracted from *Gynura procumbens* inhibited hydrocortisone 4 mg/ear (mouse) at 35.0%. These tests revealed that steroids could be one type of anti-inflammatory compound found in this plant [35]. The cortisol concentration in plasma or serum is the most frequently measured indicator of the stress response in fish. Like our findings support other studies that also found the cortisol concentration is increased in stress condition and remained unchanged till 24 hours [36]–[38].

In my 12 weeks chronic experiment stressed group had shown significantly higher level of cortisol compared to control and prevention (n=3; F= 15.778; p=0.034) (Figure 5.3). Prevention group had a cortisol level as low as control while the treatment group was in between control and the stressed group. Specifically**,** up to 6 weeks we treated the stress treatment group with cortisol only. Therefore, the cortisol level of stress and treatment group was higher than the control group which was not treated with any stress. Prevention group was also exhibited higher than the control since it was also treated with the cortisol along with *Gynura*. However, at week 8, it has been seen that all the cortisol treated groups tried to acclimate. At week 10, the prevention group mitigated the release of cortisol significantly (n=3; F= 5.699; p=0.034) than the stress group. During our final week of experiment the control group come back to the baseline concentration. The prevention (n=3; F= 15.778; p=0.006) and the treatment (n=3; F= 15.778; p=0.002) groups were significantly lower than the stress group and the baseline concentration. The anti-inflammatory effects of *Gynura* species are known to act through the modulation of inflammatory cytokine production, inhibition of prostaglandin E_2 and nitric oxide production, cellular inflammatory-related parameters, and inflammation in animal models [39]. The results of the biological evaluation of *G. procumbens* have supported the medical usage of this plant as a topical anti-inflammatory agent in Thai traditional medicine.

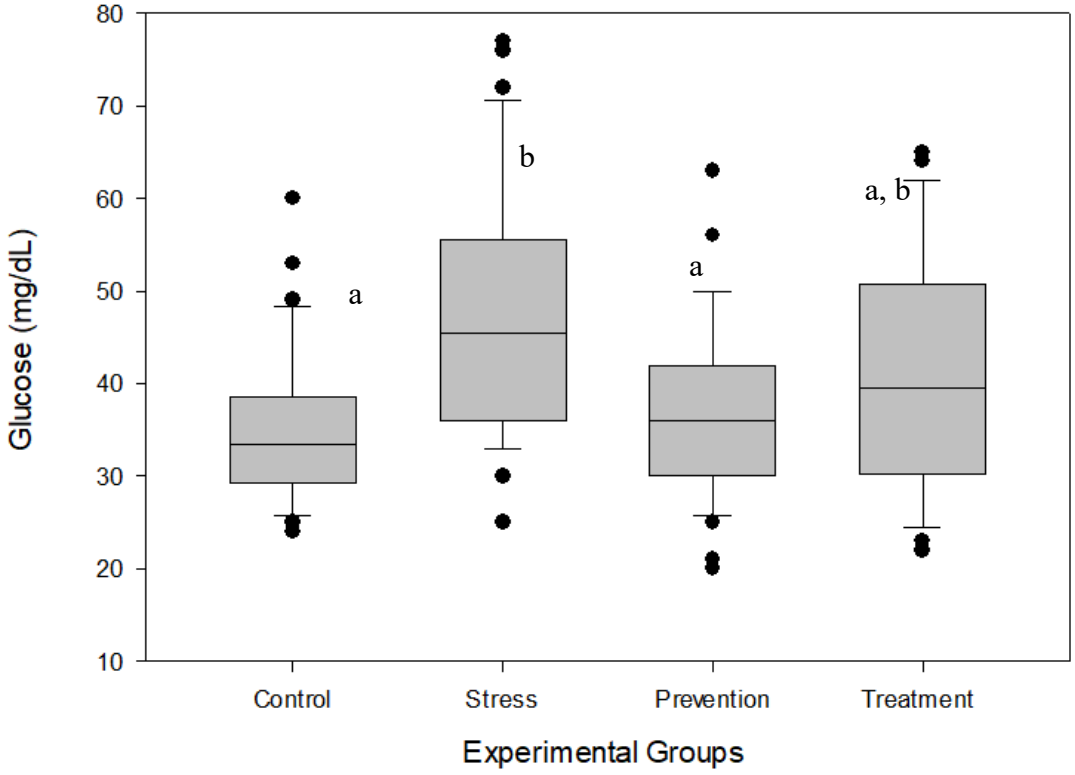

Figure 5.5 Glucose level in different experimental groups over the 12 weeks experimental period. Boxplot indicates the median (the midline in the box) and the 5th, 25th and 75th percentile from the bottom to top. Filled circles are outliers. Different alphabets are significantly different than each other (p<0.05).

