Evaluation of Adaptogenic Activity of Dietary (Bitter Melon) *Momordica charantia* Seed Powder on (Nile tilapia) *Oreochromis niloticus*

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A thesis submitted in partial fulfillment of the requirements for the Degree of

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IN

FISHERIES AND AQUACULTURE

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Declaration

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

Andleeb Ashraf

Dedication

This thesis is dedicated to my affectionate parents, supportive and encouraging siblings especially my eledest sister Ayesha Ashraf whose advice, unflagging support, and faith in me have been priceless.

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List of Abbreviations

°C	Celsius
µL	Microliter
µM	Micromolar
AH	Altered hepatocytes
ALT	Alanine transaminase
ANOVA	Analysis of variance
AST	Aspartate transaminase
BGS	Bitter gourd seed
C	control
CAT	Catalase
CMC	Carboxy methylcellulase
CP	Crude protein
DEPC	Diethyl Pyrocarbonate
DO	Dissolved oxygen
EAA	essential amino acids
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and agriculture organization
FBW	Final body weight
g	Gram
g/dl	Grams per deciliter
g/ml	Gram per milliliter
gKg^{-1}	Grams per kilogram
H	Hepatocytes
H_2O	Water
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HCT	Hematocrit
HFD	High fat diet
HN	Hepatocyte nucleus
Hp	Hyperplasia

hr	Hour
HT	Hypertrophy
IBW	Initial body weight
Ig M	Immunoglobulin
ISS	Increased sinusoidal space
Kg	Kilogram
L	Liter
LPO	Lipid peroxidation
LSD	Least significant difference
M	Molarity
M cm	Molar coefficient
m/v	Mass per volume
MC	Momordia charantia
Mg	Milligram
mg/L	Milligram per liter
Min	Minute
Mm	Millimeter
mM	Millimolar
MS-222	tricaine methanesulfonate
MT	Matric tonn
N	Necrosis
$NADH^+$	Nicotinamide adenine dinucleotide hydrogen
NBT	Nitro blue tetrazolium
NCBI	National center for biotechnology information.
ND	Hepatocyte nuclear degeneration
ND.	Nanodrop
Nm	Nanometer
nmol	Nanomole
O_2	Oxygen
P	Probability
pg	picogram
pH	Power of hydrogen

POD	Peroxidase
PUFA	polyunsaturated fatty acids
RNA	Ribonucleic acid
ROS	Reactive oxygen specie
rpm	Revolution per minute
SE	Standard error
SEM	Standard error mean
SOD	Superoxide dismutase
SST	Serum separating tubes
TBARS	Thiobarbituric acid reactive substances
U/L	Units per liter

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In the name of Allah who is the most Beneficent and the most Merciful. All praises to Almighty Allah, the creator of universe. I bear witness that Holy Prophet Muhammad (SAW) is the messenger, whose life is a perfect model for the whole mankind till the Day of Judgment. Allah blessed me with knowledge related to earth. Allah enabled me to complete my work. Without the blessings of Allah, I would not be able to complete my work and to be at such a place.

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ABSTRACT

The primary objective of this investigation was to explore the potential relationship between adaptogenic constituents inherent to dietary *Momordica charantia* (bitter gourd) seeds and their ameliorative role during an increase in cortisol, a biomarker indicative of stress in *Oreochromis niloticus* (Nile tilapia). *M. charantia* plant is used for medicine and has substantial pharmacological effects due to its bio-actives and phytochemical composition and also an adaptogens source. Six discrete dietary protocols were implemented. One functioned as the control group (denoted as C), while the remaining five involved the supplementation of MC seed powder at levels of 0.2, 0.4, 0.6, 0.8, and 1 g per 100g feed (g/100g). Among the groups, one showing the most substantial growth response (S10) was chosen for confinement stress protocol incorporation. Fish from both the C and the S10 group were exposed to a 1-hour confinement stressor, to evaluate the potential adaptogenic or anti-stress properties of MC seed in mitigating the stress-induced alterations in plasma cortisol levels and hepatic antioxidant activity level, since various oxidative processes are known to be involved in stress. The fish were subsequently allocated certain release times, including 0 (with immediate sampling), 0.5, 1, 2, 4, 8, 12, 24, and 48 hours. Then the fish were dissected for further examination after the end of their respective recovery periods. We employed an instantaneous sampling protocol to investigate the impact of a 1-hour confinement stress. This stress resulted in more elevation of plasma cortisol levels in C group but significantly ($P< 0.001$) less in S10 group fish treated with MC seed powder in diet, along with this significantly (0.001) enhanced activity levels of hepatic antioxidant enzymes(SOD, POD, CAT) and decrease in LPO level in S10 fish. Histological study also indicate pronounced protective effect on *O.niloticus* spleen fed with MC seed supplemented feeds. For *O. niloticus*, dietary MC seed supplementation can be advised for its adaptogenic and boosting immunostimulatory defense.

INTRODUCTION

According to UN (2019), estimations, the population of the globe will be around 8.5 billion by 2030 and 9.7 billion by 2050. Malnutrition on one hand, is a prevalent global challenge (Golden *et al.,* 2021), feeding more than 9 billion people will be a significant issue by 2050. Food must be produced in methods that ensure a sustainable supply (Grafton *et al.,* 2015). Fish, a significant protein and essential oil source, is often underestimated in meeting future food needs, despite its potential for substantial growth in farmed fish production (Béné et al., 2015).

Fisheries and Aquaculture as an advancing sustainable food source

A major source of food is provided by fisheries, especially aquaculture (FAO, 2021). Fish is a nutritious food with minimal levels of cholesterol, carbohydrates, and saturated fats. It is a good source of high-quality protein and includes many vital micronutrients (Thilsted *et al.,* 2016), including essential amino acids (EAA) and polyunsaturated fatty acids (PUFAs) vitamins, and minerals (Maulu et al., 2021). Nearly 20% of the average per capita animal protein consumption of nearly 3.3 billion people came from fish (FAO, 2020). Between 1961 and 2017, the average annual growth rate of global fish consumption was 3.1 percent, above the yearly growth rate of 1.6 percent of the human population (FAO, 2020). With an emphasis on supplying the growing population with high-quality protein, aquaculture is a rapidly growing sector (Herforth *et al.,* 2020). More than half of the fish used for human consumption are now raised in aquaculture worldwide, surpassing capture fisheries in under four decades (FAO, 2019; Houston *et al.,* 2020). In order to increase fish production rates, a number of global projects and initiatives have been launched, including the Blue Revolution, the State of World Fisheries and Aquaculture, intensive aquaculture, the use of specific metabolic boosters and growth enhancers, and others (FAO, 2022; Belton *et al.,* 2020; Jiang *et al.,* 2022). When catch fisheries are unable to meet demand, farmed fish, in particular, can help stabilize overall supplies of fish and fish products and provide safe food that is less impacted by pollution (Tacon *et al.,* 2020).

As the world's human population continues to increase, global capture fisheries harvest has stabilized and remained constant at approximately 90 million tons since the 1990s. Aquaculture is already the primary source of aquatic food production and is expected to become an even more significant and necessary part of the global food system in the future. Aquaculture

is currently providing the world with high-quality and affordable aquatic food for human consumption (FAO, 2020a). According to (Metian *et al.,* 2019), the most versatile food-producing industry is aquaculture.

Dominant aquaculture food species

According to FAO's (2020) report, from 2006 to 2018, there was a 31.8 percent increase in the total number of commercially produced aquaculture species items, with production rising from 472 MT in 2006 to 622 MT in 2018 (FAO, 2020). Three seaweeds, four freshwater fish (grass carp, silver carp, Nile tilapia, and common carp), two molluscs, and one crustacean are among the ten most produced ASFIS list of aquatic species (Aquatic Sciences and Fisheries Information System) species in 2017. Nile tilapia and common carp were the two most widely farmed species among these top 10 species in 2017, and they were followed by two carp species: grass carp and silver carp, which were farmed in 38 and 37 nations, respectively (Cai *et al.,* 2019). Seaweeds, carps, bivalves, tilapia, and catfish were among the major species groupings that contributed to the top 75% of aquaculture production in 2017, according to (Naylor *et al.,* 2021). According to (Fitzsimmons *et al.,* 2011), tilapia will eventually surpass carp as the most significant farmed fish crop. According to (Fisheries, F. A. O. 2011), the Nile tilapia *Oreochromis niloticus* is one of the most significant commercial fishes in the world, with annual production globally leading to more than 2.6 million metric tons in 2014.

Tilapia status

Tilapia is the second most important farmed fish in the world, after carps (El-Sayed, 2002). The family Cichlidae of fishes is collectively known as tilapia. There are 1524 species of tilapia (Eli, 2004). The Nile tilapia, *O. niloticus*, the Mozambique tilapia, *O. mossambicus*, the blue tilapia, *O. aureus*, and *O. urolepis hornorum* are among the species of the genus *Oreochromis* that are crucial for aquaculture. With farms opening and expanding all over the world and demand surpassing even the most ambitious plans for farm construction, tilapia has established itself as aquaculture's brightest star and has earned the name of aquatic chicken. The production of farmed tilapia surpassed 3.2 million metric tons yearly in 2010, surpassing that of the salmon and catfish industries, due to its exceptional nutritional and commercial value, rapid growth rates, tolerance to poor water quality, disease resistance, efficient feed conversion, and high consumer

acceptance, O. niloticus (Nile tilapia) has been the primary focus of numerous intensive breeding initiatives (Fitzsimmons et al., 2011; Barcellos et al., 1999; Figuereido-Fernandes et al., 2006). Furthermore, it serves as a significant fish model for investigating stress responses (Barreto & Volpato, 2004).

Constraints in aquaculture

The introduction of stress from various sources is a significant barrier for the aquaculture industry (Oliva-Teles, 2012). Common stresses that include physical and emotional suffering related to capture, transportation, handling, and confinement are regularly faced by captive fish. Malnutrition, changes in water parameters like temperature, oxygen content, and salinity, as well as side effects brought on by contact with pollutants or infectious diseases are all included in this list of stressors (Harper & Wolf, 2009). Fish experience increased stress in situations of intensive culture (crowding or confinement) as a result of a combination of environmental conditions (including water quality and hypoxia) as well as health-related problems (such as parasite and infectious disease vulnerability) This leads to a higher occurrence of diseases.(Montero, Izquierdo, Tort, Robaina & Vergara 1999) and poor growth (Abou, Fiogbe & Micha 2007). Notably, stress susceptibility consistently remains elevated under conditions of overcrowding (Oliva-Teles, 2012).

