Effect of Ashwagandha (*Withania somnifera*) Root Powder on Thermal Induced Stress Responses in Nile Tilapia (*Oreochromis niloticus*)

By

Ashiq Hussain

Department of Zoology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad
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A thesis submitted in partial fulfillment of the requirements for the Degree of

MASTER OF PHILOSOPHY
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.

Ashiq Hussain

CERTIFICATE

This dissertation ‘‘**Effect of Ashwagandha (*Withania somnifera*) Root Powder on Thermal Induced Stress Responses in Nile Tilapia (*Oreochromis niloticus*)**’’ submitted by **Ashiq Hussain** is accepted in its present form by the Department of Zoology, Faculty of Biological sciences, Quaid-I-Azam University, Islamabad, as satisfying the thesis requirement for the degree of Master of Philosophy in Zoology.

Supervisor:

_____

Prof Dr. Amina Zuberi

External Examiner: _____

Chairperson_____

Prof Dr. Amina Zuberi

Date: _____

Dedication

I dedicated my dissertation work to my family and teachers, special feeling of gratitude to my loving parents (Adday and Dadak) &my beloved uncle (Baqir Hussain).

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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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List of Abbreviations

°C	Celsius
µL	Microliter
µM	Micromolar
ALT	Alanine transaminase
ANOVA	Analysis of variance
AST	Aspartate transaminase
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
cm	Centimeter
DEPC	Diethyl Pyro-carbonate
DO	Dissolved oxygen
EDTA	Ethylenediaminetetraacetic acid
gm	Gram
g/ml	Gram per milliliter
H_2O	Water
H_2O_2	Hydrogen peroxidase
Hb	Hemoglobin
HCT	Hematocrit
HN	Hepatocyte nucleus
Hp	Hyperplasia
hrs	Hours
HT	Hypertrophy
Ig M	Immunoglobulin
IL-1β	Interleukin 1 beta
Kg	Kilogram
L	Liter

W. somnifera	*Withania somnifera*
O. niloticus	*Oreochromis niloticus*
LDH	Lactate dehydrogenase.
LPO	Lipid peroxidation
LSD	Least significant difference
M	Molarity
M cm	Molar coefficient
m/v	Mass per volume
mg	Milligram
mg/L	Milligram per liter
min	Minute
mM	Millimolar
mm	Millimeter
mRNA	Messenger ribonucleic acid
ISS	Increased sinusoidal space
AH	Altered hepatocytes
N	Necrosis
$NADH^+$	Nicotinamide adenine dinucleotide hydrogen
NBT	Nitro blue tetrazolium
NCBI	National center for biotechnology information.
HND	Hepatocyte nuclear degeneration
ND.	Nanodrop
Nm	Nanometer
Nmol	Nanomole
O_2	Oxygen
OE	Edema
P	Probability
PCR	Polymerase chain reaction

pH	Power of hydrogen
POD	Peroxidase
RNA	Ribonucleic acid
ROS	Reactive oxygen specie
Rpm	Revolution per minute
SE	Standard error
SOD	Superoxide dismutase
D_1	Control group
D_2	Control group
TBARS	Thio-barbituric acid reactive substances
TNF-α	*Tumor necrosis factor*
U/L	Units per liter
V	Vacuolization

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Abstract

High growth rate with efficient tolerance against environmental stress agents are the two most important character acknowledged in modern aquaculture. *O. niloticus* is the most cultured species after carps worldwide, which is known for its lower tolerance to cold shock. Certain studies have been conducted to address this issue by incorporating various feed additive and plant extracts in the fish feed. The present study was conducted to evaluate the possible role of Ashwagandha (*Withania somnifera*) root powder in enhancing stress tolerance of *O. niloticus* to cold shock. A 80 days trial was conducted, and a total of 90 fish (Initial body mass=90g) were randomly distributed in 6 fiberglass tanks capacity,100L stocking density (1.5g/L). The tanks were divided at random into two groups and experiment was conducted in triplicates. One group was fed with basal diet without Ashwagandha root powder (D_1), while second group was fed with Feed having 15% Ashwagandha root power inclusion (D_2). After 80 days growth performance was noted and then both groups were subjected to cold shock (15°C.) for a period of 2 h. The fish were the anesthetized with MS-222, taking blood from the caudal vein for hematological analysis while dissection was done in the cold chamber for taking samples for the assessment of antioxidant enzymes activity. The water quality parameter including water temperature (27-29°C), pH (6.5), ammonia, was maintained throughout the experimental trial by changing 20% water having same temperature after every 3 days. Results indicated significant improvement in growth performance; FBW (Final Body Weight), TWG (total weight gain), and SGR (specific growth rate) along with improved FCR in D_2 compared to D_1. A Post cold shock hematological indices have shown a decrease in in RBC hemoglobin (Hb), hematocrit (Hct), mean corpuscular hemoglobin (MCH) and (%), mean corpuscular volume (MCV) count in D_1 fish compare to D_2. Furthermore, activity of metabolic enzymes ALT, AST and LDH increased in D_1 compared to D_2. Owing to antioxidant activity in liver and kidney univariate analysis has revealed a significant Organ× diet interaction. Significantly high SOD and CAT activities were observed in the liver and kidney of D_2 group followed by D_1 while POD was enhanced in liver and kidney of D_1 followed by D_2. However, LPO activity was higher in D_1 (both liver and kidney) compared to D_2 group. Similarly, cortisol releasing hormone (CRH) expression in the brain tissue as well as blood plasma cortisol level was significantly higher in D_2 compared to D_1. These results suggest phenomenal impact of Ashwagandha root powder in reducing stress in *O. niloticus* which is evident from the decreased

plasma cortisol level in blood after cold shock in D_2 compared to D_1. However, further study regarding the optimum inclusion rate and molecular study related to stress genes, is recommended.

Key words: Cold shock, Thermal tolerance, *Withania somnifera, Oreochromis niloticus,* antioxidant activity.

INTRODUCTION

Aquaculture is a rapidly developing industry with the focus to meet the need of high-quality protein to the increasing population (Herforth *et al*., 2020). Certain projects and initiatives have been started worldwide like blue revolution, State of World Fisheries and Aquaculture to enhance fish production rate by introducing new culturing techniques, intensive aquaculture, use of certain metabolic boosters and growth enhancers etc. (FAO, 2022; Belton *et al.*, 2020; Jiang *et al.*, 2022). One of the major limitation in the aquaculture sector in this regard is the stress caused by various sources include thermal stress (Oliva-Teles, 2012). Certain species which are highly considered in aquaculture often possess internal thermal shock resistance system to withstand. However, other species like *O. niloticus* do not withstand such stress. Certain studies have revealed use of feed additives to improve the thermal tolerance capabilities but no such study has been reported for *O. niloticus*. The present study was designed to study the potential role of ashwagandha on thermal stress tolerance and growth in Nile tilapia, incorporating it as feed additive (Mumu & Mustafa, 2022).

The Nile tilapia (*Oreochromis niloticus*) is now more widely produced, and many populations rely on it as a significant source of protein (Herforth *et al*., 2020). Farming of *O. niloticus* (245 MT), which contributes most to Pakistan's yearly total production of freshwater aquaculture (200,003 MT), accounts for most of this production (Food, 2019). Warm-water fish such as tilapias likes warm-water habitats (Sun *et al*., 1992). In intensive or semi-intensive cultures, instead of being kept at their ideal temperature of 28 °C, *O. niloticus* is raised at a chilly suboptimal temperature of 22 °C, which causes them to consume less feed and experience a considerable decline in growth. Some of its body processes, such food consumption and immunity, can be affected by temperatures below 16 °C, whereas temperatures below 10 °C are lethal. (Wohlfarth & Hulata, 1981; Dellagostin *et al*., 2022). *O. niloticus* is a tropical fish, yet it is produced in many places with subtropical climates, such as Egypt, China, Brazil, and Asian countries (Herforth *et al*., 2020), where production is limited in the winter.

Tilapias have a good adaptation to warm water environments however they have a lower level of resistance to cold stress (Sun *et al*., 1992; Dellagostin *et al*., 2022). There was a significant economic loss because of the widespread deaths of cultured *O. niloticus* in several subtropical

areas during the winter season due to the drop in water temperature (Hassan *et al*., 2013). Therefore, overcoming this difficulty in subtropical *O. niloticus* farming by increasing their resistance to cold would help to lengthen their growth period, reduce mortality rates, and ultimately minimize production costs (Nobrega *et al*., 2020).

Numerous studies have been done on the nutritional needs of tilapia, but there hasn't been much done explicitly on the nutritional needs to reduce low temperature stress (Nobrega *et al*., 2020). Functional feed additives are nutritive and nonnutritive substances added to fish and shrimp diets for a variety of reasons, such as improving the physicochemical characteristics of feed or the performance of the target species (Bai *et al*., 2015). Diets rich in chia seed powder (CSP) help *O. niloticus* to become more resilient to cold and heat stress (Mahmoud *et al*., 2023). As observed in many of fish species, including channel catfish, *Ictalurus punctatus*, diet may enhance fish immune, illnesses resistances, and stress tolerance, especially for fish (Murray *et al*., 1977).