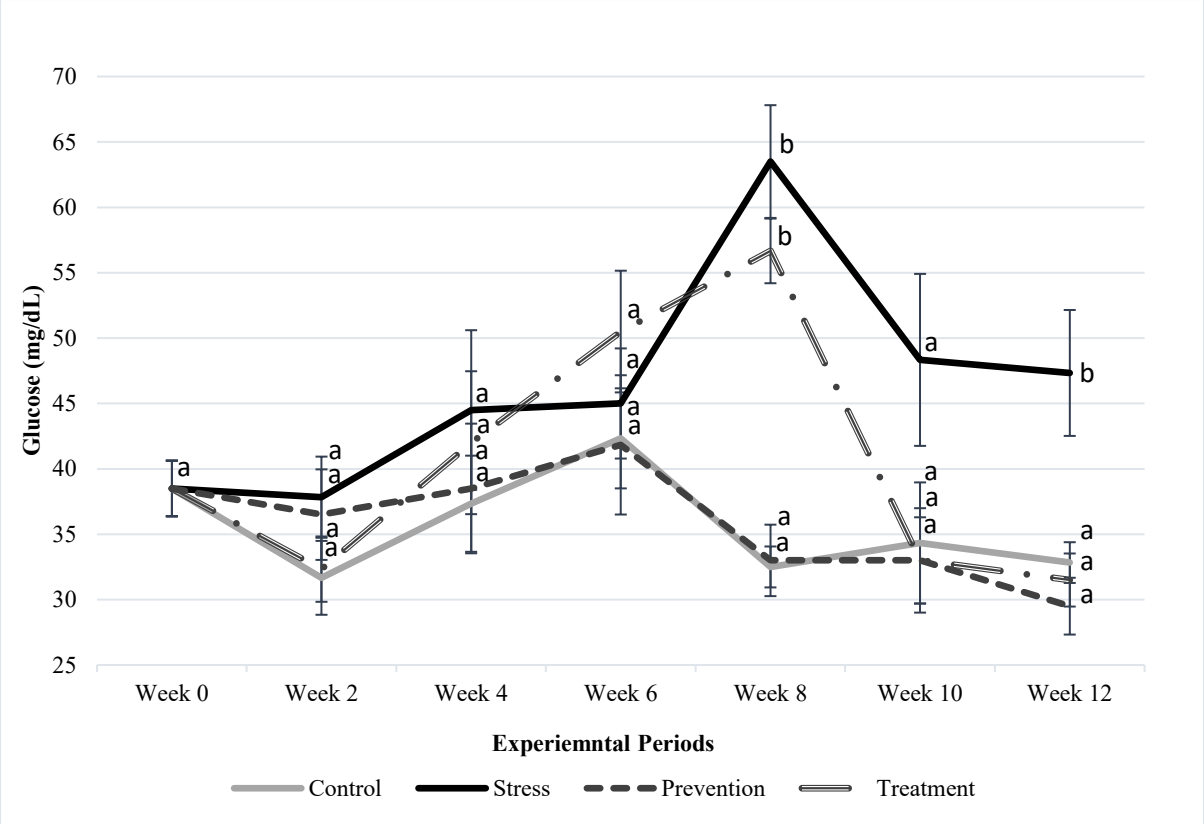

Figure 5.6 Blood Glucose concentration in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

Glucocorticoids are recognized as key stress hormones across vertebrates. Released into the circulation following activation of the neuroendocrine stress axis in response to actual or perceived threats, glucocorticoids have a broad number of physiological effects on multiple target tissues [40]. Since these glucocorticoid-mediated effects help vertebrates counter the impact of stressors and maintain their homeostatic state, they are generally considered to be adaptive. However, it is also well known that sustained levels of glucocorticoids can have deleterious effects [41]. Glucocorticoids promote gluconeogenesis in liver, whereas in skeletal muscle and white adipose tissue they decrease glucose uptake and utilization by antagonizing insulin response. Therefore, excess glucocorticoid exposure causes hyperglycemia and insulin resistance. Glucocorticoids also regulate glycogen metabolism [42]. Mean baseline glucose levels were 38.51 mg/dL, within the range reported by others, that were between 34.54 and 130