Physiological stress and its response

According to Iwama *et al. (*2004), stress is an adaptive physiological, biochemical, and behavioral reaction to a stimulus. In the realms of vertebrates, including fish, typical physiological reaction to challenging circumstances is commonly known as stress. This stress response is set on instantaneously upon detecting a stress-inducing stimulus (Schrek & Tort, 2016). The three main types of stress reactions are primary (changes in the levels of the hormones cortisol and catecholamines in the bloodstream) (Barton, 2002). (According to (Pawar & Shivakumar, 2012) continual stimulation of these stress hormone has potential to lead to the deterioration of vital organ), secondary (metabolic changes to meet higher energy demands, such as changes in blood glucose), and tertiary (which affect growth, the condition of reproductive capacity, disease resistance, and survival) (Barton, 2002). According to (Braithwaite & Ebbesson, 2014), stress is a coordinated set of physiological and behavioral reactions to any

perceived threat to homeostasis or allostasis. Because fish and other aquatic creature's homeostatic processes are strongly dependent on the environmental conditions in their immediate surroundings, they are vulnerable to a wide range of stresses (Harper & Wolf, 2009).

Physiological stress in intensive fish culture; Mechanism and its mitigation strategies

The expanding aquaculture sector necessitates increased emphasis on fish welfare, as it significantly affects stress responses, health, and disease resistance, ultimately shaping the sector's sustainability (Ashley, 2007). Stress events in intensive fish farming, including stocking density, management and environmental changes, can negatively impact fish growth due to typical stress response (Barton & Iwama., 1991). So, Fish must be provided with enough diets that satisfy all of their nutritional needs for sufficient growth and resistance to stress and illness problems (Trichet, 2010). Diets significantly impact stress tolerance and health, with overly rich diets enhancing health and illness resistance. Functional ingredients like probiotics, prebiotics, and immunostimulants enhance fish diets (Oliva-Teles, 2012). Brekhman and Dardymov discovered adaptogens, plant-derived agents that increase non-specific resistance to stress, offering a solution to the significant therapeutic issue of stress disorders.(Pawar & Shivakumar, 2012). Therefore since ancient times, some adaptogenic plants have been utilized in Ayurveda and traditional Chinese medicine to increase longevity, improve physical and mental health, and strengthen the body's defenses in humans and animlas (Panossian, 2017).

Adaptogens

The Soviet scientist Lazarev coined the term "adaptogen" in 1947, making it comparatively recent. Adaptogens are compounds that cause non-specific resistance in living things, according to this definition both people and animals benefit from adaptogens (Todorova *et al.,* 2021). Adaptogens are generally referred as the medicinal plants, nutrients, and phytochemicals that non-specifically increase resistance, adaptation to challenging conditions, and chances of survival by activation of signaling pathways in affected cells (Panossian et al., 2021). By enhancing an organism's capacity for adaptation and survival, adaptogens are stress-response moderators that raise an organism's non-specific resistance to stress (Panossian, 2017). Herbs or plant-derived adaptogens (Esmaealzadeh *et al.,* 2022) possess a diverse array of phytochemical components (Todorova *et al.,* 2021) and must meet four requirements in order to

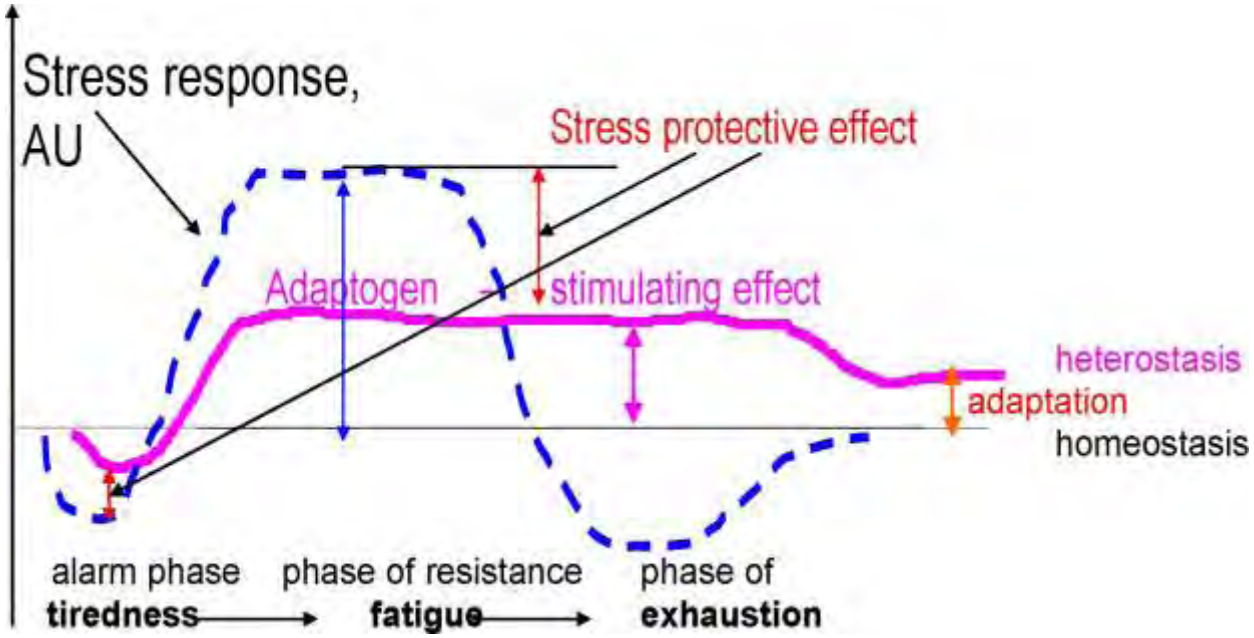
be useful to stressed organisms. First, it has a protective impact against infections, tiredness, and depression all stressful circumstances. Second, they ought not to disrupt body's regular processes in any way. Thirdly, adaptogens are anticipated to have some excitatory effects. Finally, the excitatory impact should be advantageous; undesirable side effects include increased energy expenditure or sleeplessness is not considered favorable (Liao *et al.,* 2018). Some of the most important phytochemicals with adaptogenic properties are: triterpenoid, saponins, phytosterols and ecdysone, lignans, alkaloids, flavonoids, vitamins, etc (Todorova *et al.,* 2021). Ancient literature reports potent antistress activity in herbs, which are gaining popularity for their adaptogenic properties and being investigated for various disorders (Ernst, 1998).

Mode of action

The mechanism of action of plant adaptogens is complicated and still not entirely understood (Todorova *et al.,* 2021). One notable trait of adaptogens is their ability to function as eustressors, often referred to as "beneficial stressors" and as mild stress mimics or "stress vaccines" that trigger stress-protective responses (Panossian, 2017). Since plant-derived adaptogens can both prevent and fight stress these chemicals can stimulate the release of cortisol and nitric oxide in the plasma, enabling the body to adapt to greater loads. Exercises(stress) that are performed after taking plant-derived adaptogens do not result in a rise in the levels of cortisol and NO in the body; rather, the levels fall relative to those that existed before the exercise (Liao *et al*., 2018).

Adaptogens modify the body's response to stress through endocrine, immunological, and neurological systems, affecting the hypothalamic/pituitary/adrenal axis (HPA), sympatho-adrenal system, and metabolic regulators like cytokines and stress hormones (Pawar & Shivakumar, 2012). Since fish lacks adrenal glands, the interrenal cells(HPI) and chromaffin tissues, which are both generally found in the piscine anterior kidney, produce and release adrenal cortex and medullary hormones in comparable ways (Harper & Wolf, 2009). Adaptogens work by enhancing the sensitivity of hypothalamic and peripheral receptors to cortisol and other adrenal hormones. This enables the body to initiate an effective stress response with reduced levels of cortisol compared to what would typically be necessary, as explained by (Dean, 2001).

Adaptogens lengthen the phase of resistance (stimulatory effect), enhance the state of non-specific resistance to stress and increase sensitivity of hypothalamic and peripheral receptors to cortisol and other adrenal hormones (Dean, 2001), and decrease sensitivity to stressors, resulting in stress protection. This enables the body to initiate an effective stress response with reduced levels of cortisol compared to what would typically be necessary, as explained by (Dean, 2001). Rather than exhaustion a greater level of equilibrium (the homeostasis) is achieved. The better the adaptation to stress, the higher it is. As a result, both in humans and in animals, adaptogens have been shown to have stimulating and anti-fatigue effects. (Explained with Figure: 1).



(Panossian & Wikman, 2010).

Adaptogenic plants

According to a literature review, the plants listed in (Panossian *et al.,* 2021; Pawar & Shivakumar, 2012) show adaptogenic (anti-stress) properties and used in traditional medicinal systems as rejuvenating medicinal plants (Panossian *et al.,* 2021). One such plant that has been utilized for medical purposes frequently is Momordica charantia (MC) (Grover & Yadav, 2014), and we have studied its adaptogenic effect in this research.

Momordica charantia

Momordica charantia (Karela) commonly known as Bitter melon or Bitter gourd is tropical and subtropical climber of the family Cucurbitaceae (Taylor, 2002). The generic name "*Momordica*" comes from Latin, meaning "to bite", which refers to its leaf with serrated edges that look as if it has been bitten (Subratty, A. H *et al.,* 2005). In many countries and regions, *M. charantia* also has been used as herbal medicine. The whole plant, especially the seeds and fruit, have significant pharmacological effects. Phytochemicals including proteins, polysaccharides, flavonoids and phenols Wu & Ng. (2008) in Mc extract., triterpenes, saponins, (Grover & Yadav, 2004), momorchins, momordinol, momordicins, charantin, cucurbitacins, diosgenin, goyaglycosides, and goyasaponins, among others (Xie et al., 1998) ascorbic acid and steroids have been found in this plant. Various biological activities of *M. charantia* have been reported, such as immunomodulation, antioxidant, hepatoprotective, antidiabetic, (Basch et al., 2003) anti-hyperglycemic, antibacterial, antiviral, antitumor, antiulcer, anti-inflammatory anthelmintic, antimutagenic, antilipolytic, adipogenesis-reducing antifertility and anticancer (Jia *et al.,* 2017).

Bitter melon has been used in various Asian traditional medicine systems for a long time (Kumar *et al.,* 2010). Momordica charantia glycosides, saponins, triterpines, steroids, vitamin C and A, as well as phytochemicals like momorchins, momordinol, momordicins, charantin, cucurbitacins, diosgenin, goyaglycosides, and goyasaponins, among others (Xie *et al.,* 1998) and the presence contains of flavonoid and phenolic phytochemicals in MC extracts Wu & Ng. (2008).