The alternative herbal bio-medical products used in aquaculture operations work as appetite stimulants and have the properties of stimulating growth and strengthening the immune system (Dawood *et al*., 2018). They will be quite useful in the cultivation of shrimp and other fin fishes since they boost consumption, induce maturity, have antibacterial capabilities and anti-stress qualities, and do so without posing any environmental or dangerous issues (Citarasu, 2010). There is less life expectancy of sick fish administered different immunostimulants, vaccines, and probiotics rose after being challenged with pathogens, according to several research (Dawood *et al*., 2018; Wendelaar Bonga, 1997). Recently, there has been an increase in interest in using medicinal plants that have gained widespread attention and are currently the focus of active scientific research (Bai *et al*., 2015; Richard *et al*., 2016). Due to their affordability, ease of preparation, effectiveness in treating diseases, and lack of any negative environmental or hazardous effects (Citarasu, 2010; Dawood *et al*., 2018). Examples of medical plants include herbs, seaweeds, herbal extracts, spices, conventional Chinese medicines, and commercial products made from plants (Van Hai, 2015).

Additionally, to being utilized as chemotherapeutics and feed additives in aquaculture, medicinal plants (Pathak *et al*., 2000). They can encourage growth, acting as an immune system booster, being antimicrobial, enhancing hunger, and possessing anti-stress qualities (Citarasu,

2010). In comparison to synthetic antibiotics, chemicals, vaccines, and other compounds, a large number of phenolic, alkaloid, terpenoid, polyphenolic, lectin, quinone and polypeptide molecules present in plants and associated byproducts are better (Harikrishnan *et al*., 2011).They can be administered as a whole plant, as leaf, root, or seed isolates, as extract compounds, as water or feed additives, either alone or in combination with some other extracted compounds, and even in conjunction with immunostimulants or other prebiotics (Dawood *et al*., 2018).

According to Ardó *et al*. (2008), herbs may contain a wide variety of active substances, including polysaccharides, alkaloids, and flavonoids. The herbal-compound extracts are immunostimulants that boost fish immunological responses. They also contain growth-promoting, immune system-improving, antimicrobial, appetite-stimulating, and anti-stress effects (Citarasu, 2010). Antiprotease, complement, viz. lysozyme, myeloperoxidase, reactive nitrogen species, ROS (reactive oxygen species), phagocytosis, respiratory response, and reactive nitrogen species (Harikrishnan *et al*., 2011). Numerous studies have examined the effectiveness of different plant products in aquafeeds, including herbs (Ardó *et al*., 2008), roots (Sharma *et al*., 2017), seed meal (Ahmad & Abdel-Tawwab, 2011), Curcuma longa (Turmeric) (Mahmoud *et al*., 2017), ginger (*Zingiber officinale* Roscoe) (Nya & Austin, 2009; Panase *et al*., 2018). As one of the most significant herbs in Ayurveda, ashwagandha (*Withania somnifera*), often known as "Indian Ginseng," is a subtropical perennial shrub with significant medicinal characteristics. It is utilized for its many health benefits and as a tonic to improve vigor and longevity (Saleem *et al*., 2020; Sandhya & Sushil, 1998).

Ashwagandha, also known as *Withania somnifera* (WS) Dunal (family, Solanaceae), has been used for more than 2500 years in Ayurveda, the traditional Hindu medical system. The attributes ascribed to ayurvedic rasayanas are remarkably similar to those asserted toward being found in adaptogens like PG (Panax ginseng), that appear to increase generalized body resistance against diverse stresses and support the promotion of physical and psychological states of the individual (Bhattacharya & Muruganandam, 2003). There are around 35 chemical components in ashwagandha (Mishra *et al*., 2000). Alkaloids (isopellertierine, anferine), steroidal lactones (withanolides, withaferins), flavonoids, tannins, starch, reducing sugars, saponins, and other biologically active substances are all present, and it is also a source of iron (Mishra *et al*., 2000) (Srivastava *et al*., 2020).

Ashwagandha is well known for its immunomodulatory, anti-inflammatory, anti-stress, antioxidant, adaptogen, antibiotic, aphrodisiac, astringent, deobstruent, diuretic, and sedative activities (Singh *et al*., 1982; Bhattacharya *et al*., 1997; Bhattacharya & Muruganandam, 2003; Srivastava *et al*., 2020). Active substances with substantial anti-stress activity include withaferin-A and sitoindosides VII-X (Bhattacharya *et al*., 1997).

Among the several tactics used, dietary interventions seem to be the most practical and promising way for aqua culturists to keep aquatic animals' homeostasis against various stressors (Huang *et al*., 2012). Different dietary components and nutraceuticals have already been shown to improve immunity and temperature tolerance in several fish species (Gupta *et al*., 2010).

It is well known that diet can increase a fish's ability to withstand heat, as evidenced by reports for numerous fish species, including the channel catfish (*Ictalurus punctatus*) (Murray *et al*., 1977; Nobrega *et al*., 2020). There are few dietary needs to reduce low temperature stress (Nobrega *et al*., 2020). Several elements, including environmental temperature, which has a significant impact on fish reproductive, behavior, and immunological response, make aquaculture a challenging operation (Elkatatny *et al*., 2020). One of the most crucial abiotic elements in aquaculture is water temperature because it directly influences fish growth and survival (Engy *et al., 2017)*). Since fish are ectothermic animals, changes in ambient temperature have a significant impact on their feed intake (FI), metabolism, growth, immunological response, and survival (Mahmoud *et al*., 2023).

One of the most crucial abiotic elements in aquaculture is water temperature because it directly influences fish growth and survival (Fatma & Ahmed, 2020). Fish raise serum cortisol and catecholamine levels in response to low temperatures; as a secondary reaction, metabolic alterations may take place (Barton, 2002). These adaptations are frequently employed as markers of transient cold reactions (Barton, 2002). Low water temperatures might cause secondary reactions that can cause physiological abnormalities and even death if they continue for an extended period (Donaldson *et al*., 2008; Reid *et al*., 2022). Temperature carefully controls almost all biological mechanisms, particularly fish physiological stress responses (Reid *et al*., 2022).

According to one definition of stress, it is the "physiological chain of events that take place while the organism struggles to fend off death or restore homeostatic norms in the face of

injury"(Schreck, 2000). Stress, according to him, is the "physiological cascade of events that occur as the organism attempts to reduce the morbidity and mortality or reestablish homeostatic standards in the face of attack." Primary, secondary, and tertiary reactions are three roughly consecutive categories that can be used to categories the systemic response to stress in fish and other aquatic creatures (Reid *et al*., 2022; Wendelaar Bonga, 1997). In response to a stressor, the brain and neuroendocrine system release catecholamines (such as dopamine, norepinephrine and epinephrine) and corticosteroids (like cortisol) (Iwama *et al*., 1999). Secondary stress reactions include alterations in metabolic activities, osmoregulation, blood chemistry, immunological and cardiovascular performance at the molecular and cellular level, along with cellular responses (Skomal & Mandelman, 2012). Changes in predation and foraging behavior, oxygen uptake and metabolic activity, entire immunologic function, and population level modifications including changes in development and growth are all examples of tertiary stress responses (Barton, 2002; Reid *et al*., 2022).

Stress is also typically categorized as either chronic or acute, but the distinctions among the two and how it influences an organism (e.g., creating crisis vs. stress strategies) are not evident in the setting of several or contemporaneous stressors (Schreck & Tort, 2016). Along with additional physiological modifications that are not always a component of the overall stress response, such changes in metabolic rate (Tseng *et al*., 2014) and changes in temperature may cause a neuroendocrine response to stress with accompanying physiological changes, as well as cellular membrane fluidity (e.g., cortisol increase) (Buhariwalla *et al*., 2012). The major stress response in teleost includes rapid changes in blood levels of corticosteroids and catecholamines. These hormones are the byproducts of the brain sympathetic-chromaffin cells (BSC) and hypothalamus pituitary-inter-renal (HPI) axis, respectively (Schreck & Tort, 2016; Zuberi *et al*., 2020).

1.1 Hypothesis

It is hypothesized that the inclusion of ashwagandha as feed additive in the diet of *O. niloticus* will improve tolerance to cold shock**.**

1.2 Aim and Objectives

The main aim of the present study is to evaluate the thermal tolerance responses of *O. niloticus* after feeding supplemented diet with Ashwagandha (*W. somnifera*). The objectives are as follows:

- To evaluate the effect of ashwagandha on growth performances.
- To evaluate the effect of ashwagandha on blood hematological indices.
- To investigate the effect of ashwagandha on antioxidant activity.
- To evaluate the effect of ashwagandha on plasma cortisol concentration.