mg/dL [43]. Algariri *et al* in their experiment found that n-butanol fraction (n-BF) of *G. procumbens* ethanolic extract showed the highest dose-dependent glucose-lowering action (51.2% and 62.0% at 500 mg/kg and 1000 mg/kg, respectively) in mice model, which is closest to metformin effect (63.6%, $p<0.05$). Moreover, they also found that *Gynura* leaves have a no-observed-adverse-effect-level in mice [44]. The GPE group significantly reduced the increase in postprandial blood glucose levels compared to the control group of streptozotocin (STZ)-induced diabetic mice. In STZ-induced diabetic mice, *Gynura* extract administration significantly reduced the area under the curve. These findings imply that *G. procumbens* extract may aid in the treatment of postprandial hyperglycemia by inhibiting carbohydrate digesting enzymes [45].

My observation agreed with previous studies had done on mice with *Gynura procumbens* extract. In my experiment the effects on blood glucose were also studied following 12 weeks of repeated oral administration of the *Gynura procumbens* extract with 25% ethanol at dose levels 1.5g/kg. The stress and treatment groups had over all higher glucose levels than the control and prevention groups (Figure 5.5). In week specific analysis, it was not significantly different from the comparable groups at 6 weeks (Figure 5.6). The prevention (n=6; F= 7.703; p=0.002) and treatment (n=6; F= 7.703; p=0.006) group successively decreased the glucose level over the study duration (12 weeks) compared to stress group. Week 8 data exhibited that the average glucose level of stress group was at 63 mg/dL whereas both prevention and treatment group were able to decrease the level average at 33 mg/dL. As experiment progress at 12 weeks glucose level representation maintained similar pattern. In the last sampling day (12 weeks) the level of glucose recorded for prevention and treatment group respectively at 29.5 mg/dL and 31.5 mg/dL which are significantly ($p<0.05$) lower than the stress group (47.3 mg/dL). In my experimental design the prevention group was treated with GPE from the very beginning of the experiment along with cortisol exposure, however the treatment group were started treating with *Gynura* after 6 weeks while its glucose level reached to the peak. Despite the fact their starting time of the treatment is different but they showed the similar results in terms of decreasing the level of blood glucose. Therefore, after 12 weeks of repeated oral treatment, the *Gynura* extract has shown to be the probable treatment of active glucose lowering nutraceutical as in chronic antihyperglycemic experiments.

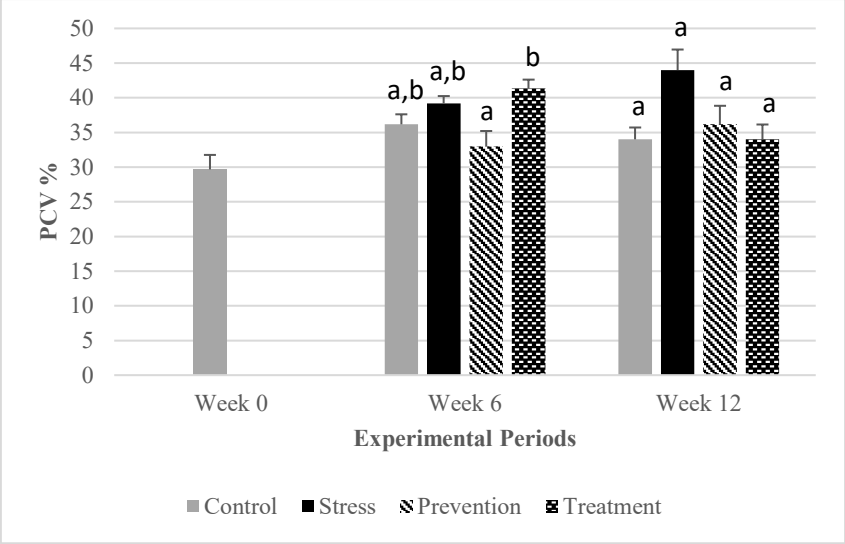

Figure 5.7 Percentage of packed cell volume in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