Since the Nile tilapia has higher requirement in global market, it is reared in intensive cultures (at higher stocking densities). So, we chose it as a test subject to see how well we could manage the stress generated in fish while in intensive culture. To enhance tilapia stress tolerance,

the incorporation of bitter gourd (*Momordica charantia*) seed powder into the diet is opted for its potential adaptogenic properties. This study aims to investigate the adaptogenic effects of bitter gourd seed powder on fish subjected to confinement stress, mimicking the elevated stocking densities observed in intensive aquaculture systems."

Hypothesis

We hypothesized that *O. niloticus'* ability to tolerate stress may be improved by adding *M. charantia* (bitter gourd) seed powder to dietary supplements because bitter gourd seeds are source of adaptogens.

Aim and objectives

This study aims to investigate the adaptogenic effect and anti-oxidant potential of Momordica charantia (MC) seed powder on stress-induced cortisol alterations in tilapia fingerlings.

Following objectives are established to investigate the aim of adaptogenic or stress-resilient properties of bitter gourd seed powder on *O. niloticus* fingerlings:

- To access the growth performance.
- Hematological indices (RBCs, WBCs, Hb) and hematocrit.
- RBC related indices (MCV, MCH, MCHC)
- MC seed antioxidant (SOD, POD, CAT) capacity in stressed fish
- Attenuating effect of MC seed on hepatic lipid peroxidation (LPO).
- To measure and compare cortisol level in fish fed with basal and bitter gourd supplemented diet and
- To evaluate the effect of MC seed powder on spleen morphology.

MATERIALS AND METHODS

Procurement of *Momordica charantia* seed (MC seed)

Ripe fruits of a local variety (from district Swat) were procured in the month of April from the nearby vegetable market. The fruits were sliced in two halves; seeds were manually extracted from the fruits and underwent thorough washing using water to ensure complete elimination of any plant residues or foreign matter. Afterward, the seeds were air-dried in shade at room temperature/in an electric oven (SANFA DHG-9053A) on stainless steel trays until moisture-free seeds were attained. Once dried, the seeds were stored in air-tight jars for future utilization in experiment.

Feed preparation

The seeds of *Momordica charantia*, an air dried bitter gourd, were ground into a powder using a standard household electric grinder (MXBAOHENG Model HC-500). A predetermined ratio of all the dried ingredients and bitter melon seed powder (as shown in Table 1) were properly mixed to provide a 35% protein basal diet for fingerling tilapia *(Oreochromis niloticus).* Fish oil was then added. Water was added to this combination, and the resulting dough was processed through a feed mill to make pellets. After being dried in an oven (SANFA DHG-9053A) at 60 ^{o}C for 24 hours, feed pellets were then placed in storage to be used on a daily basis. Fresh feed was prepared each week.

Supplementation

35% CP supplemented feed with bitter gourd seed powder as additive were as;

Group 1 (C): The fish were fed with controlled basal diet

Group 2 (S2): The fish were fed with 2 g kg^{-1} MC seed powder supplemented diet

Group 3 (S 4): The fish were fed with 4 g kg^{-1} MC seed powder supplemented diet

Group 4 (S 6): The fish were fed with 6 g kg^{-1} MC seed powder supplemented diet

Group 5 (S 8): The fish were fed with 8 g kg^{-1} MC seed powder supplemented diet

Group 6 (S 10): The fish were fed with 10 g kg^{-1} MC seed powder supplemented diet

Fish procurement and Acclimatization

In May, around 200 tilapia (*Oreochromis niloticus*) fingerlings were transported from the National Agriculture Research Centre (NARC) to the Fisheries and Aquaculture Research Facility at Quaid-i-Azam University Islamabad. The fingerlings were carefully packed in leak-proof polyethylene bags containing a controlled mixture of one-third water and two-thirds air, to ensure sufficient dissolved oxygen levels during the 30-minute transportation period. Before fish transfer, the raceways were thoroughly cleaned and limed to prevent any potential infection and to create a suitable environment for the tilapia fingerlings. Upon arrival, the tilapia fingerlings were gently placed in cemented raceways and subjected to acclimatization period of at least two weeks. Throughout this acclimatization phase, the water quality parameters were regularly monitored and were within optimal ranges to facilitate their physiological adjustment to the new environment. After the acclimatization period, uniform-sized fingerlings were transferred to fiber tanks and chosen randomly for 80-day experimental feeding trial. It involved closely observing and recording the growth performance, feed efficiency, and other relevant parameters to assess the impact of the experimental diet on the tilapia fingerlings' growth and overall health.

Experimental design

Experiment 1

Before being transferred into outdoor fiber tanks (30 fish per tank; 10.03±0.3g, stocking density of 1g/L) and after acclimatization fish were given precautionary $KMnO_4$ bath before the experiment was started. The rearing and experiments were performed in duplicate (by placing separator in tanks) in semi-static conditions in tanks (supplied with oxygenated water from reservoir), under naturally occurring temperature and photo-period conditions observed in Islamabad, Pakistan during the months of May–July. This consisted of daily light-dark cycle of 13 hr light: 11 hr darkness (mean temperature: 29-31°C). All tanks were placed in same vicinity, to ensure consisted environmental conditions to all fish. . The fish were fed experimental feed twice daily, with each feeding amount given, being 3% of their body weight

Experiment 2: For confinement stress

After completion of 80 days feeding trial, 15 fish each from control and best (S-10) treatment group were shifted from tank to 6 glass aquarium (60 x 30 x 30) at stocking density of 1g/L (5 fish in each aquarium)for confinement stress protocol. For pre-stress (without confinement stress) analysis fish only from controlled group was sampled. Stress was induced by placing 4 fish, 2 from each control and treatment group for period of 1 hr in 2 borosilicate-glass beakers (2 fish/beaker) of 2L capacity separately (labeled as control and treated), both were filed with 1L of water (half the capacity of beaker)and sampled immediately giving no release time(0 hr). Next 4 fish, 2 from each group were confinement-stressed in 2 beakers for 1 hr, and then released back in other separate aquariums assigned to control and treated fish for period of 30min. After release period of 30 min all fish were anesthetized and sampled. Every time 4 fish, both from control and treatment group were stressed for 1 hr and then sampled after completion of their respective release time(post-stress) of 0, 1, 2, 4, 8, 12, 24, and 48 hr. (Figure 2)

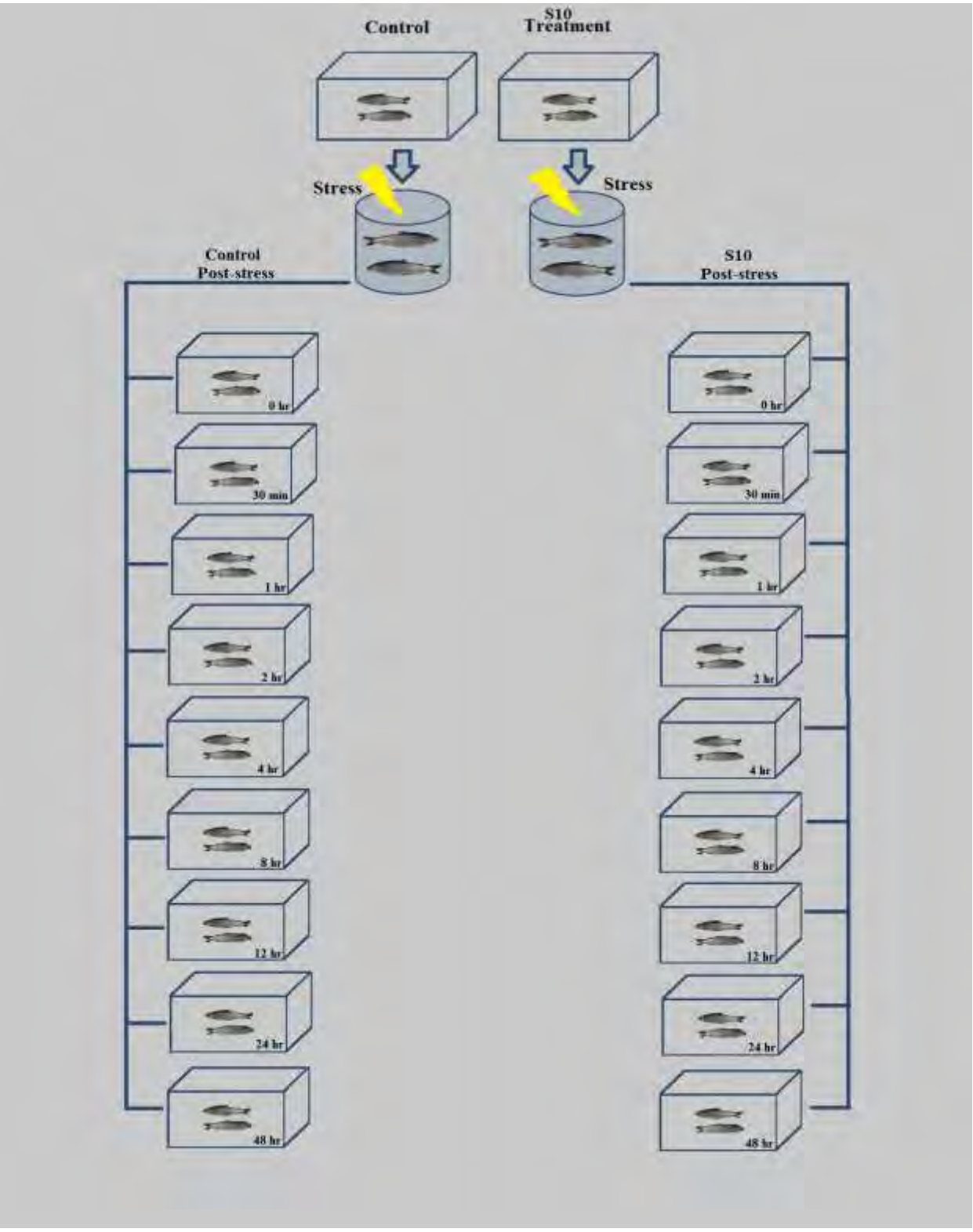


Figure 2: Sampling and handling protocol for experiment 2 (confinement stress). 2 fish each from control and S10 group were kept in 2L beakers filled with 1L water for duration of 1hr. After stress period fish were moved back into aquarims , and were sampled at 0, 1, 2, 4, 8, 12, 24, 48 hr post stress.

Proximate analysis

In partnership with the Pakistan Poultry Research Institute (PPRI) in Islamabad, a proximate analysis was carried out on the muscle ash content, crude fats, and crude protein of Nile tilapia fingerlings, employing standard methods (AOAC, 2000). The measurement of crude fats and protein was conducted using the Soxhlet apparatus and the micro Kjeldahl technique, respectively (Sutharshiny & Sivashanthini, 2011).

Growth Performance

After 90 days of feeding, fish from each fiber tank were captured and quickly weighed using weighing balance. Growth performance and other nutritional parameters [weight gain (WG), specific growth rate (SGR) and FCR] were calculated using formula.