MATERIALS AND METHODS

2.1 Transportation & Acclimatization of Experimental Fish

Fish, Nile tilapia (*O. niloticus*) having average mean weight of 90±4 grams were collected from National Agriculture Research Center, Islamabad. The fish were transferred using live hauling technique, in polyethylene bags partially filled with water (30%) and oxygen (70%). to the Department of Zoology, Fisheries and Aquaculture Research facility, Quaid-i-Azam University Islamabad, Pakistan. Fish were acclimatized to laboratory condition for two weeks before starting experiment.

2.2 Feed formulation and Preparation

Basal diet was formulated in accordance with nutritional requirement *O. niloticus* (Van Trung *et al*., 2011), 32%CP (crude protein) diet was formulated (Table 1), using dry ingredients; Fish meal, soybean, rice polish, wheat flour, sun flower, corn Gluten. All the ingredients were finely grinded using electric grinder (MXBAOHENG Model HC-500) and weighed using digital balance (KS106) China. Ashwagandha (*Withania somnifera)* plant was collected from District Swabi during summer season, Roots were separated and sun dried. The roots were also grinded using electric grinder, (MXBAOHENG Model HC-500). Two experimental diets were prepared; Control diet (D_1) with no supplementation of Ashwagandha and Experimental diet (D_2) which was supplemented Ashwagandha Root Powder. For both test diets, all feed ingredient were mixed as per formulation and water was added, the mass was thoroughly mixed till a dough was appeared. After that the mass was processed using a meat grinder to form a 3mm thick noodle which was then dried in oven (SANFA DHG-9053A) at 60°C for 24 hours. The noodles were crumbled into small pellets and stored in fridge further usage. Feed was prepared for 20 days and after every 20 days fresh batch of feed was prepared.

2.3 Experimental Design

Followed by acclimatization, completely randomized 80 days experimental trial was performed under semi-static laboratory conditions. Irrespective of sex 90 healthy fish were randomly distributed in 6 fiberglass tanks (180×90×76 cm) at stocking density1.5 g/L (15 fish per tank). Formulated feed was given twice a day with feeding rate of 3 percent of body weight. The feeding rate was adjusted after every 15 days. Experiment was caried out in triplicate comprising

two groups i.e., control feed with basal diet containing no Ashwagandha (D_1) and treated group feed with formulated feed having same proximate value as basal diet but having 15% (15mg per 100g of basal diet) Ashwagandha root powder (D_2). During feeding trial, water quality parameters were observed daily and found in range temperature (24.5 - 26.2°C), dissolved oxygen (5.0 – .5.5 mg L^{-1}) and pH (7.5 – 7.7) suitable for the growth of Nile *tilapia*. All tanks were in the same vicinity, adjacent to each other, thus no significant difference among different tanks was observed.

2.4 Growth Performance:

At the end of feeding trial, fish were starved for 24 hours before sampling. For growth performance, fish of each tank were collected and weighed and by using following standard formulas growth performance was evaluated.

$$Weight\ gain = Wf - Wi$$

Wf =Final body weight of fish

Wi=Initial body weight of fish

$$\%WG = Wf - \frac{Wi}{Wi}$$

WG% (Percentage weight gain)

$$SGR\% = (\mathrm{I}n\ final\ weight - In\ initial\ weight)No\ of\ days\ of\ experiment\ \times 100$$

SGR (Specific Growth Rate)

The FRC was calculated using the formula below

$$FCR = \frac{Total\ feed\ consumed\ (g)}{Total\ wet\ weight\ gain\ (g)}$$

FCR = Feed Conversion ratio

FCE (Feed conversion efficacy): The formula used to compute it is as follows:

$$FCE(\%) = \frac{1}{FCR} \times 100$$

2.5 Cold Shock

At the end of feeding trail, 9 fish each from control and treated groups were collected at random and exposed to cold shock, i.e., exposure to low temperature 15°C for 2 hrs. For cold shock treatment, the water temperature was gradually down by adding cold water and when reached to desired temperature (15°C), then fish were kept at this temperature for 2 hours. After cold shock treatment, fish were anesthetized with MS222 (25mg/L).

2.6 Sampling

Blood of each fish was drawn from caudal vein by using 3 ml disposable heparinized syringe and collected in EDTA tube for hematology and Eppendorf for separation of plasma. After that fish dissection was conducted on cold chamber using sterilized dissection kit and disposable gloves, syringes and surgical blades. Fish were dissected on ice boxes, and their kidney and liver were collected immediately deep frozen in liquid nitrogen, and then kept at -20 °C for antioxidant enzymes assays.

The blood was centrifuged for 15 minutes at 20000×g for separation of plasma. After centrifugation of all samples supernatant of each sample was collected in separate tube and stored at 4°C for further analysis of Alanine aminotransferase (ALT), Aspartate aminotransferase AST, lactate dehydrogenase LDH and cortisol.

2.7 Hematological Analysis:

Blood samples in VACUETTE® ETDA tubes were processed for a complete blood profile using a hematocrit analyzer. A complete blood profile provides estimates of red blood cells (× 103/µL), white blood cells (× 103/µL), hemoglobin (Hb) (g/dL), PCV hematocrit (%), corpuscular volume (MCV volume (MCV)(Fl), mean corpuscular hemoglobin (MCH) (pg), and mean corpuscular hemoglobin concentration (g/dL) MCHC.

2.8 Antioxidants

2.8.1 Superoxide Dismutase

Assay Modified Kakkar *et al*. (1984) method was used for the measurement of SOD activity. The reaction mixture contains 0.1 mL of 186 M phenazine methosulphate solution, 1.2 mL of 0.052 mM sodium pyrophosphate buffer (pH=7.0), and 0.3 ml of precipitate. After 1 minute, the reaction was halted by introducing 1 mL of the glacial acetic acid to 0.2 mL of 780 µM NADH+ solution. The chromogen obtained as a result was measured through spectrophotometer set at 560 nm absorbance wavelength. SOD's one unit is the quantity of enzymes/mg protein that inhabits the oxidation reaction of quercetin via 50% maximal inhibition. Results for SOD activity, measured in moles per minute per milligrams of protein, are represented using a molar coefficient of 6.22 103/M cm.

2.8.2 Catalase Assay

The Chance & Maehly. (1955) technique was used to assess the specific activity of catalase. Briefly, 5.9 mM hydrogen peroxide (H2O2), 50 mM phosphate buffer (PBS; pH=5.0) of 2.5 ml and 0.1 ml (100 l) of supernatant were combined. At one minute interval interval the reaction mixture's absorbance, was measured via a spectrophotometer set at 240 nm wavelength. The result of CAT was stated as nmol/ min/ mg protein employing 46/ M cm as a molar coefficient.

2.8.3 Peroxidase

For determination of POD activity, The Chance & Maehly. (1955) technique was employed. The reaction mixture consisted of 0.3 ml of 40 mM H2O2, 0.1 ml of 20 mM Guaiacol, 2.5 ml of 50 mM PBS (pH 5.0), and 0.1 ml of supernatant. Using a UV/Visible spectrophotometer, absorbance at 470 nm was measured after a 1-minute break. A measurement of POD activity was made as nmol min-1mg-1 protein using 2.66×104/ M cm as a molar coefficient 0.3 M^{-1} cm^{-1}.

2.8.4 Lipid peroxidation

Assay The Wright *et al*. (1981) technique was used to determine LPO activity. Supernatant, ferric chloride, ascorbic acid, and 0.58 ml (580 l) of tris buffer (0.1 M, pH 7.4) were combined to create 1.0 ml of the reaction mixture, which was then incubated at 37°C in a water bath. 10% trichloroacetic acid is added after 1.0 ml as a stop reaction. After that, 1.0 ml of thiobarbituric acid was added, and the mixture was once more incubated for 20 minutes in a water bath. The solution was centrifuged at 2500 g for 10 minutes after being cooled to a low temperature. Using a UV/VIS spectrophotometer, absorbance at 1-min intervals was measured at 535 nm wavelength. LPO activity was calculated as nM TBARS/min/mg tissue at 37°C using a molar extinction value of 1.56 105 / M cm.

2.9 Measurement of plasma Cortisol Levels

Serum cortisol level were analyzed by ELISA (CO368S, CALBIOTECH), according to the manufacturer's instructions.

2.10 Gene Expression Analysis

CRH gene expression was analyse by Real Time PCR System. In Brief, tissue samples were removed from the RNA later, homogenized and 0.5ml chilled TRIzol reagent was added. Then the incubation was done at ambient temperature for 5 minutes before being applied to chloroform (0.1ml) and vigorously agitated for 15 seconds. The sample was again incubated for 5 minutes at room temperature before being centrifuged at 12,000 rpm for 15 minutes at 4℃. Afterwards, the upper colourless aqueous layer was separated and transferred to a new microcentrifuge tube. The separated aqueous phase was vortexed briefly with chilled isopropyl alcohol (absolute). Prior to centrifugation, the samples were again incubated for 10 minutes at room temperature and then centrifuged at 12,000 rpm for 10 minutes. The pellets were washed twice in 75% ethanol (0.5ml) (prepared in DEPC treated water), and the liquid phase was discarded. After that, air-drying of pellets was done and then dissolved in 50 µl of nuclease-free water (Hi-media, India). The isolated RNA was stored at -80℃ analysis.