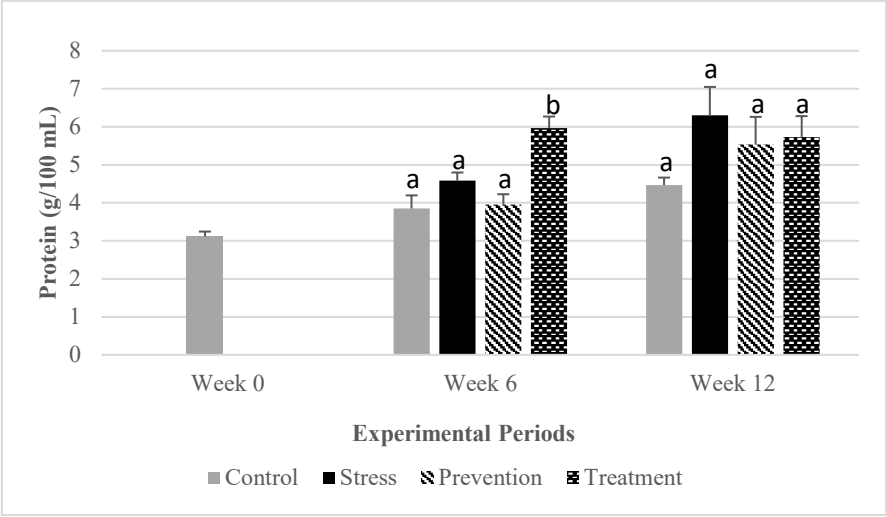

Figure 5.8 Plasma protein concentration in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

Because blood cells perform major physiological functions in the body as well as immune modulation, hematocrit or packed cell volume (PCV) is a good indicator of physiological and immunological function. As a result, in stressful situations, there will be more blood cells to compensate for the increased demand on the body, resulting in a higher PCV percentage [46]. Such is seen in this results in Figure 5.7, where at week 6 stress and treatment (also only treated with cortisol until 6 weeks) had the highest % PCV. On the other hand, control and prevention group had the lowest % PCV and there was a significant difference between treatment and prevention at week 6 (n=6; F= 5.389; p=0.007). It can be stated that during primary responses of stress 2 of the 3 stress groups exhibited higher PCV. However, the prevention group was able to mitigate the stress as this group expressed lower PCV. I started to treat the treatment group after week 6 sampling with same amount of GPE as prevention group. Analyzing the 12 weeks data it can be said that the stress group remained higher but the prevention and treatment group showed lower PCV level than the stress group.

The plasma protein level is another important secondary stress biomarker. As previously stated, when under stress, organisms increase their plasma protein levels in order to supply proteins throughout the body to repair any damage done to various tissues as a result of increased activity caused by stress [47]. So, under stressed conditions, higher protein levels are expected in the blood. In Figure 5.8, at week 6, we can see that all the control and prevention group exhibited the lower concentration of protein which is expected. Thus, in prevention group which was treated with *Gynura* along with stress were able to mitigate the stress. Another 2 stress groups at 6 weeks should have released increased amount of protein, however the stress group did not as much as the treatment group released although they were treated exactly same until week 6. At week 6 treatment was significantly different from other 3 groups: control, stress and prevention (n=6; F= 11.351; p<0.001). Furthermore, week 12 data showed that there were no significant differences between groups. Nevertheless, prevention and treatment group were released higher and lower amount of protein than the stress and control group respectively. The amount of protein is not always reliable to conclude the level of stress and physical condition. George Iwama (1998) in his paper titled “Stress in Fish” reviews among other things, the effect of stress on plasma proteins. He reviews studies in this paper that show that stress does increase blood plasma protein, which could be used as a stress biomarker. Because of the wide variability in results, plasma protein is not a reliable stress biomarker on its own and must be used in conjunction with other biomarkers [48].