Weight gain (g) = Wf -Wi

WG% = Wf – Wi /Wi × 100

SGR% = *ln* Wf - *ln* Wi / Number of days of experiment ×100

FCR= Total feed consumed (g) / Total wet weight gain (g)

(Where **Wf** is Final Body Weight, **Wi** is Initial Body Weight, **SGR** is Specific Growth Rate & **FCR** is Feed Conversion Ratio)

Sample collection

At the end of the trial, to mitigate the stress response associated with sampling, the fish were anesthetized using tricaine methanesulfonate (MS-222) at a concentration of 100 mg/liter, with an induction time of 2 minutes at 100 mg/L. Prior to any sampling procedures, individual fish (one fish per operator) were anesthetized to minimize the potential effects of handling stress. Blood samples were obtained by puncturing the caudal sinus with a 1-ml plastic syringe and subsequently transferred to 1.5 ml VACUETTE® EDTA tubes containing lithium heparin as the anticoagulant for hematological analyses. Additionally, blood was collected in SST tubes for serum extraction.

Liver and spleen samples from each stress group were rapidly dissected and preserved in liquid nitrogen for antioxidant studies and 10% formalin for histological examinations, respectively. After chilling the samples in a refrigerator for six hours, they were centrifuged at 1500 rpm at 4°C for 10 minutes. The resulting supernatant was collected and stored at -80°C for subsequent biochemical parameter measurements. For liver tissue, 0.1 g was taken and homogenized in 0.9 mL of 0.65% normal saline to create a 10% tissue homogenate. Following centrifugation at 4000 rpm for 10 minutes at 4°C, the supernatant was collected and stored at -80°C. Total protein content in each liver sample was determined for the assessment of enzyme activity.

Hematological Parameters

Blood samples were obtained from the caudal vein of both C and S10 groups of *O.niloticus* using 1ml heparinized syringes. These samples were then collected in EDTA VACUETTE® K3 tubes and subsequently analyzed using an automatic analyzer to determine various hematological parameters, including RBC count, WBC count, hematocrit, hemoglobin levels, mean corpuscular volume, mean corpuscular hemoglobin concentration, and mean corpuscular hemoglobin. The blood from nine fish (n=9) in each aquarium was combined to assess the complete hematological indices.

Serum stress-related Parameters

A commercially available ELISA kit (CO368S, CALBIOTECH, delivered by General Scientific Traders, Pakistan). All samples were analyzed in duplicate. On the day of analysis, both reagents and samples were thawed and allowed to reach room temperature. The reagents were gently shaken on vortex for 15 seconds before use. A total of 25µl of cortisol standards and samples were carefully pipetted and transferred to 96 wells on an ELISA plate. Subsequently, 200 µL of cortisol enzyme conjugate was added to each well and left to stand for 10 seconds. The solution was then incubated at room temperature for 60 minutes.

After the incubation period, the liquid from all wells was aspirated, and the ELISA plate was washed. This washing process was performed first by adding 300 µL of a 1X solution to

each well and then repeating it with washing buffer three times. Any remaining water droplets were removed by gently tapping the plate on absorbent paper.

Following the removal of excess water, 100 µL of TMB substrate was pipetted into each well and incubated at room temperature for 15 minutes. The enzyme reaction was halted by adding 50 µL of stop solution (1M-HCl) to each well. Subsequently, the plate was read at 450 nm using a plate reader (Microplate Reader; AMPPlots 496, AMEDA Labordianosik). The standard logarithmic curve was created by graphing the concentrations of cortisol standards on the horizontal axis against their corresponding absorbance values on the vertical axis. Subsequently, the concentrations of cortisol in serum samples were determined by referencing this curve.

The accuracy of cortisol concentration determined using the ELISA Kit was validated by ensuring that the slopes of curves produced from serial dilutions (0, 20, 40, 60, 80%) of samples with EIA buffer were consistent with the curve generated by cortisol standards (slope, 0.94; R2 = 0.999). To assess the precision of the kit (intra-assay), the coefficient of variance was computed from five repeated assays of two samples with distinct cortisol concentrations, yielding a value of 7.63%. The reproducibility of the kit (inter-assay, CV) was evaluated by comparing the results of three samples repeated in each assay, resulting in a value of 9.3%.

Antioxidants Enzymes Determination

To assess antioxidant enzymes in the liver, tissue samples were subjected to homogenization using a Dounce manual homogenizer (Sigma, Aldrich) in a 100 mmol potassium phosphate buffer containing 1 mmol EDTA. Following homogenization, the next step was centrifugation at 12,000×g for 30 minutes at 4°C. The resulting pellets were then thrown away, and the supernatant was cautiously collected. Subsequently, various aliquot were prepared in eppendorf tubes and stored at -20°C until the determination of antioxidant enzymes activity.

Superoxide Dismutase (SOD) Assay

A modified approach based on Kakkar et al. (1984) was used to measure the SOD activity. The reaction mixture consisted of 0.3 ml of supernatant, 1.2 mL of 0.052 mM sodium

pyrophosphate buffer (pH=7.0), and 0.1 mL of a 186 µM phenazine methosulphate solution. A 0.2 mL addition of a 780 M NADH+ solution started the reaction, and a 1 mL addition of glacial acetic acid brought it to an end after one minute. A UV-3100PC spectrophotometer was used to measure the absorbance of produced chromogen at a 560 nm wavelength. The amount of enzyme per milligram of protein that inhibits the quercetin oxidation reaction by 50% of its maximum inhibition is referred to as one unit of SOD. SOD activity is measured in moles per minute per milligram.

Per-oxidase (POD) assay

POD activity was assessed using the technique developed by Chance and Maehly in 1955, and then modified by Bibi in 2012. 2.5 ml of 50 mM PBS (pH 5.0), 0.3 ml of 40 mM H2O2, 0.1 ml of 20 mM Guaiacol, and 0.1 ml of supernatant were combined for the reaction. After 1 minute, the absorbance was measured at 470 nm with a spectrophotometer. POD activity was measured using a molar coefficient of 2.66 104 / M cm and expressed as nmol per minute per milligram of protein.

Catalase (CAT) Assay

The Chance and Maehly method (1955) was used to measure catalase activity. Supernatant, 2.5 ml of 50 mM phosphate buffer (pH 5.0), and 0.4 ml of 5.9 mM hydrogen peroxides were combined with 0.1 ml (100µl) of supernatant. After a 1-minute interval, the reaction mixture's absorbance was measured using a spectrophotometer calibrated to 240 nm wavelength. The CAT result was represented as nmol/ min/ mg protein using a molar coefficient of 43.6/ M cm.

Lipid peroxidation (LPO/ TBARS) Assay

Following the approach of Wright et al. (1981), LPO activity was evaluated. The following ingredients were combined to make 1.0 ml of the reaction mixture: 0.2 ml (200µl) of supernatant, 0.58 ml (580µl) of phosphate buffer (0.1 M, pH 7.4), 0.02 ml (20µl) of ferric chloride (100 mM), and 0.2 ml (200µl) of ascorbic acid (100 mM). After one hour at 37 degrees Celsius incubation in the water bath, the reaction was stopped by adding 1.0 ml of 10% trichloroacetic acid. The tubes were all heated for 20 minutes in a water bath after 1.0 ml of thio-

barbituric acid was added. The tubes were then centrifuged at 2500 g for 10 min. following being chilled in an ice bath. After a 1-minute break, a spectrophotometer was used to measure the absorbance at 535 nm. LPO activity was measured as nM TBARS/ min/ mg tissue at 37°C with a molar extinction coefficient of 1.56 x 105 / M cm.

Histological studies

Spleen samples of 6 fish were collected post-stress immediately and separated into two parts along the long axis. All samples were fixed in 10% formaldehy buffered with monobasic and dibasic sodium phosphate buffre (pH 7.2) for 24h, dehydrated in increasing cocn. of ethanol(50%, 70%, 80%, 90%, 100%), washed with 100% xylene and then embedded in paraffin wax. Each block was sliced into longitudinal sections, which were mounted on glass slides, rehydrated, and then stained with haematoxylin-eosin stains [H&E)] (Drury & Wallington, 1980). Slides were then examined using a digital light microscope and photographed using an AIPTEK digital camera.

Statistical analysis

Prior to conducting statistical analyses, all data underwent normalization. The results for growth performance, hematological indices, antioxidants, and cortisol levels were presented as mean ± SEM. Growth performance and hematological indices data, as well as antioxidant results, were subjected to statistical analysis using One-way ANOVA, followed by the LSD post hoc test, utilizing IBM SPSS Statistics 21 and Statistics Version 8.1 software.

Cortisol data, on the other hand, underwent analysis using paired two-tailed T-Test and ANOVA with a double factorial(for time and concentration comparison) in a complete randomized design, followed by post hoc Duncan's HSD, utilizing SPSS software. Statistical significance was defined as $P<0.05$. Graphs was generated using GraphPad Prism software version 8.0.2."

RESULTS

Weight gain

The inclusion of MC seed powder in the diets had a noteworthy impact on the growth performance of *O. niloticus* fingerlings, as evidenced in Table 3. Fish in each group, which initially had the same weight (mean ± SE body mass 10.03 ± 0.3 g), were weighed again. . The increase was determined by dividing the average total final weight by the average total initial weight of each group. Statistical analysis using One-way ANOVA demonstrated a significant influence of MC seed supplementation at various dosage levels on final body weight (FBW)($n=3$, $F_{5,12}=7044$; $P<0.001$), WG($n=3$ $F_{5,12}=145088$; $P<0.001$), %WG ($n=3$, $F_{5,12}=2.2$; $P<0.001$), %SGR ($n=3$, $F_{5,12}=620$; $P<0.001$) and FCR ($n=3$, $F_{5,12}=3594$; $P<0.001$) among all experimental groups of fish (Table 3).

Pair-wise comparisons of the results revealed that the group of fish supplemented with S10 exhibited the highest percentage of weight gain (%WG), followed by S8, S6, S4, and S2, with the lowest %WG observed in the control group of fish (C < S2 < S4 < S6 < S8 < S10).

Hematological Indices

The inclusion of MC seed powder in the diets had a significant impact on the hematological indices of *O. niloticu*s fingerlings. Statistical analysis, employing one-way ANOVA, revealed a notable effect of MC seed supplementation at various dosage levels on the RBC count ($n=9$, $F_{5,48}=17.6$; $P<0.05$), WBCs count ($n=9$, $F_{5,48}=412$; $P<0.001$), Hb ($n=9$, $F_{5,48}=19.1$; $P<0.001$), PCV%($n=9$, $F_{5,48}=6707$; $P<0.001$), MCV ($n=9$, $F_{5,48}=4508$; $P<0.001$), MCH ($n=9$, $F_{5,48}=16892$; $P<0.001$) and MCHC ($n=9$, $F_{5,48}=1550$; $P<0.001$) among all MC seed supplemented diet groups of fish. Pair-wise comparisons indicated that the hematological indices were highest in fish supplemented with S10, followed by those supplemented with S8, with the lowest values observed in the control group of fish (S10 > S8 > S6 > S4 > S2 >C)(Table 5).