2.10.1 Quantification of RNA

Nanodrop ND1000 (Thermo scientific, USA) was used to evaluate the quality and quantity of isolated RNA. The quantification of total RNA conc. in the sample was performed by measuring

absorbance at 260nm while to check the purity of the samples the ratio of ND at 260 and 280nm was used with estimated values between 1.9 and 2.0.

2.10.2 Synthesis of cDNA

Each isolated RNA was reverse transcribed to cDNA by using the method as reported earlier by Amir *et al.* (2019). In brief, the reaction mixture of 20 µl was prepared by mixing 8 µl RNA, 4 µl Buffer, 1 µl of dNTPs, 1 µl of MMLV-RT, 2.5 µl random primers, 0.5 µl (RNasin® Ribonuclease inhibitor) and 3 µl DEPC water. Incubation of the reaction mixture in a water bath at 37°C for an hour and then in PCR Machine (BIO-RAD T100 TM thermal cycler) at 55°C for 5 min. Nanodrop ND1000 (Thermo-scientific, USA) was used to evaluate the concentration of synthesized cDNA in each sample. The prepared cDNA was immediately stored at a temperature of -20°c.

2.10.3 Primer Designing

Primers shown in (Table 8) were self-designed and Oligo Primer Analysis Software version 1.1.2 was used for Primers designing and manufactured from Humanizing Genomics Macrogen. The nucleotide sequence of the corresponding genes of Nile tilapia was obtained from the gene bank NCBI (www.ncbi.nlm.nih.gov). The quality of cDNA and its compatibility with primers was checked by performing a simple PCR followed by gel electrophoresis of each sample in duplicate. Afterwards, qPCR was performed by following the protocol previously, reported by Ahmad et al. (2020). Briefly, a 20 µL reaction mixture was prepared by mixing 0.4µl of reverse and 0.4µl of forward primer with 7.6µl of syringe water, 10µl of SYBER green and 1.6µl of diluted cDNA. The PCR conditions were optimized along with cycle numbers (initial denaturation at 95°C for 4 min followed by 40 cycles at 95°C for 15 seconds and subsequently, 62°C for 15 seconds). The efficiency of PCR reaction for each gene was measured by the slope of a standard curve using serial dilutions of cDNA of a control sample. The mRNA levels of each gene were compared with the expression of β-actin reference gene of Nile tilapia. The relative variations in gene expression were calculated by the standard ΔΔCT method (Pfaff, 2001).

2.11 Statistical Analysis

The results of the experiment were expressed as mean ±SE. The unpaired T-test was used to determine the effect of Ashwagandha roots powder supplemented on growth performance and cold shock effect on hematological indices, metabolic enzymes and cortisol level. Moreover, one way ANOVA followed by LSD test was used to compare the effect of Ashwagandha roots powder supplemented diet on the antioxidant enzymes status in liver and kidney of the fish.). Graph Pad Prism Version 8 was used to plot graphs.

RESULTS

3.1 Growth

The presently study reveal significant effect of Ashwagandha root supplementation on the growth performance of *O. niloticus*. The results of Unpaired T test indicated a significantly higher net weight gain, specific growth rate and final weight gain in D_2 compared to D_1 (Table 3).

3.2 Hematological analysis

Analysis of blood of the treated group (cold shock for 2 h) showed a significant positive effect of Ashwagandha root powder on hematology. Unpaired T-test revealed significantly ($p>0.001$) high RBC hemoglobin (Hb), hematocrit (Hct), mean corpuscular hemoglobin (MCH) and (%), mean corpuscular volume (MCV) in the D_2 group, however, MCH in D_1 and D_2 show no significant difference which suggest the positive impact of Ashwagandha inclusion on the overall hematological indices (Table4).

3.3 Blood biochemical Indices

The ashwagandha root powder supplemented diet showed significant positive effect on the liver metabolic enzymes, AST, ALT and LDH. The D_2 group showed significantly low level of these enzymes compared to D_1 group.

3.4 Antioxidant activity

Antioxidant activity in both kidney and liver revealed significant variation in response to the experimental feed.

3.4.1 Catalase

The study revealed significant Diet×organ interaction in term of CAT release, ($p<0.001$). Furthermore, release of CAT was also significantly related to diet ($p<0.001$). All pairwise comparison indicated significantly higher level of CAT in the both kidney and liver tissues of D_2 group compared to D_1 group. Similarly, organ wise comparison indicated higher level of CAT in liver compared to kidney.

3.4.2 Superoxide Dismutase (SOD)

One way ANOVA revealed a significant Diet× organ interaction in terms of SOD release ($p>0.001$). Furthermore, diet showed significant effect on the SOD release (0.001). Significantly

low level of SOD was observed in the both tissues of D_2 group compared to D_1 group. Moreover, pairwise comparison among organs indicated higher level in liver of both groups compared to kidney.

3.4.3 Peroxidase

In term of POD release, our study showed significant diet ×organ interaction (Univariate Analysis ($p<0.001$). Likewise, release of POD was also found significantly related to diet ($p<0.001$) POD release in both liver and kidney of D_1 group was significantly ($p<0.001$) low level compared to D_2 group. The pairwise comparison among organs indicated significantly low level in kidney compared to liver tissues.

3.4.4 Lipid Peroxidase

In terms of LPO release, the study found a significant interaction between diet and organs ($p< 0.001$). Additionally, feed was substantially correlated with LPO release ($p<0.001$). In addition, LPO release in both organs was altered significantly ($p<0.001$). Significant variation in LPO was found in all groups according to one-way Anova ($p=0.01$). The results of the Multiple Comparison Tucky test indicated that LPO level was significantly higher in the liver of D_1 group followed by kidney of the D_1 group compared to LPO level in kidney and liver of D_2 group.

3.5 Plasma Cortisol

In the present study, results showed significant variation in plasma cortisol level in response to addition of ashwagandha root powder in basal diet of *O. niloticus*. Unpaired T- test revealed significantly ($P<0.0001$) high cortisol level in D_1 compared to D_2, which suggest the positive impact of ashwagandha root powder on cortisol release in fish in response to cold shock while releasing less stress hormone cortisol in treated group.

3.6 Brain CRH expression

The inclusion of ashwagandha in the feed of Nile tilapia has significantly effected CRH expression in the brain. Significantly, higher CRH expression was observed in absence of ashwagandha D_1 while in D_2 the CRH was observed comparatively low (Fig 8).

Table 1. Ingredient and Feed Formulation for Experimental Study

Ingredient	CP per inclusion	D_1	D_2
Fish meal	9.6	16	16
Soybean	7.2	15	15
Rice polish	1.8	15	15
Wheat flour	1.8	15	15
Sun flower	5.1	15	15
Corn Gluten	7.2	12	12
Fish oil	0	6	6
Min and Vit mix	0	2	2
Vit C	0	2	2
CMC		2	2
Root powder		0	15

In D_2 1.5g of Ashwagandha root powder was added per 100g of basal diet

Table 2. Proximate Composition Analysis of the basal and experimental diets

Proximate composition	**D_1 (g)**	**D_2 (g)**
Moisture	10.23	10.27
Crude protein	32.7	32.24
Crude lipids	13.43	13.32
Crude fiber	7.06	7.34
Total ash	14.14	14.11
NFE	22.44	22.72

Table 3. Growth performance of *O. niloticus* after feeding test diets for 80 days

Parameter	D_1	D_2	F value	P-value	SEM
Initial body weight (g)	90.1	90.3	0.75	<0.01	0.40
Final body weight (g)	132.4	172.4	2.801	<0.01	1.41
Weight gain (g)	42.2	82.1	2557	<0.01	0.001
SGR (%)	0.01	0.14	1681	<0.01	0.05
FCR	0.933	0.716	2113	<0.01	0.101

Table 4. Hematological Analysis of *O. niloticus* after feeding Ashwagandha Root Powder supplemented diet for 80 days and then exposure to cold Shock for 2 hours

Parameters	D_1	D_2	P-value	SEM
RBCs($\times 10^{6/\mu l}$)	2.818	6.159	0.01	0.008
WBCs ($\times 10^{3/\mu l}$)	11.69	11.9	0.001	0.16
HCT (%)	35.17	42.78	0.01	0.11
HB(g/dl)	11.18	15.89	0.01	0.03
MCH (pg)	27.63	37.81	0.01	0.02
MCV (fL)	74.30	76.38	0.01	0.02
MCHC (g/dl)	30.14	30.14	0.99	0.07

Table 5. Blood Serum Biochemical Indices of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposure to cold Shock for 2 hours

Groups	D_1	D_2	P-value	SEM
AST(U/L)	21.15	19.09	0.01	0.019
ALT(U/L)	71.20	68.03	0.01	0.03
LDH(U/L)	23.42	22.35	0.04	0.21

Data is presented as Mean $\pm$ SE (n=3). Unpaired T-test is applied on AST (Aspartate amin transferase), ALT (Alanine amino transferase) and LDH (Lactate dehydrogenase) activity of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old *s*hock for 2 hours.