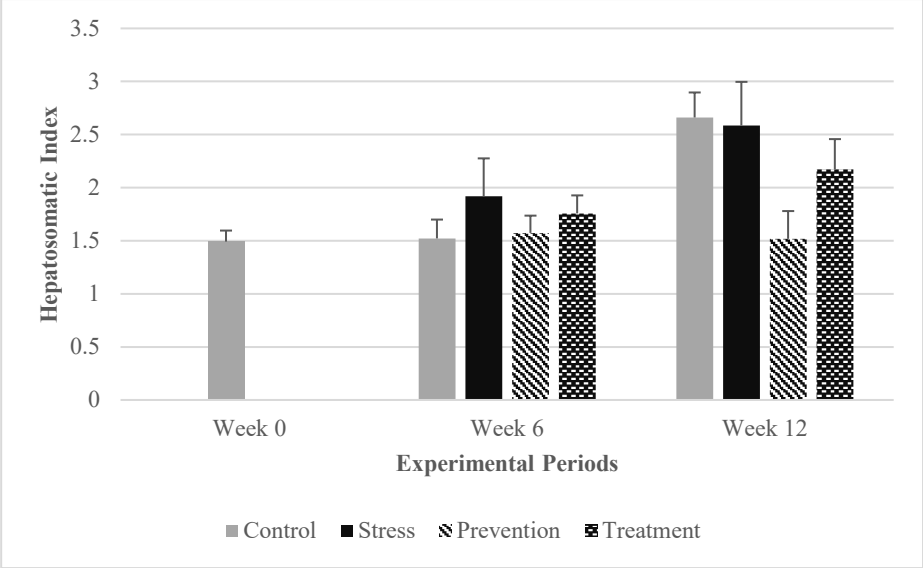

Figure 5.9 Hepatosomatic index in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

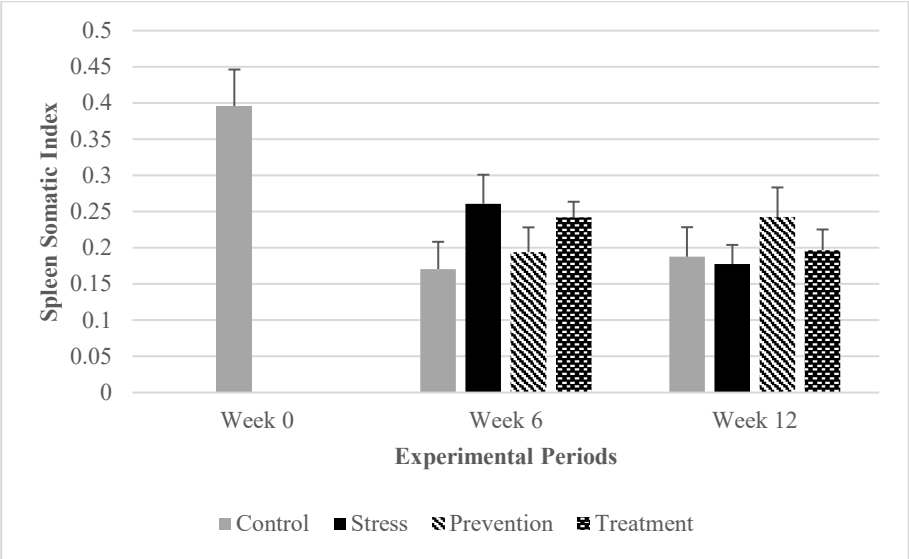

Figure 5.10 Spleen somatic index in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

Continuing to look at the secondary stress biomarkers, hepatosomatic index (HSI) is the weight of the liver in proportion to the fish body weight. Hepatosomatic index should be low in organisms under acute stress as the glycogen stores in the liver get mobilized to form blood glucose under stress [14]. This glucose is then used to meet the high energy demands of a stressed condition as mentioned earlier. However, as stress is prolonged for a long time (chronic stress), the body stores energy as glycogen in the liver to deal with stress and this becomes the new basal HSI level. Higher metabolic activity in the liver due to stress can lead to higher HSI in chronic stress. This phenomenon is observed in previous research [49]. In figure 5.9 there were no significant differences between the groups in week 6 and 12. The prevention maintained the lower HSI throughout the experiment which reflect that this group was able to control the stress. The treatment group was at week 6 was higher than the control and prevention and lower than the stress at week 12 but not as low as the prevention. However, at week 6 and 12 stress group remained consistently higher, the control group was lowest in the week 6 and highest at week 12. As every week, we sampled different fish from each group it might be possible that the selected control group fish for week 12 was responsible for higher HSI. This is indicative of the ability of GPE to reduce stress. To my knowledge, no other study was found to have investigated this parameter.