Antioxidant activity

The inclusion of MC seed powder in the diets has shown a noteworthy impact on the hepatic antioxidant enzyme activity of *O. niloticus* fingerlings.Statistical analysis using One-way ANOVA revealed a significant effect ($P<0.05$) of MC seed supplementation at different dosage levels on key antioxidant enzymes, including SOD ($n=3$, $F_{5,12}=652$; $P<0.001$), POD ($n=3$, $F_{5,12}=3176$; $P<0.001$), CAT ($n=3$, $F_{5,12}=1195$; $P<0.001$), and LPO ($n=3$, $F_{5,12}=5928$; $P<0.001$). Pair-wise comparison showed significantly high SOD, POD, CAT and lowest LPO activity in S10 group of fish when compare to C group fish post stress which had comparatively low SOD, POD, CAT and high LPO activity as evidenced in Table 5.

Plasma Cortisol

In the present study, results showed significant variation in plasma cortisol level $[F_{1,36}]=1018.439$; $P=<0.001$ among groups control and treated S10, exhibiting the effect of supplementation. Further it was also noted that recovery time (hr) $[F_{8,36}]=7016.417$; $p=<0.001$ significant effect too. Plasma cortisol measurement in two fish group was done using micro-plate ELISA reader. Results showed a significant ($P<0.001$) elevation in plasma cortisol concentration of C group fish post stress, when compare to the basal (pre-stress) group. While MC seed pretreated fish (S10) showed significant ($P<0.001$) low levels of serum cortisol when compare to the C- group(given same stress of 1 hr confinement), S10 which suggest the positive impact of MC seed powder on cortisol release in fish in response to stress while releasing less cortisol hormone in treated group.

Table 1: Formulation of 35% CP feed for *O. niloticus* fingerlings supplemented with bitter melon (*Momordica charantia*) seed powder at different dosage levels

Ingredients	Control	S2	S4	S6	S8	S10
Fish	22	22	22	22	22	22
Soya bean meal	14.5	14.5	14.5	14.5	14.5	14.5
Rice polish	15	15	15	15	15	15
Wheat flour	14.5	14.3	14.1	13.9	13.7	13.5
Sunflower meal	10	10	10	10	10	10
Corn gluten	14.5	14.5	14.5	14.5	14.5	14.5
Fish oil	05	05	05	05	05	05
[a]Vitamin and mineral premix	02	02	02	02	02	02
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5
CMC	02	02	02	02	02	02
MC seed powder	00	0.2	0.4	0.6	0.8	1.0
Total	100	100	100	100	100	100

[a]Composition of the vitamin-mineral premix kg^{-1} diet: vitamin A: 500,000 UI, vitamin D3, 250,000 UI, vitamin E 5000 mg, vitamin K3, 500 mg, vitamin B1 1000 mg, vitamin B2: 1000 mg, vitamin B6: 1,000 mg, vitamin B12: 2000 mg, niacin: 2,500, folic acid: 500 mg, biotin: 10 mg, vitamin C 10,000 mg, choline: 100,000 mg, Inositol: 1000 mg: selenium: 30 mg, iron: 5000 mg, copper: 1000 mg, manganese: 5000 mg, zinc: 9000 mg, cobalt: 50 mg, iodine: 200 mg.
All ingredients were bought from local markets.

Table2: Proximate composition of basal and supplemented feeds fed to *O. niloticus* fingerlings

Proximate composition (%)	Control	S2	S4	S6	S8	S10
Crude protein	33.80	34.21	34.24	34.53	35.29	35.67
Crude lipid	12.21	12.32	12.50	12.52	12.97	12.98
Crude fiber	7.09	8.01	8.03	8.04	8.06	8.13
Total ash	12.70	12.94	13.23	13.26	13.27	13.28
Moisture	10.08	10.26	10.25	10.31	10.32	10.29
***M. charantia* seed(g)**	0	2	4	6	8	10

Control= *M. charantia* seed free diet. **S2** = (2g seed/kg diet), **S4=** (4g seed/kg diet), **S6=** (6g seed/kg diet), **S8=** (8g seed/kg diet), **S10**= (10g seed/kg diet).

.

Table 3: Growth performance of *Oreochromis niloticus* fingerlings after feeding trial of 90-days with basal & experimental diets supplemented with graded levels of *Momordica charantia* (bitter gourd) seed powder

Parameter	Control	S2	S4	S6	S8	S10	F value	P value
IBW	10.38±0.2	10.46±0.3	10.04±0.4	10.02±0.4	10.32±0.4	10.62±0.2	_____	____
FBW	19.4 ±0.4^{f}	21.1±0.5^{f}	222.5±0.4^{d}	25.0±0.2^{e}	30.7±0.5^{b}	33.8±0.3^{a}	7044	<0.001
WG	9.3±0.4^{f}	11.24±0.3^{e}	12.5±0.1^{b}	15.0±0.1^{e}	20.5±0.8^{b}	23.8±0.1^{a}	145088	<0.001
WG%	93.7±0.6^{f}	111.8±0.7^{e}	125.3±0.8^{d}	150.1±0.4^{e}	203.7±0.9^{b}	238.9±0.3^{a}	2.2	<0.001
SGR	0.40±0.03^{f}	0.48±0.01^{e}	0.55±0.01^{e}	0.65±0.02^{e}	0.84±0.05^{b}	0.96±0.02^{a}	620	<0.001
FCR	2.2±0.02^{a}	2.1±0.01^{b}	2.06±0.03^{e}	1.8±0.03^{d}	1.4±0.09^{e}	1.3±0.01^{f}	3594	<0.001

Data is presented as Mean ± SEM (n=3) One-way ANOVA followed by LSD post hoc test shows a comparison between growth performance of fish fed BGS (bitter gourd seed) powder supplements at five graded levels. Lowercase superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.001$).

Control= bitter gourd seed free diet. **S2** = (2g seed/kg diet), **S4=** (4g seed/kg diet), **S6=** (6g seed/kg diet), **S8=** (8g seed/kg diet), **S10**= (10g seed/kg diet).

IBW= Initial Body Weight, **FBW**= Final Body Weight, **WG**= Weight Gain, **WG%**= Weight Gain Percent, **SGR**= Specific Growth Rate, **ADWG**= Average Daily Weight Gain, **FCR**= Feed Conversion Ratio.

Table 4: Hematological parameters evaluation following 90-day feeding trial of *O. niloticus* with basal and *M. charantia* seed powder supplemented feeds

Group	C	S2	S4	S6	S8	S10	F value	P value
RBCs(10^6/ul)	1.81 ± 0.02^{d}	1.85 ± 0.04^{cd}	1.88 ± 0.06^{c}	1.93 ± 0.05^{b}	1.96 ± 0.03^{ab}	2.00 ± 0.08^{a}	17.6	<0.05
WBCs(10^3/ul)	3.30 ± 0.05^{f}	3.50 ± 003^{e}	3.70 ± 0.04^{d}	4.10 ± 0.01^{c}	4.24 ± 0.06^{b}	4.34 ± 0.02^{a}	412	<0.001
Hb(g/dl)	4.71 ± 0.08^{d}	4.75 ± 0.09^{cd}	$4.77\pm0.03b^{c}$	$4.78\pm0.07b^{c}$	4.80 ± 0.03^{b}	4.90 ± 0.04^{a}	19.1	<0.001
PCV%	27.61 ± 0.07^{f}	28.60 ± 0.04^{e}	29.84 ± 0.09^{d}	30.84 ± 0.03^{c}	31.78 ± 0.06^{b}	32.27 ± 0.03^{a}	6707	<0.001
MCV (um^3)	110.2 ± 0.02^{f}	115.5 ± 0.01^{e}	$122.2\pm0.0z^{d}$	127.4 ± 0.03^{c}	132.7 ± 0.03^{b}	134.0 ± 0.01^{a}	4508	<0.001
MCH (pg)	51.7 ± 0.09^{f}	56.4 ± 0.06^{e}	60.2 ± 0.04^{d}	64.5 ± 0.07^{c}	68.1 ± 0.01^{b}	69.2±0.05a	16892	<0.001
MCHC(g/dl)	33.5 ± 0.08^{f}	34.7 ± 0.07^{e}	36.5 ± 0.03^{d}	38.1 ± 0.06^{c}	40.1 ± 0.02^{b}	41.6 ± 0.01^{a}	1550	<0.001

The data in Table (4) is provided as Mean ± SE (n=9). One-way ANOVA and the post-hoc LSD test were employed to demonstrate the pairwise comparison between groups. The difference was significant (P=<0.001) for the lower-case superscript. **C (Control) =** MC seed free diet. **S2=** (2g seed/kg diet), **S4=** (4g seed/kg diet), **S6=** (6g seed/kg diet), **S8=** (8g seed/kg diet), **S10=** (10g seed/kg diet).