Table 6. Antioxidant Activity in Liver of *O. niloticus* after feeding Ashwagandha Root Powder supplemented diet for 80 days and then exposed to cold shock for 2 hours

Groups	D_1	D_2	P-value	SEM
CAT (µmol/min/mg)	258.7	324.4	0.01	± 0.40
SOD (µmol/min/mg)	535.4	1139	0.01	± 4.23
POD (µmol/min/mg)	2498	2458	0.01	± 0.57
LPO (µmol/min/mg)	159.9	114.7	0.01	± 0.19

Represents the CAT (Catalase), SOD (Super oxide dismutase), POD (peroxidase), and LPO (Lipid peroxidase) activity in liver tissues of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old *s*hock for 2 hours.

Table 7. Antioxidant Activity in Kidney of *O. niloticus* after feeding *Ashwagandha root* powder supplemented diet for 80 days and then exposed to cold shock for 2 hours

Groups	**D_1**	**D_2**	**P-value**	**SEM**
CAT (µmol/min/mg)	202.2	230.0	0.01	± 0.3
SOD (µmol/min/mg)	438.9	923.0	0.01	± 5.8
POD (µmol/min/mg)	808.5	660.2	0.01	± 0.40
LPO (µmol/min/mg)	111.3	106.1	0.01	± 0.13

Represents the CAT (Catalase), SOD (Super oxide dismutase), POD (peroxidase) and LPO (Lipid peroxidase) activity in kidney of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old *s*hock for 2 hours. Unpaired t-test applied to show the comparison between groups.

Table 8. Target genes and sequence of Primers

GENE	SEQUENCE	Tm	GC%	ACCESSION NO
B-actin	F<AAGGGAGGTATTGTGGGTAAAC	57.69	45.45	**XM_051108628.1**
	R<GTTGTCCTGGCACTCAATCT	57.69	50	
CRH	F< GACTCGAACTCTTTCCCATCAA	58.07	45.45	**XM 003443615.5**
	R<TTCCCAACTTTGCCCTGTAA	57.24	45	

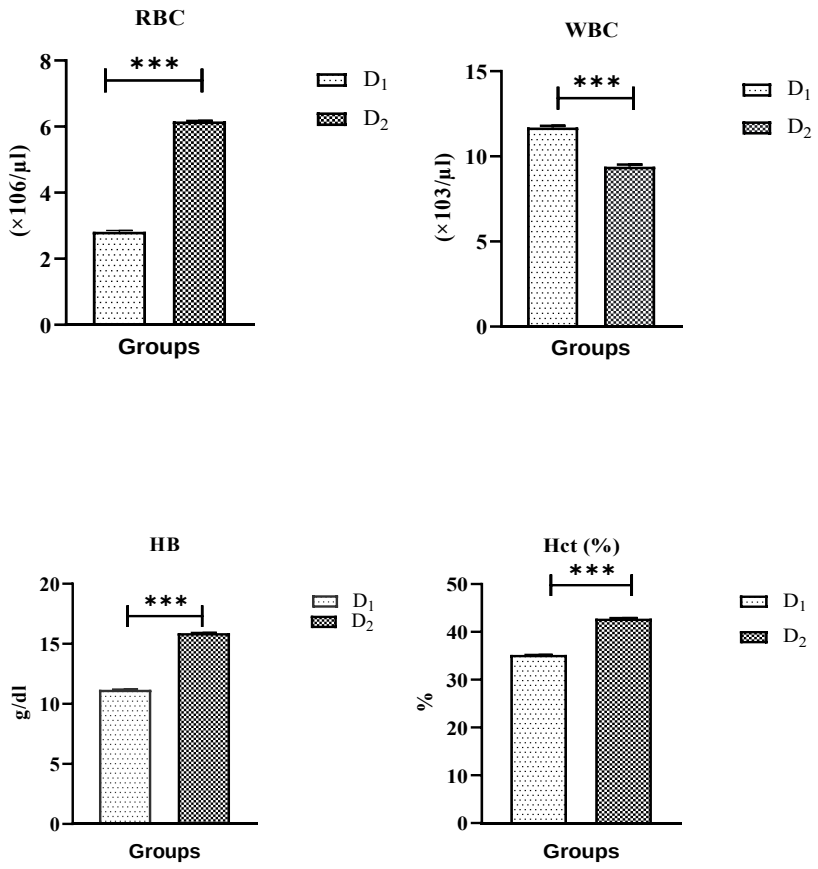

Figure 1. Represents the Hematological indices i.e., RBC (Red blood cells), WBC (White blood cells), HB (Hemoglobin), Hct% (Hematocrit) of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old *s*hock for 2 hours. Mean ± SE (n=3) *represent level of significance where: *** is P<0.001.

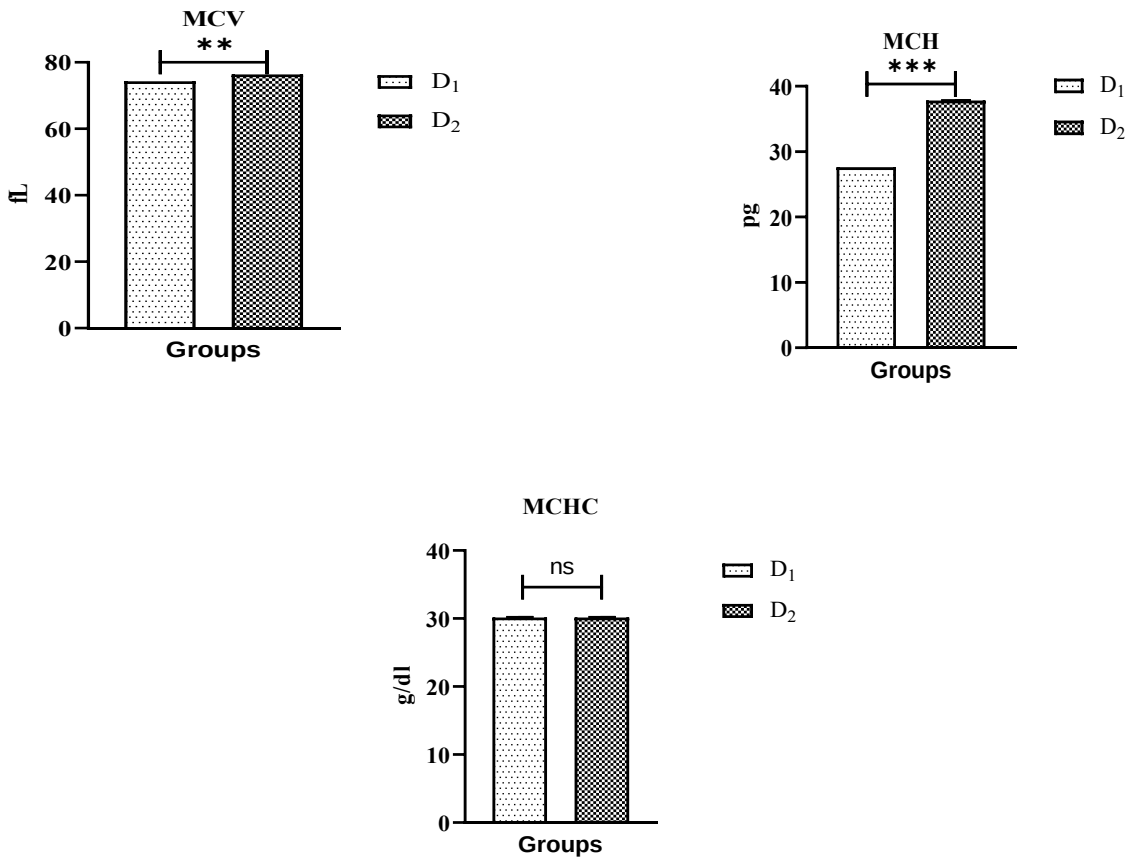

Figure 2. Represents RBC indices i.e., MCV (Mean corpuscular volume), MCH (Mean corpuscular hemoglobin), and MCHC (Mean corpuscular hemoglobin concentration) of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old *s*hock for 2 hours.

Mean ± SE (n=3), ns (non-significant), **represent $p<0.01$ and *** represent $p<0.001$.