Just like the HSI, the spleen somatic index (SSI) is the weight of the spleen in proportion to its total body weight. It is a good stress biomarker since the spleen stores immune cells and red blood cells. Stress prompts the release of blood cells and spleen cells (T and B cells, macrophages) from the spleen to supply for the increased respiratory demands and to fight the perceived threat [30]. In prolonged stress (chronic), just like in the case of the HSI, the spleen becomes larger to hold larger quantity of immune cells ready to be released in the case of another infection [50]. In a study published in 2017, it was found that mice exposed to chronic stress from crowding were more likely to physically bite each other. This in turn led to an increase in spleen weight over a period of 19 days due to the activation of spleen immune cells to fight off any invading pathogens from such bites [51]. However, an increased production of immune cells may also affect the overall quality of the immune cells and their ability to fight infections [52]. Figure 5.10 exhibits that all the groups including control, stress, prevention and treatment had no significant difference between the groups at week 6 and 12. The control group maintained lower at week 6 and 12 both. However, the stress group was higher at week 6 and lowest at week 12 which is expected when the stress is prolonged as at week 12 it reached at exhaustion phase. The prevention and treatment maintained the SSI at primary and secondary level of stress with the provided GPE treatment.

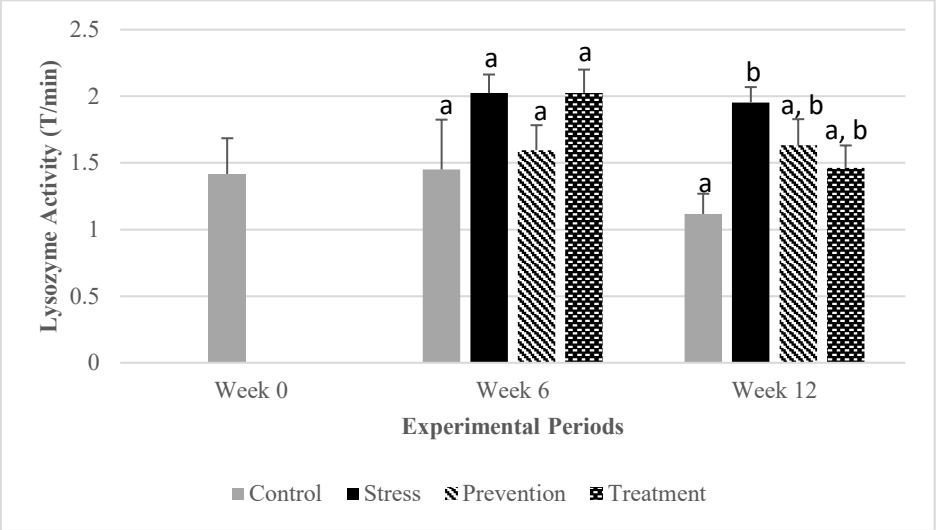

Figure 5.11 Lysozyme activity in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

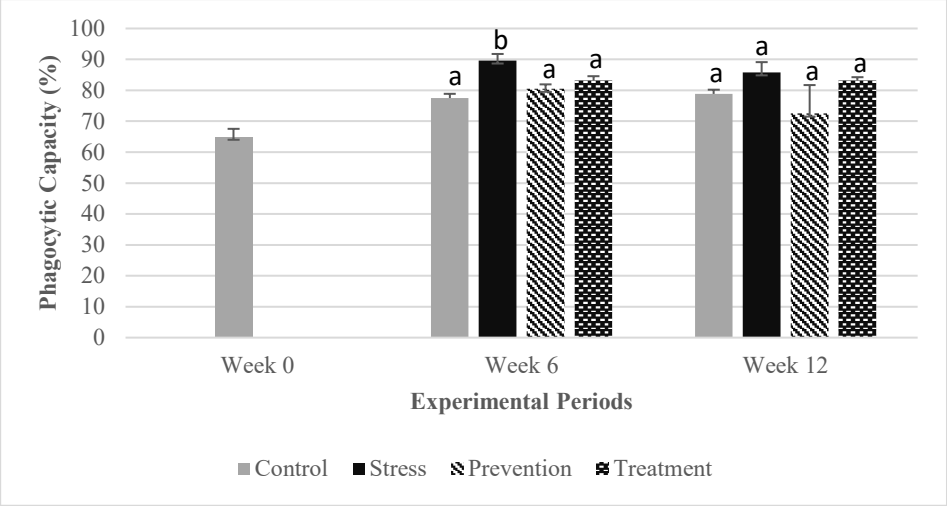

Figure 5.12 Phagocytic capacity in different experimental treatments in tilapia. Concentrations (mean ± SEM) are represented in groups: Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks). Different alphabets are significantly different than each other at a specific sampling period (p<0.05).