RBC (Red Blood Cell), **WBC** (White Blood Cell), **Hb** (Haemoglobin), **MCV** (Mean Corpuscular Volume), **MCH** (Mean Corpuscular Hemoglobin), and **MCHC** (Mean Corpuscular Hemoglobin Concentration) **ul**(micro litre)**, g/dl**(gram/ deci-litre)**, um^3**(cubic micrometer)**, pg**(pico gram)

Table 5: Hepatic antioxidant enzyme activity of *O. niloticus* after 90-day feeding trial (with basal and *Momordica charantia* seed supplemented feeds) and after exposure to stress

Parameter	Basal	Control	Treatment	F value	P value
SOD (µmol/min/mg protein)	340.92 ± 0.2^{c}	409.35 ± 0.3^{b}	$445.21 \pm^{a}$	652	<0.001
POD (µmol/min/mg protein)	288.19 ± 0.9^{c}	369.31 ± 0.6^{b}	373.51 ± 0.8^{a}	3176	<0.001
CAT (µmol/min/mg protein)	207.57 ± 0.7^{c}	344.04 ± 0.6^{b}	385.06 ± 0.8^{a}	1195	<0.001
LPO (µmol/min/mg protein)	40.134 ± 0.2^{c}	99.35 ± 0.5^{a}	81.484 ± 0.3^{b}	5928	<0.001

Data is presented as Mean ± SE (n=3) One-way ANOVA followed by LSD post hoc test shows a comparison between antioxidant activity in liver of *O. niloticus* fish, fed with MC seed supplemented feed and of control (C) and of basal (pre-stress) groups. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$)

Basal means (pre-stress), **Control** (with basal feed), **Treatment** (S10 group, fed with 1 g/100g of MC seed)

Table6: Serum cortisol (Mean ± SEM) (ng/ml) in control and treated *O. niloticus* subjected to chronic physical stress of 1 hr confinement and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Groups		Statistics
	Control(ng/ml)	Treated(ng/ml)	(P-value)
Basal	36.14±0.5^{g}	36.12±0.3^{g}	0.97
0 hr	134.60±1.3^{c}	121.27±0.7^{c}	0.001
0.5	197.88±1.7^{b}	180.65±1.9^{b}	0.001
1	244.97±1.7^{a}	203.10±1.6^{a}	0.001
2	220.75±1.6^{b}	159.42±0.9^{d}	0.001
4	102.94±1.7^{d}	94.47±0.9^{e}	0.01
8	73.57±1.2e	62.77±1.1^{f}	0.001
12	50.70±0.4^{f}	38.27±0.4^{g}	0.001
24	38.57±0.7^{g}	37.19±0.1^{g}	0.13
48	36.90±0.3^{g}	36.81±0.4^{g}	0.87

P values in the column is from T-TEST, pairwise comparison of results between groups, cortisol level of control and treated *O. niloticus* Means with different superscript are significantly different (P<0.05) in the columns represent within group variation . Comparison of serum cortisol (ng/ml) after stress with their respective level of control group. **Control**= without supplementation,**Treated**= supplemented with 1 g/100g of *M. charantia* seed powder.

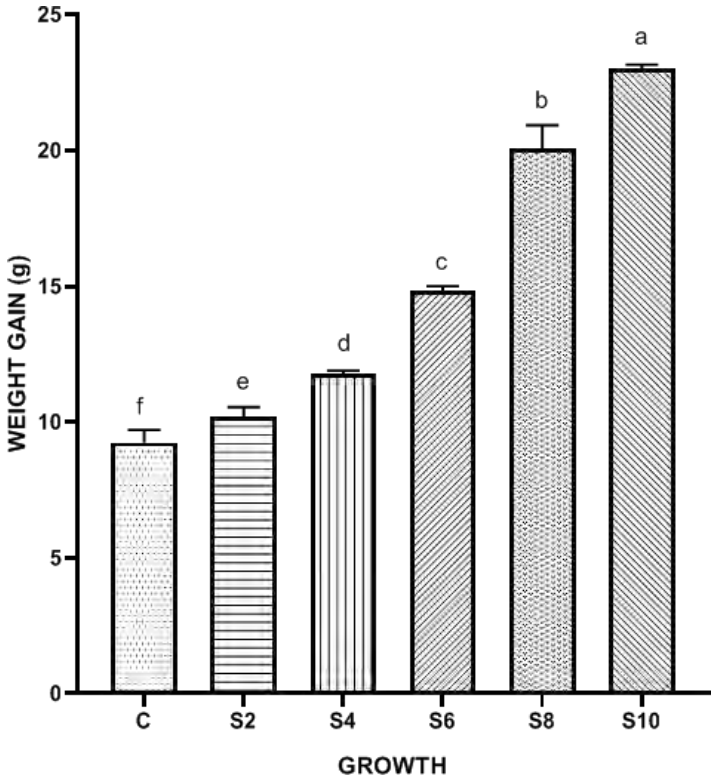
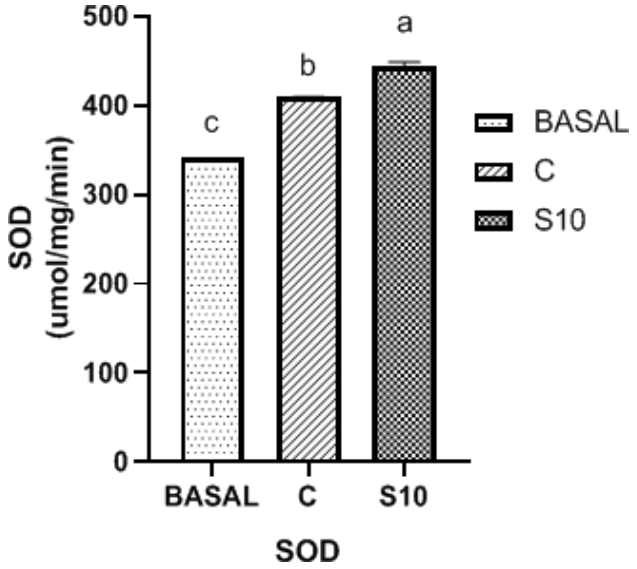


Figure 3: Comparative effect of *M.charantia* seed powder supplemented diet on weight gain of *O.niloticus* fingerlings. Superscript letters show a comparison between groups.

C (Control) = MC seed free diet. **S2=** (2g seed/kg diet), **S4=** (4g seed/kg diet), **S6=** (6g seed/kg diet), **S8=** (8g seed/kg diet), **S10=** (10g seed/kg diet).



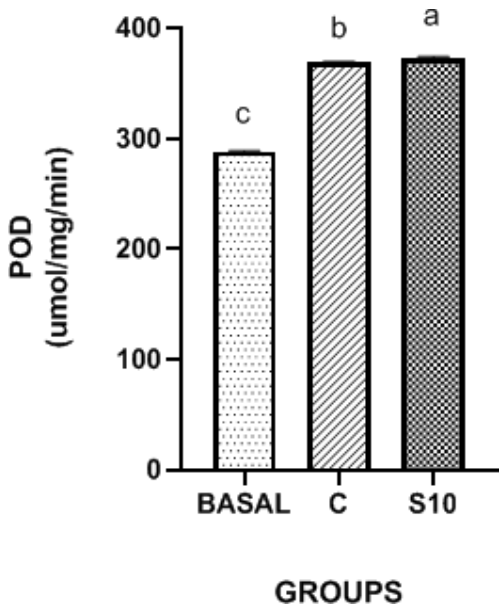


Figure 4: Hepatic SOD and POD antioxidants enzyme activity of *O. niloticus* after feeding MC seed supplemented diet for 90 days and then exposed to 1 hr confinement stress. Mean ± SE(n=3). Superscript letters show a comparison between groups.

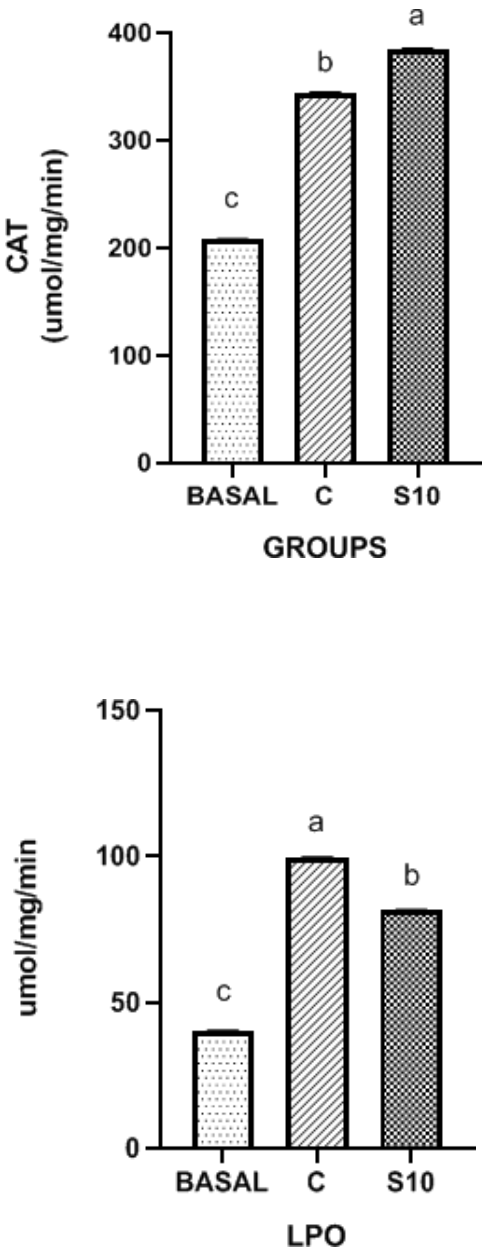


Figure 5: Hepatic CAT and LPO antioxidants enzyme activity of *O. niloticus* after feeding MC seed supplemented diet for 90 days and then exposed to 1 hr confinement stress. Mean ± SE(n=3). Superscript letters show a comparison between groups.

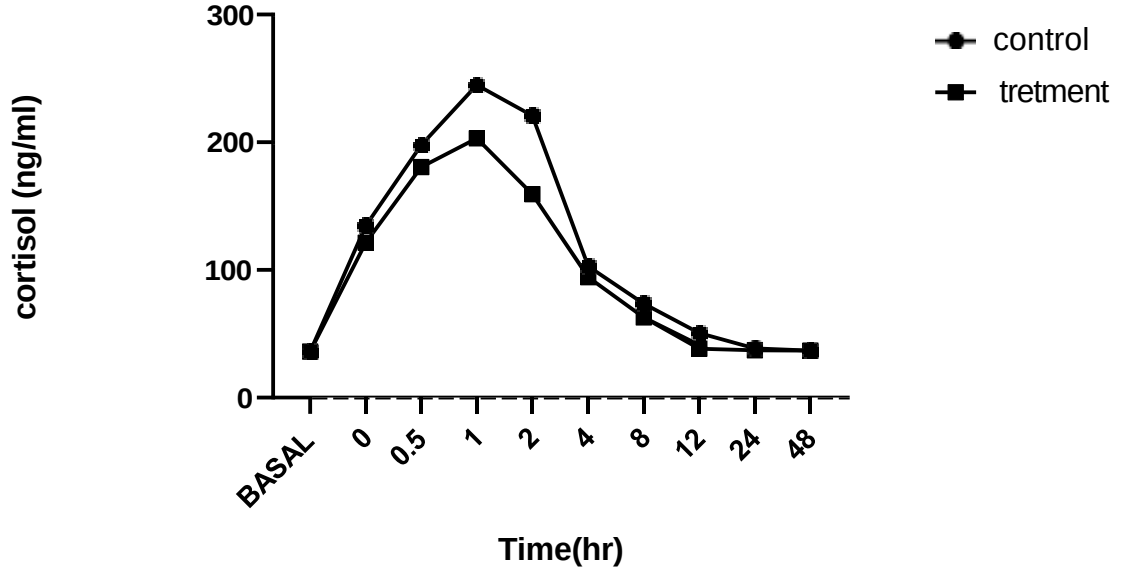


Figure 6: Cortisol concentration (ng/ml) of control and treatment S10 group of *Oreochromis niloticus* subjected to 1 hr confinement stress. Sampled at various time inetrvals. Basal means pre stress level.