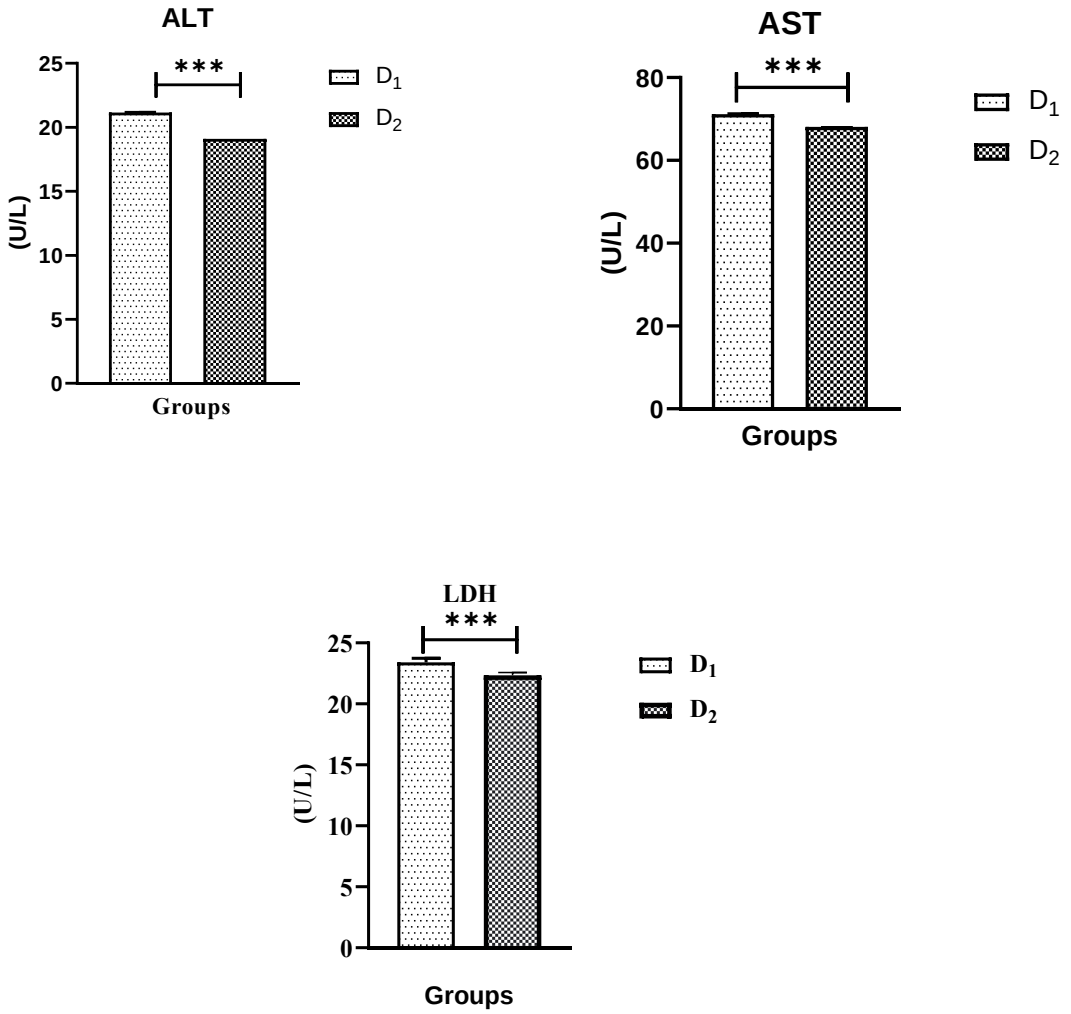

Figure 3. Represents the biochemical indices i.e., AST (Aspartate amin transferase), LDH (Lactate dehydrogenase), and AST (Alanine amino transferase) activity of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old *s*hock for 2 hours.

Mean ± SE(n=3) *represent level of significance where: *** is $p<0.001$.

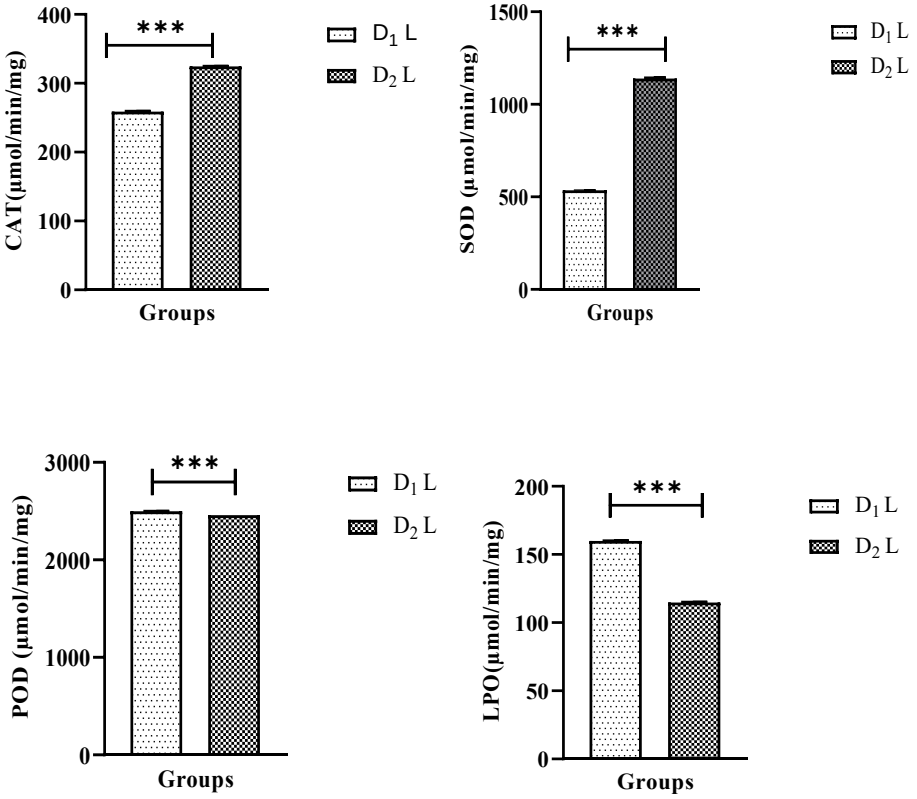

Figure *4.* Represents antioxidants activity in liver of *O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old *s*hock for 2 hours Mean ± SE(n=3) *represent level of significance where: *** is P<0.001.

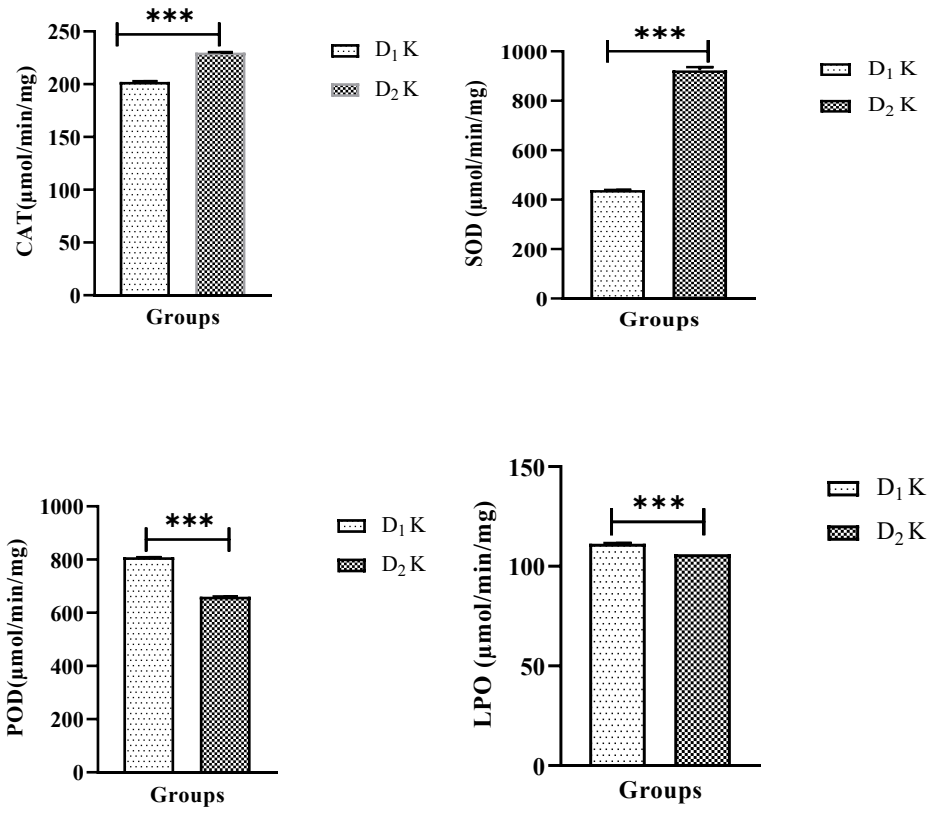

Figure 5. Represents the antioxidants activity in kidney of *O. niloticus after feeding* Ashwagandha root powder supplemented diet for 80 days and then exposed to *c*old shock for 2 hours.

Mean ± SE(n=3), *represent level of significance where: *** is P<0.001.