Lysozyme and phagocytic activities are humoral aspects of the innate immune system that target the peptidoglycan layers of Gram-positive bacteria's cell wall. They are affected by a variety of stresses [53]. In hybrid tilapia exposed to Cadmium, there was an increase in lysozyme activity and a decrease in other humoral immune components. Pollutants may enhance innate immunity while also reducing humoral mediated immunity, enabling pathogen susceptibility in pollutant stressed fish, according to the research [54]. The tertiary stress biomarker, lysozyme activity demonstrates the ability of blood lysozyme to clear out bacterial solutions. Clearer the solution, higher the amount of light that passes through it in comparison to the light emitted by the spectrophotometer. Therefore, higher the transmittance (T). In stressed conditions, organisms produce increased amounts of lysozyme to fight off any perceived infectious threat [55]. This gives a higher transmittance value. Such is seen in figure 5.11, where the stressed group had the highest transmittance value 2.02 T/ min, higher than the control group at 1.45 T/ min. This is indicative of stress. In another study it was demonstrated that handling stress in rainbow trout, *Oncorhynchus mykiss*, led to an increase in lysozyme activity of the fish [56]. Notably, at week 6, the prevention group showed reduced lysozyme activity than both stressed group which portray that GPE was able to reduce the lysozyme activity significantly from 2.02 to 1.5 T/ min; this is indicative of a reduction in stress. However, at week 12 with the introduced GPE treatment in treatment group reduced the lysozyme activity than stress. Phagocytic activities are humoral aspects of the innate immune system that target the peptidoglycan layers of Gram-positive bacteria's cell wall. They are affected by a variety of stresses [53]. Another study revealed that in common carp, cortisol induced mRNA quantity increase the phagocytic activity [57]. Parallel to lysozyme activity in phagocytic capacity there were no significant difference in groups. However, in week 6 stress groups (stress and treatment) had higher phagocytic capacity than control and prevention. With *Gynura* the treatment group had lower phagocytic capacity than the stress group and prevention group showed the lowest capacity. With this change alongside previous analysis I can conclude that the GPE was able to reduce the stress in Nile tilapia.

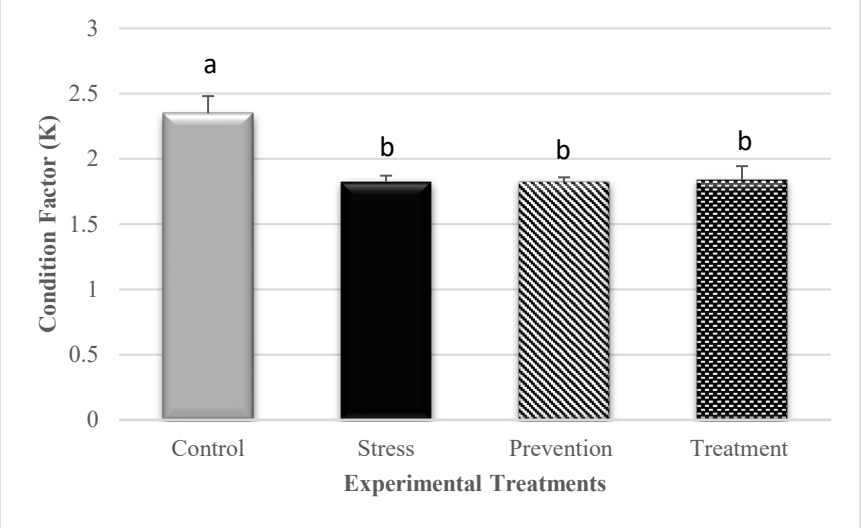

Figure 5.13 Condition factor (K) of Nile tilapia fed with four different treatments represented by Control= Feed without stress and treatment, Stress= Feed with stress but without treatment, Prevention= Feed with stress and treatment, and Treatment= Feed with stress but without treatment for first part of experiment (first six weeks) and feed with stress and *Gynura* treatment for the other half of the experiment (last six weeks) after 12 weeks of experiment. Results are presented as means ± SEM. Different alphabets are significantly different than each other (p <0.05).