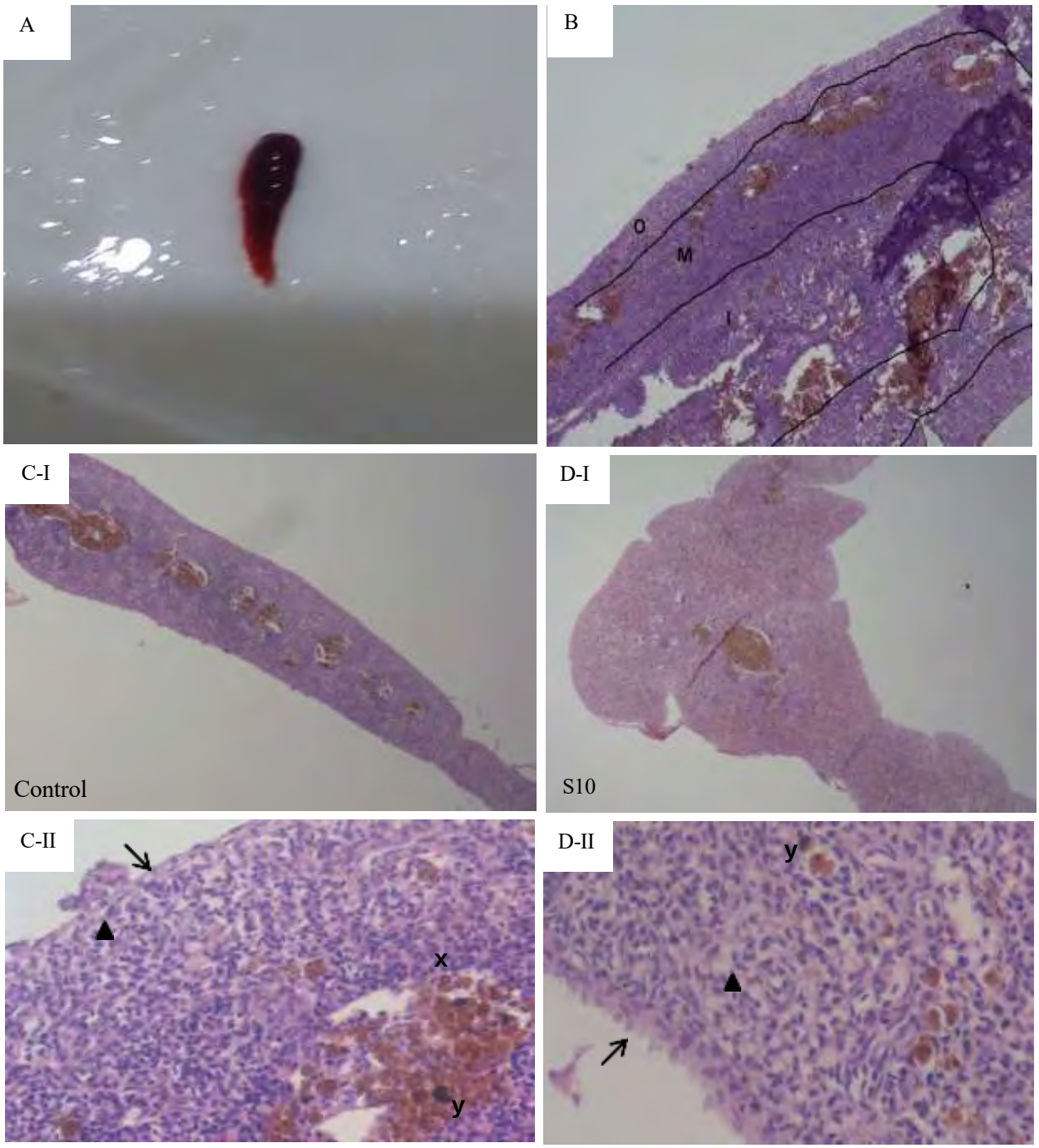


Figure 7: Spleen histology of *Oreochrmis niloticus* fingerling after 90 days of trial, feeding MC seed powder supplemented diets. The components of spleen (H&E staining). (➔showing thin spleenic-capsule, and (▲) showing the poorly developed trabecular, (**x**) The MMC melano-macrophage centers ,surrounded by single layer of flat cells and (**y**) showing melanin pigmentin MMCs.

DISCUSSION

On a global scale aquaculture continues to demonstrate its rapid growth as a crucial food-producing sector, possessing significant potential to support the global demand for aquatic food, (FAO, 2016). In freshwater aquaculture, tilapias are recognized as the second most significant category of cultivated fin fish, ranking closely behind carps (Waite *et al.,* 2014). This global aquaculture production performance is basically attributed to the widespread adoption of intensive production systems, characterized by increased stocking densities (Basha *et al.,* 2013). As a result, a variety of issues, such as impaired metabolism (Santos *et al.,* 2010), poor growth (Gabriel, 2019), substandard meat (Jittinandana *et al.,* 2003), a greater vulnerability to infections (Wu et al., 2013a), and in the worst situations, death occur (Mckenzie *et al.,* 2012). There have been numerous reports of medicinal plant extracts/powders promoting feed utilization, development as well as survival rates in tilapia(Gabriel, 2019). Adaptogens are a class of herbal nutritional, medicinal, and therapeutic agents that support the resilience, adaptability, and survival of living things under stress (Panossian *et al.,* 2021). At low dosages, they operate as moderate stress mimics, activating the signaling pathways for the adaptive stress response to deal with extreme stress (Panossian *et al.,* 2021), and in order to prevent the usage of chemotherapeutic medications; adaptogenic herbals treatments are ascribed to their ample bio-actives, which include saponin, polysaccharides, flavonoids, and phenols (Gabriel, 2019) which can have beneficial effects on growth, immune responses and overall welfare (Van Doan et al., 2020). The current study sought to understand how the adaptogens in *Momordica charantia* seed powder affected the levels of cortisol and antioxidant activity in the liver of stressed *Oreochromis niloticus*.

Momordica charantia contains various bioactive compounds, including glycosides, saponins, alkaloids, flavonoids and triterpenes, as well as phytochemicals like momorcharins, charantin, and cryptoxanthin (Grover & Yadav 2004).

The inclusion of MC seed powder resulted in tilapia growth that was dose-dependent in terms of weight gain (WG), feed conversion ratio (FCR), and specific growth rate (SGR), with the highest growth and other growth-related parameters being recorded in the group S10 that included 1 g MC seeds powder per100g of feed. These results for the growth are in accordance

with the results presented by other studies, with extracts derived from various sources, including Aloe vera (Gabriel *et al.,* 2015), the camphor tree, Neem tree, Garden spurge, and papaya (Kareem *et al.,* 2016), Garlic (Shalaby *et al.,* 2006), and the Tea plant (Abdel-Tawwab *et al.,* 2010), in Nile tilapia. In a related study on rainbow trout, adding 1.5% Mespilus germanica extracts (ME) supplemanteds diet had growth-promoting effects on FW, WG, SGR, and FCR (Patra *et al.,* 2023). The enhanced growth performance and improved utilization of feed observed in fish after the administration of medicinal and adaptogenic herbal extracts are thought to be linked to their diverse immuno-nutritional components, including complex sugars like polysaccharides (Zahran *et al.,* 2014). Polysaccharides are believed to exhibit prebiotic characteristics (Singdevsachan *et al.,* 2016), which can enhance the animal's ability to digest, absorb, and assimilate nutrients more easily (Heidarieh *et al.,* 2013). In current study enhanced growth effect that is exhibited by MC seed treated fish may be due to the presence of phytochemicals which according to Grover & Yadav (2004) include proteins, polysaccharides, flavonoids, triterpenes, saponins, cucurbitins, ascorbic acid, and steroids.

Blood is the most often investigated tissue in vertebrates, including fish, in an effort to determine their physiological or general health status (Fadeifared *et al.,* 2018). In present study results in table 4 revealed a significant improvement in hematological parameters including WBCs, MCHC, MCV, and MCH. The M. charantia seed inclusion significantly ($P<0.001$) improved the hematological values for PCV, MCH, MCHC, and WBCs, but they had less still significant ($P<0.05$) impact on RBCs and Hb, despite still being substantial. The increase in PVC is consistent with the results reported by Obaroh *et al.* (2014), who fed tilapia diets containing *Magnifera indica* at dosages of 0.5 to 8 mg kg-1. Additionally, the RBC results are consistent with (Gabriel *et al.,* 2015), who reported that supplementing with Aloe Vera did not significantly affect the RBCs in tilapia. Current findings, however, differ from those made by Fafioye *et al.* (2012) on tilpia givent a dose of A. indica at rate of (0.5–0.1 g L-1), which showed a significant decrease in Hb, MCHC, MCV, and MCH. Similar to this, Saravanan *et al.* (2011) noted a decrease in Hb, MCV, MCHC, and MCH in *C. mirigala* after exposure to *A. indica* (1.0g L-1). These findings imply that MC seed supplemented diets (at dosages of 2–10 g/kg) improve O. niloticus hematological indices because the values listed in Table 4 are consistent with the range of numerous hematological parameters determined for healthy *O. niloticus* by Bitterncourt *et al.*

(2003). Herbal extracts have the potential to stimulate erythropoiesis (production of red blood cells). This, in turn, increases the capacity for oxygen transport and strengthens the body's defense mechanisms against physiological stress. This effect is believed to be due to the extracts' abundant nutritional components, particularly polysaccharides, essential vitamins like riboflavin, thiamine, and folic acid, as well as nonessential amino acids, primarily necessary for the synthesis of hemoglobin (Latona *et al.,* 2012; Hamman, 2008).

In the present study, following one hour of confinement stress, the C group of fish exhibited notably higher plasma cortisol levels compared to the S10 group. The cortisol concentration was observed to increase from the baseline at 0 hours post-stress, reaching its peak at ½ hour and remaining elevated for the subsequent hour before gradually declining. After a 4-hour recovery period, the group supplemented with MC seeds showed cortisol levels closer to those of the basal group than the C group. Throughout this entire timeframe, the S10 group consistently demonstrated significantly lower values ($P<0.001$) than the C group, until both groups returned to basal (pre-stress) cortisol levels. This timely decrease in stress hormone concentration aligns with the findings of Aupérin *et al.* (1997), who also observed a similar trend in cortisol levels in O. niloticus. Fish that underwent pre-treatment with MC seed (S10 group) at a concentration of 1 g/100g successfully reversed the effects of stress. Similar studies to evaluate the MC adaptogenic activity were done by (kavitah *et al.,* 2011; (Meera & Nagarjuna, 2009). In wisbtar albino rats (180-200 g), who were pre-treated with MC ethanolic extract at doses of 200 and 400 mg/kg exhibit less elevation in plasma cortisol levels on receiving acute stress of being immobile for 150 minutes and chronic stress (kavitah *et al.,* 2011). In another study on Swiss albino mice, Momordica charantia (MC) show its adaptogenic or antistress properties. This was evaluated by measuring the swimming time and the stress-inducing effects of cold immobilization in mice. At both the low (450 mg/kg) and high (900 mg/kg) doses MC aqueous extract significantly (P 0.001) increased swim time and counteracted the effects of cold immobilization showing better results on higher dose level(Meera & Nagarjuna, 2009).