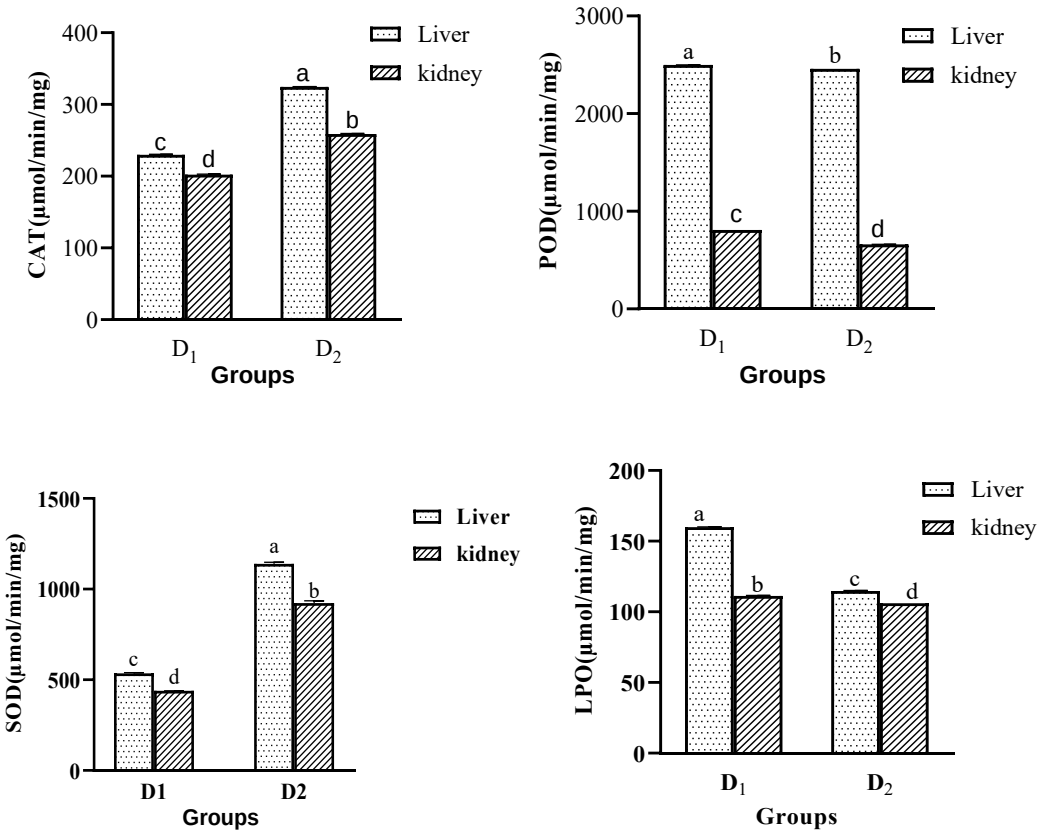

Figure 6. Comparative antioxidant activity in liver and kidney *of O. niloticus* after feeding Ashwagandha root powder supplemented diet for 80 days and then exposed to cold shock for 2 hours.

D_1 without Ashwagandha and D_2 with supplementation of Ashwagandha. one-way ANOVA was conducted to compare the overall comparative activity. alphabets above bar graphs represent Tuckey test analysis with significant difference ($p<0.05$).

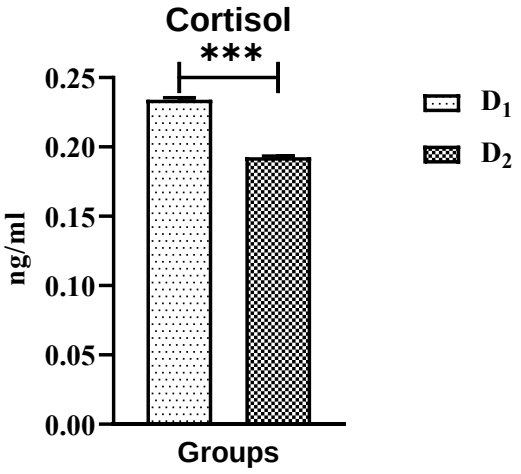

Figure* 7*: Represents the plasma cortisol concentration *O. niloticus* after feeding Ashwagandha Root Powder supplemented diet for 80 days and then exposed to cold shock for 2 hours. n= 9*represent level of significance where: *** is P<0.001.

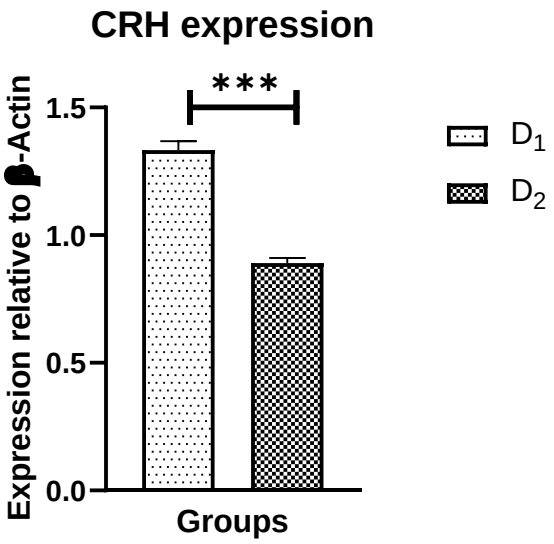

Figure 8: Represents the CRH expressions of *O. niloticus* after feeding Ashwagandha Root Powder supplemented diet for 80 days and then exposed to cold shock for 2 hours.

n= 9*represent level of significance where: *** is P<0.001

DISCUSSION

Global warming is associated with almost all life activity in one way or another. Thermal change of water is one of such issue which severely affect aquatic species (Reid *et al*., 2022). Seasonal variation in temperature has been shifting with time where water temperature caused the most of concern, as many of the species cannot tolerate thermal change, cold stress, heat shock etc. *O. niloticus* is the prominent model species which cannot survive cold environmental conditions (Zhou *et al*., 2019). Temperature variations can affect several elements of fish metabolism and physiology, blood parameters, growth, and survival (Dellagostin *et al*., 2022). Certain techniques has been studied/applied including, breeding studies, encorporation of various dietary additives (Shi *et al*., 2015; Sifa *et al*., 2002; Van Hai, 2015). Solving this issue with dietary manipulation is the most simple, applicable and easy technique. Various plants has been used in this regard; Propolis (Islam *et al*., 2021), linseed, sunflower, olive and coconut oils (Correa *et al*., 2017), astragalus membranaceus (Elabd et al., 2020), propylene glycol (Soaudy *et al*., 2021), curcumin (Mahmoud *et al*., 2017) and chia seed powder (Mahmoud *et al*., 2023), ashwagandha, Indian herb, is reported to have anabolizing and virilizing effects (Raju et al., 2017; Dar *et al*., 2016).

The present study was conducted to evaluate the pottential role of Ashwaganda root podwer as dietary additive in feed and determine their effects on growth performance, hematoloical indices, biochmeical indices, antioxidant activity and plasma cortisol hormone release under cold stressed condition in *O. niloticus.* Ashwagandha root was previously reported as principal phytoconstituent contain Withanolides, a group of Phyto-steroid present, have been reported to possess immunostimulatory activity. Compounds such as flavonoids and alkaloids associated with antioxidant activity, saponin and glycosides associated with growth, hematological modulation and stimulation of hormone secretion, triterpenoids associated with promoting muscle growth and phytosterol associated with elevating plasma vitellogenin level were observed to be present in different solvent extracts of *W. somnifera* roots (Lopresti *et al*., 2019).

The positive impact of Ashwagandha root on growth (observed in D_2) may be associated with the presence of extracts, especially tannin, saponin, alkaloids, steroids, and terpenoids, which were reported as promoter to muscular growth (Sharma *et al*., 2017). Similar positive impact on

growth parameters were found in *L. rohita* to *W. somnifera* fed at 0, 0.1, 0.2 and 0.3 g/100 g feed for 42 days (Sharma *et al*., 2017). Engy *et al*. (2017) recorded significant higher growth rate in a group of *W. somnifera* 4% supplemented feed during the 28-day experimental trail in *O. niloticus.* Our results are in agreement with previous study conducted by Borkar *et al*. (2014), who observed an improvement in body weight of fresh water fish Channa punctatus supplemented with different levels of *W. somnifera*. Mukherjee *et al*. (2019) found that Nile tilapia fed on diets supplemented with varying doses of ethanolic and methanolic extracts of *W. somnifera* roots for 30 days significantly enhanced growth parameters, especially at the treatment of 0.7 g/kg feed. Those authors linked their results with the beneficial components present in WS extracts, especially tannin, saponin, alkaloids, steroids, and terpenoids, which are associated with promoting muscle growth.