The condition factor (K) of a fish reflects physical and biological circumstances and fluctuations by interaction among feeding conditions, parasitic infections and physiological factors. This also indicates the changes in food reserves and therefore an indicator of the general fish condition. Above 1 is considered as condition factor [33]. The specific growth rate period is defined as the rate of increase of biomass of a cell population per unit of biomass concentration [58]. In my study the growth parameters as Fulton's condition factor (Figure 5.13) of tilapia showed significant differences between the treatments (n=6; F= 8.096; p=0.001). Control group was significantly different from stress, prevention and treatment group with P value 0.003, 0.004, and 0.003 accordingly. However, with externally induced chemical stress all the fish group had condition factor higher than 1.5, an indicator of good health in terms of weight to length ratio. Considering all the factors, I can conclude from the condition factor results that *Gynura* is beneficial to overall health because they were able to maintain a good condition factor despite being subjected to chemical stress.

5.5 Conclusion

Aquaculture's role in providing a healthy, lean protein source for the world's growing population is essential. Stress in fish is a considerable issue due to the extensive husbandry methods used in aquaculture. Crowding, handling, and vaccinating are common husbandry methods that can stress the fish, resulting in decreased productivity. Farmers frequently use antibiotics and chemical drugs to alleviate the stress response. When antibiotics and other chemical drugs are released into the environment, they can create superbugs or harm non-target species. Antibiotic and other chemical exposure has the potential to harm human health if the chemicals remain in the fish after consumption. We need to find a solution to the stress problem in the aquaculture industry that is not harmful to the animals, consumers, or the environment. Based on our findings, *Gynura procumbens* may be a more environmentally friendly and cost-effective alternative to using commercial drugs to reduce stress in fish. All thing together, *G. procumbens*, as a preventative medicine, has the potential to reduce stress and improve immunity in fish without affecting growth and metabolic parameters. Since this is the first chronic stress study with *G.* procumbens on fish, additional research with this plant could be beneficial in stress medication in aquatic organisms.

5.6 References

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CHAPTER 6. OVERALL CONCLUSION

Nature provides a plethora of plants that serve as important source of traditional medicines that can be used to treat a variety of diseases. Because of their increased abundance and lower cost, they have enormous potential. Despite the fact that many of the medicinal properties of this plant are known, *G. procumbens* is one of many species that have yet to be discovered. I investigated the plant's microbiological, immunological, and physiological effects. Using an antimicrobial assay, we intended to inhibit the growth of several pathogenic bacteria. With the presence of a zone of inhibition, an ethanolic extract of *Gynura* demonstrated sensitivity against *E. feacalis* and *P. aeruginosa* in the well diffusion method. *Gynura* in ethanol extract demonstrated significant sensitivity against *S. aureus* and *S. epidermidis* in the disc diffusion method. *Gynura* in methanol extract has also demonstrated significant sensitivity to *B. subtilis* and *S. aureus*. Based on mjy microbiological study I can summarize that *Gynura* is more effective against gram positive bacteria than gram negative bacteria.

To investigate *Gynura's* immunological effects, I examined the effect of different dilutions of *G. procumbens* ethanolic and aqueous extracts on the inhibition of a melanoma cell line. Furthermore, I evaluated the immune stimulating potential of *G. procumbens* in the presence of a specific mitogen to stimulate the immune cells. In *in-vitro* analysis, found that a 4 g/mL and 0.8 g/mL of ethanolic extract significantly inhibited melanoma cell growth. However, there was no significant proliferation or inhibition in spleen cell proliferation assays with 25% ethanol or aqueous extract of *Gynura.* The 4 g/mL and 0.8 g/mL concentration of *Gynura* for inhibiting melanoma cell proliferation did not interfere with T or B cell growth. As a result, this plant may be useful in melanoma therapy. The other concentrations of *Gynura* 0.16 g/mL, 0.032 g/mL and 0.062 g/mL with both ethanol and methanol were able to inhibit the T cell proliferation. Thus, with such effect it has potential to use as transplant rejection, asthma, and a variety of other immune diseases. Extensive research is required to identify the active compounds which are responsible for these inhibitory effects. It is also clear from acute and chronic stress-physiology studies that extracts of *G. procumbens* contain active principles with anti-hyperglycemic properties showing reductions of blood glucose in stressed fish *in-vivo*. These results suggest that the plant may be able to reduce stress in the fish population when propagated in farms. Given the above-mentioned potentials, *G. procumbens* should be thoroughly investigated to determine its nutritional repercussions.