In a related study for fish Patra *et al*. (2023), reported that serum glucose and cortisol concentrations in rainbow trout *(Oncorhynchus mykiss)* exposed to crowding stress and fed various doses of dietary medlar *(Mespilus germanica)* extract, when compared to the C group post-challenge, groups T4 (3%) and T5 (4%) showed significantly ($P<0.05$) lower cortisol and

glucose values. The primary response against stress involves the increases in plasma cortisol (Barton *et al.,* 2002). This hormone induces secondary stress responses, characterized by increased glucose levels, mobilizing glucose to tissues for homeostasis to cope with energy-demanding processes of restoration (Barton *et al.,* 2002). Our theory is supported by literature studies in which Sen *et al.* (2000) speculated that the plants' antistress properties may be due to a range of chemicals present in them and their antioxidant activity.

Plasma corticosterone levels were significantly raised during the acute stress (AS) and chronic unpredictable stress (CUS) regimens. Pretreatment with 200 mg and 400 mg/kg body weight of the ethanolic extract of *M. charantia* significantly (P 0.01) inhibited both AS- and CUS-induced rise of plasma corticosterone. By reducing the lipid peroxidation in rat brain, MC extract demonstrated excellent antioxidant activity in this study, pointing to a reduction in oxidative damage (kavitah *et al.,* 2011).

Stressors like high stocking density are causes of oxidative stress (Yousefi *et al.,* 2018), and numerous pathologies have been linked to oxidative stress (Youdim *et al.,* 2001), which ends in a decline of SOD and CAT activities. SOD functions as an enzyme, facilitating the conversion of the superoxide anion into molecular oxygen and hydrogen peroxide, as described by Malmstrom *et al.* (1975). Additionally, lipid peroxidation stands as a recognized mechanism for cellular damage in both plant and animal systems, serving as an indicator of oxidative stress within cells and tissues, as elucidated by Yagi, (1998). These two enzymes are the first line of defense enzymes (Yousefi *et al.,* 2020) in response to the free radicals' attacks (Ighodaro and Akinloye, 2018) and decompose pro-oxidant molecules (O2- and H2O2). Lipid peroxidation results from decreases in these enzymes' activity (Yousefi *et al.,* 2018).

In the current study of M. charantia post-stress, fish in the S10 group had significantly ($P<0.001$) higher levels of hepatic POD, CAT and SOD activity and lower levels of LPO activity, whereas stress caused the control group's fish to have comparatively higher levels of LPO and lower levels of antioxidant enzymes, indicating more oxidative damage to their liver. Theses results are analogous to that found in *Cypinus carpio* which show high level of SOD and CAT while low levels of MDA after feeding of diets with bitter melon extract in dose dependant manner (0%, 0.25%, 0.5%, and 1%)(Qin *et al*.,2022), though similar experiments utilizing *M. charantia* for its

antioxidant impact have been described in mice. The findings are consistent with a study by Kavitah *et al.* (2011) who found that *M. charantia* had attenuating effect on lipid peroxidation in rat brain tissue. Rat brain homogenate was pre-incubated with the MC extract, and this demonstrated strong antioxidant activity by reducing the amount of lipid peroxidation in the rat brain and perhaps reducing oxidative damage. Horax et al (2005) has reported the antioxidant activity of phenolic compounds extracted from bitter melon..The research conducted by Sathishsekar and Subramanian (2005) investigated the antioxidant properties of *Momordica charantia* (Karela) seeds on diabetic rats induced with streptozotocin. The findings strongly indicate that the seeds of Momordica charantia (Karela) have the potential to restore the compromised antioxidant status in streptozotocin-induced diabetes.

The administration of other herbal adaptogens is also used in several fish species. Rainbow trout fed 1.5% and 2% ME (Medlar extract) diets in a recent study by Patra *et al.* (2023) showed reduced MDA and significantly higher SOD, CAT, and GPx levels than the control group following the stress. The antioxidant properties, due to phenolic, and flavonoid components of ME medicinal herb may be responsible for this beneficial outcome. In a different study employing lavender extracts (LE) as a plant adaptogen for Leonardo DiCaprio's healthy diet, liver SOD and CAT activities revealed a substantial rise ($P<0.001$) when LE supplementation fish was consumed. Hepatic SOD decreased in the control group following the crowding stress, but not in the LE-supplemented animals. Although CAT activity decreased in all treatments, it increased in tandem with LE levels, peaking in fish fed 1.5% LE (Yousefi *et al.,* 2020).

According to Ramchandani et al. (2014), phytochemicals like flavonoids and polyphenols demonstrate cell-protecting function by recovering defense enzymes. They are also known to promote the inhibition or suppression of the oxidation process (Esmaeilzadeh *et al.,* 2014).The presence of these components may be the cause of the MC seed powder's antioxidant effect. According to previous studies on phytochemicals, *Momordica charantia* contains glycosides, saponins, triterpines, steroids, vitamin C and A, as well as phytochemicals like momorchins, momordinol, momordicins, charantin, cucurbitacins, diosgenin, goyaglycosides, and goyasaponins, among others (Xie *et al.,* 1998) and the presence of flavonoid and phenolic phytochemicals in MC extracts Wu & Ng. (2008). The presence of the aforementioned bio-

actives and phytochemicals may also be the cause of the observed antioxidant activity of MC (Kavitah *et al.,* 2011).

In order to ascertain the effects of *Momordica charantia* on liver lipid peroxidation in mice, hepatic MDA and SOD levels were analyzed. Wang & Ryu (2015) evaluated the usage of Momordica charantia as an adaptogen source for mice study. According to Armstrong and Browne (1994), MDA is a naturally occurring byproduct of lipid peroxidation. MDA levels were considerably reduced by 41.9% in mice given a high dose of MCE compared to the HFD (high fat diet) group ($P<0.05$), although SOD levels did not differ significantly between the normal and HFD groups. SOD activity, which inhibits lipid peroxidation, was higher in mice given a high dose of MCE compared to the other groups ($P<0.05$) (Wang & Ryu, 2015). This herbal adaptogen's antioxidant property is crucial since, according to the notion put forth by Dardymov and Kirkorian for adaptogens, these substances work largely as antioxidants and free radical scavengers (Pawar & Shivakumar, 2012).

Now, it is generally accepted that the improvement of antioxidant enzyme and hepatoprotective activity in fish by herbal extract is frequently connected with specific phytochemicals.The presence of phytochemicals in herbal extracts, including phenol/polyphenols, enzymes (SOD, CAT), vitamins (C, E, and carotenoids), and flavonoids, is known to help the inhibition or suppression of the oxidation process (Esmaeilzadeh *et al.,* 2014).

To the best of our knowledge, there has not been any research on the impact of MC seed powder on fish under stressful or confined conditions' antioxidant status. Dietary MC Seed powder has anti-stress/adaptogenic properties because it was able to reduce blood cortisol and glucose. There are no studies on this subject, however earlier research has shown that other herbal substances such as *Rheum officinale* extract and rosemary leaf can reduce stress in a variety of species (Xie *et al.,* 2008; Yousefi *et al.,* 2019). Dietary MC's anti-stress properties could be attributed to the presence of vitamin C, flavonoids, and phenols /polyphenols in M. charantia. A significant increase in serum cortisol and glucose was observed in Gilthead seabream(*Sparus aurata L.*) after short-term crowding (Ortuño *et al.,* 2001).There are strong linkages between immunological responses and the antioxidant system, therefore the drop in

cortisol levels and higher antioxidant defense may explain increased fish immune responses in MC-fed fish (especially 1 g/100g dosage).

Confinement is a stressful practice for fish, which can culminate in tissue damage (Streckert *et al.,* 2018) The spleen is a vital immune organ containing lymphocytes, macrophages, and granulocytes. (Wang *et al.,* 2006). Melano-macrophage centers, function as metabolic sites for the removal of waste from damaged cells (Agius, 1980). Certain authors have suggested that melano-macrophage centers may serve as sensitive indicators of stressful conditions in aquatic environments (Blazer et al., 1987; Fournie et al., 2001). In our study, the control group as compare to S10 treated group showed an increase in Melano-Macrophage Centers (MMC) and melanin deposition in an attempt to defend cell from stress. An increase in the number and size of melano-macrophage centers(MMC) indicates tissue/cell damage (Agius and Roberts, 2003). Our result aligns with Li et al.'s (2023) research on grass carp, high salinity induced a similar MMC increase, indicating stress.Likewise, Tellez-Bañuelos et al. (2009) found endosulfan exposure in Oreochromis mossambicus reduced spleen weight but increased macrophage activity. Xu et al.'s (2018) study showed that 16% salinity induced larger and more diffuse ultra-structures of macrophages in the spleen, characterized by irregular lysosomes and deposition of granules.High MMC in control group is indicative of cell damage while less MMC in S10 suggest strong imunomodulatory action of MC seed powder supplemented diet. New research indicates that a variety of foods can contribute to the immunomodulatory properties. The key components responsible for these benefits include vitamins, minerals, carotene, and flavonoids (Jadaun et al., 2022)Therefore, in our study the less MMC in S10 group may be the sign of less cell damage due to *Momordica charantia* active phytochemicals(flavonoids) exhibiting immunomodulatory effect.

Momordica charantia is a versatile plant used for both culinary and medicinal purposes. Packed with triterpenes, proteins, steroids, alkaloids, and phenolics, it exhibits diverse biological activities, including anti-diabetic, antioxidant, anti-cancerous, growth promoting, and immunomodulatory effects. This makes *Momordica charantia* a valuable source for nutrition and adaptogens.

Conclusion

Momordica charantia (MC) seeds show significant adaptogenic/anti-stress, growth-promoting, and antioxidant benefits in *O.niloticus,* according to the study's findings. Boosted immunological responses in the fish could be explained by dietary MC seed powder's ability to lower cortisol levels and increase antioxidant defense. Tilapia supplementation with MC seeds at 0.8–1 g/100g is advised to reduce stress and oxidative conditions in the fish. These sustainable, natural, and cost-efficient alternatives to chemicals can be used in commercial fish feeds.

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