Thermal fluctuations can trigger compensatory metabolic changes at the cellular and molecular levels, resulting in considerable alterations in blood chemistry (Portner & Peck, 2010). One of the natural stressors is change in water temperature that directly affect the survival of aquatic animals such as fish, shell, shrimp *etc*. When the fish face the water temperature change. In responses, most fish exhibited altering physiological and ethological functions (Panase *et al*., 2018). In our results, the variations in blood hematological indices including erythrocytes, hemoglobin concentration, hematocrit concentration, MCH and MCV showed significant($p<0.001$) increase in D_2 while MCHC indices were not significantly affected by ashwagandha supplementation. the same trend was also observed by Sharma *et al*. (2017) in *Labeo rohita* fed with *W. somnifera* root powder. Significantly increased ($P<0.001$) RBC, WBC, hemoglobin concentration and hematocrit) in fish fed diet fortified with ethanol WS 0.7 g kg-1 feed was reported by (Mukherjee *et al*., 2019).

Mahmoud *et al*. (2023) also found a linear increase in red blood cells (RBCs), white blood cells (WBCs) counts and hemoglobin ($P < 0.0001$) of fish was also achieved with increasing chia seed powder level under cold stress in *O. niloticus*. In other mod\el species a similar, supportive evidence were observed, in mice liner increase in hemoglobin concentration ($P < 0.01$), red blood cell count ($P < 0.01$), white blood cell count ($P < 0.05$), platelet count ($P < 0.01$), and body weight ($P < 0.05$) was observed in Ashwagandha-treated mice as compared with untreated (control) mice investigated by (Ziauddin *et al*., 1996). Panase *et al*. (2018) reported the effect of rapid Total red

blood cell, hematocrit percentage, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin were at the lowest level at 13 °C. Then all parameters were increased meanwhile, total white blood cell showed significantly increased from 25 °C to 13 °C. likely of our results the temperature effect our control group which were feed with basal diet the blood hematological profile, however increase in blood profile parameters might be due to phytoconstituents of *W. somnifera*. To the best of our knowledge, there are no studies concerning the effect of propolis-extract and the values of Hematocrit (Hct%) and hemoglobin (Hb) in fish exposed to low temperature conditions.

In the present study, the addition of dietary *W. somnifera* root powder promoted a significant improvement in the Hematocrit and Hb levels compared to control diet. The decrease in the values of Hb in the control group may be the result of erythropoiesis or shrinking of red blood cells, as well as dependency on bound oxygen transport as a result of lower oxygen requirements at low temperature and increased solubility of oxygen in plasma. There may be other reason of the change in results as genetic factor, size, sex, population density, seasonal variations, environmental stress, and many other factors could affect hematology data (Qrun & Erdemli, 2002; Aronold, 2005; Kori-Siakpere *et al.,* 2005).

When fish are subjected to cold stress, blood serum indices such as alanine transaminase, aspartic transaminase, and lactate dehydrogenase levels rise, indicating that the liver is stressed, injured, and impaired, which causes a significant release of ALT and AST into the bloodstream. (Liu *et al*., 2007). In present study, significantly high level of ALT, AST was observed in D_1, although, LDH shows comparatively less significant (0.0004) concentration in fish under cold stressed conditions. The low level of AST and AST in D_2 group indicates positive impact of ashwagandha. In group which was feed without WS showed higher activity of ALT and AST level may indicate leakage of enzymes across damaged plasma membranes and/or increased synthesis of enzymes by the liver (Yang & Chen, 2003). Plant based diet play vital role in release of metabolic enzymes of liver. Mahmoud *et al*. (2023) investigated significantly reduced linear, ($P = 0.002$) ALT and AST serum concentrations in CSP- fed *O. niloticus* under cold stress. Hassaan *et al*. (2019) recorded significantly decreased levels of ALT ($P = .001$), AST ($P = .001$) and lactate dehydrogenase ($P = .002$) with increasing dietary propolis-extract levels in the diet.

The oxidative stress is defined as an imbalance in the presence of ROS and antioxidant capacity (i.e., the ability to neutralize ROS) in favor of the former, resulting in oxidative stress. (Biller & Takahashi, 2018; Birnie-Gauvin *et al*., 2017; Reid *et al*., 2022). Cold shock and stress are commonly related to oxidative stress (e.g., increased lipid peroxidation), which promotes tissue-specific increases in antioxidant enzyme activity and altered metabolic processes. Oxidative stress can be measured by antioxidant activity [*e.g*., activity of antioxidant enzymes such as SOD, CAT and LPO (Birnie-Gauvin *et al*., 2017).

According to He *et al*. (2015), superoxide dismutase, glutathione peroxidase, catalase, and glutathione levels in the liver of GIFT *O. niloticus* enhanced under acute low temperature stress. In contrast, heat-stressed *O. niloticus* fed diets supplemented with 1 or 3% had higher concentrations of SOD and CAT indicating a notable improvement of their oxidative status. These favorable results could be highly linked to antioxidant constituents of dried Rocket Leaves meal (DRLM) including vitamin C, carotenoids, flavonoids, glucosinolates, and volatile oils (Barillari *et al*., 2005; Hanafi *et al*., 2010). SOD activity in liver and muscle tissues exhibited significant increase in both *Withania* supplemented groups compared to the control in post challenged period. (Zahran *et al*., 2018).

In our study we have investigated the antioxidants status in liver and kidney tissues. There were significantly high levels of CAT antioxidant in D_1 L as compared to D_2 L CAT levels. Also, the D_1 K CAT concentrations were high in relation to the D_2 K CAT levels. While a significant increase ($p<0.05$) in SOD activity was observed in D_2 L when compared to D_1 L SOD levels. In addition, D_2 K SOD levels were detected to be significantly higher than that of D_1 K SOD levels. There was comparably no significant variance among D_1 L and D_2 L POD levels ($p>0.05$). Also, D_1 K and D_2 K showed no significant difference in the POD levels when compared to each other. In the present study, the highest levels of LPO were observed in D_1 L which were significantly higher than the LPO levels of D_1 K, D_2 L, and D_2 K. While the LPO levels in D_1 K, D_2 L, and D_2 K showed no significant variation ($p>0.05$). A study was performed on rats where they administered *W. somnifera* extract for 15 days showed an antistress activity and an increase in SOD levels. Also, the same pattern was observed in LPO activity as well (Bhatnagar *et al*., 2005).

In another study performed in mice, 30 days treatment with WS root powder produced a significant ($p<0.05$) decrease in LPO, and a significant increase ($p<0.05$) in SOD antioxidant activity (Panda & Kar, 1997). In yet another study the Mitomycin C induced damage in mouse bone marrow and depressed antioxidant enzymes activity (SOD) was enhanced as result of treatment with WS root powder (Khanam & Devi, 2005). A group of researchers working on iron-induced hepatotoxicity in rats found that *Withania somnifera* glycowithanolides administration caused an increase in SOD activity and a decreased activity of CAT antioxidant (Bhattacharya *et al*., 2000). The POD antioxidant activity of present study was found to be in contradiction with previously performed studies where there was seen an increase in POD antioxidant activity.

Stress responses and preservation of homeostasis increase energetic costs (Ahmad *et al*., 2011). Cortisol is the principal glucocorticoid secreted under stress condition by the internal tissue located in the head-kidney of teleost fish. Thermal stress has significant effects on cortisol levels in fish, which is a hormone that plays a key role in the stress response in these animals. Cortisol is commonly known as the "stress hormone" because its levels increase in response to various stressors, including changes in water temperature (Chadwick Jr *et al*., 2015). When fish are exposed to thermal stress, their cortisol levels typically increase. This increase in cortisol is part of the physiological response to stress that allows fish to respond to the changes in their environment. For example, cortisol can help regulate metabolism, mobilize energy stores, and modulate the immune system to help the fish cope with the stressor (Reid *et al., 2022)*. Elevated cortisol levels can indicate that a fish is under physiological stress and can lead to a number of negative impacts, including reduced growth and reproductive performance.

In this study we measured plasma cortisol as a primary stress response biomarker. In the acute cold stress condition, cortisol levels were significantly lower in D_2 compared D_1 group. In accordance to study by Khan *et al*. (2022), *O. niloticus* showed positive effect of elderberry extract under stressed condition. In our finding there was significantly ($P<0.0001$) high cortisol in D_1 compared to D_2. In support of our results, the same pattern was observed in *O. niloticus* cultured under winter thermal stress), which was nutritionally mitigated by propolis extract supplementation to the die (Hassaan *et al*., 2019). A similar trend of lower cortisol release under cold stress was observed in *O. niloticus* through nutritional modulation of via chia seed powder (Mahmoud *et al*., 2023). In our results, the low-level cortisol in fish feed with D_2 under cols stress,

might be due to stress-reducing components which effects might the hypothalamus-pituitary-adrenal axis (Lopresti *et al*., 2019).

4.1 Conclusion

From present study, it is concluded that Ashwagandha enhances growth while also improve hematological and biochemical parameters. Also, provide protection against thermal induced stress as evident from cortisol level, CRH and anti-oxidant activity in liver and kidney after feeding ashwagandha in the feed. It has been concluded that *W. somnifera* has a positive impact on the cortisol release under cold stress in *O. niloticus.*

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