Effects of Ashwagandha (W*ithania somnifera*) roots and leaves Powder on Immunity and Male Fertility of Nile tilapia (*Oreochromis niloticus)*

By

Waliullah

Department of Zoology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad
2022-2023

Effects of Ashwagandha (W*ithania somnifera*) roots and leaves Powder on Immunity and Male Fertility of Nile tilapia (*Oreochromis niloticus*)

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

MASTER OF PHILOSOPHY
IN
FISHERIES AND AQUACULTURE

By

Waliullah

Department of Zoology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad
2021-2023

CERTIFICATE

This dissertation**''Effects of Ashwagandha (W*ithania somnifera*) roots and leaves powder on immunity and male fertility of Nile tilapia (*Oreochromis niloticus*)'' submitted by Waliullah 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 Fisheries and Aquaculture.**

Supervisor:

_____

Dr. Amina Zuberi

External Examiner: _____

Chairman _____

Professor Dr. Amina Zuberi

Date: _____

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.

Waliullah

Dedicated to:

This thesis is dedicated to my loving parents, siblings, respected supervisor and my beloved friends for their advice, patience and faith in me

Table of Contents

List of Abbreviations

°C	Celsius
µL	Microliter
µM	Micromolar
ALT	Alanine transaminase
ANOVA	Analysis of variance
AST	Aspartate transaminase
BC	Blood congestion
C	Curling
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
cDNA	Complementary deoxyribose nucleic acid
CE	Cellular edema
Cm	Centimeter
COVID-19	Corona virus disease 2019
CQ	Chloroquine
CV	Central vein
DEPC	Diethyl Pyrocarbonate
DN	Degenerative necrotic
dNTPs	Deoxyribonucleotide triphosphate
DO	Dissolved oxygen
DQ	Desquamated
EDTA	Ethylenediaminetetraacetic acid
EL	Epithelial lifting
F	Fusion
g	Gram
g/ml	Gram per milliliter
GF	Gill filaments
GL	Gill lamellae
H	Hepatocytes
H_2O	Water
Hb	Hemoglobin
HCQ	Hydroxychloroquine

HCT	Hematocrit
HN	Hepatocyte nucleus
Hp	Hyperplasia
Hrs	Hours
HT	Hypertrophy
Ig M	Immunoglobulin
IL-1β	Interleukin 1 beta
Kg	Kilogram
L	Liter
L. rohita	*Labeo r*ohita
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.
ND	Hepatocyte nuclear degeneration
ND	Nanodrop
Nm	Nanometer
nmol	Nanomole
O_2	Oxygen

OE	Edema
P	Probability
PCR	Polymerize chain reaction
pH	Power of hydrogen
POD	Peroxidase
RNA	Ribonucleic acid
ROS	Reactive oxygen specie
Rpm	Revolution per minute
S	Sinusoid
SE	Standard error
SOD	Superoxide dismutase
SS	Sinusoidal space
TBARS	Thiobarbituric acid reactive substances
TNF-*α*	Tumor necrosis factor
U/L	Units per liter
V	Vacuolization
***β*- actin**	Beta actin

List of Tables

List of Figures

ACKNOWLEDGEMENTS

*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.*

*I am very grateful to my worthy Supervisor **Professor Dr Amina Zuberi** for her excellent mentorship, support, advice and encouragement. Her worthy comments in meetings increased my knowledge and experience, and her keen insight, great experience, and thoughtful reflection on the different aspect of research are outstanding abilities to which I aspire.*

*I am especially indebted to **Mr. Mashooq Ali** and **Mr. Waqar Younas** for making me this research work possible. I am also thankful to **Dr. Imdad Ullah, Muhammad Noorullah khan, Adnan Khan and Muhib Zaman** who was always with me in field work to support, encourage and appreciate me.*

*I am thankful to my class fellows, friends and juniors for their help and support. Special thanks to **Sadia Meo, Dua Laraib, Ashiq Hussain, Muhammad Alam, Muhammad Shahid, Muhammad Saeed, Andleeb, Shehla, Maryam and Samina** for their practical and moral support given to me during the M.Phil. They helped me a lot and I am unable to forget their support.*

I pay my thanks to the whole faculty of my department especially the respected teachers whose valuable knowledge, assistance, cooperation and guidance enabled me to take initiative, develop and furnish my academic carrier.

I also acknowledge the help, encouragement, support and prayers of my family especially my parents and siblings who have always been there to help and support me. I cannot forget their love, affection and care.

May ALLAH bless all of them "Ameen"!

"Waliullah"

Abstract

The Ashwagandha (*Withania somnifera)* plant contains a variety of bioactive compounds and their derivatives, making it a hematopoietic, natural antioxidant, and immunostimulant having aphrodisiac activity. In the current study, an effort was made to investigate the effect of Ashwagandha (*W. somnifera)* root and leaves powder supplemented diet for 80 days experimental trial on the immunity and fertility of male Nile tilapia (*Oreochromis niloticus*). The study was conducted in replicate of three and 21 fiber tank (500-liter capacity) were randomly divided into 7 group. A control group fed 32% CP diet without any supplement, while L5, L1. L5, L10, L15 groups were fed diets supplemented with graded level of *W. somnifera* leaves powder. Similarly, R5, R10, R15 were fed diet supplements with similar supplements of roots powder. The result indicated significant ($P<0.05$) effect of both supplemented forms (leaves and root powder) on growth performance (WG, SGR), FCR, antioxidant enzymes of testicular tissues, immunity, hematological indices and hormonal profile (Cortisol and testosterone) of *O. niloticus*. However, (R15 group) 15g of root show more significant ($P<0.05$) effect on growth performance compared to all other groups. The antioxidant enzymes of testicular tissues, immunity, hematological indices and hormonal profile were significantly ($P<0.05$) effect by 15g of root powder. Comparison between leaves and root powder indicates the most significant effect of root powder. Moreover, root powder shows dose dependent effect on all studies parameters while leaves show variable effect on different parameters. Therefore, it is concluded that root powder could be used to improve the immunity and fertility of Male Nile tilapia.

INTRODUCTION

Aquaculture is the rearing and production of aquatic organisms like fishes and some invertebrates including crustaceans, bivalves and sea weeds under controlled or semi controlled conditions. Globally it is the fast growing aquatic food production sector and is also known as aquafarming (Cai & Leung, 2017; FAO, 2018). According to Stickney, (2006) aquaculture may be defined as is the rearing of aquatic organisms under controlled or semi-controlled conditions, particularly in Asia, has made the greatest contribution to world production volumes and food security, which accounted for 92% of the fish farming and seaweeds in 2017 (Naylor *et al*., 2021) as the world's population rises food security is a top need for development and survival. According to United Nations estimates, the world's population would grow by more than 1 billion between 2017 and 2030. Over the next 20 years, it is estimated that population explosion and urbanization would increase the demand of animal protein sources including meat (Tsakiridis *et al*., 2020). Therefore, the production of aquatic food is important for ensuring cheap food security and generating income both locally and globally (Cochrane *et al*., 2009).

Global Production of Fisheries and Aquaculture

Aquaculture and fisheries output (excluding algae) has increased dramatically over the last few decades, rising from 19 million tonnes in 1950 to an all-time high of almost 179mt in 2018, with a 3.3 % annual increase. Production then fell slightly in 2019 by a decrease of 1%, before rebounding by only 0.3 percent to 178mt in 2020 (Boyd *et al.,* 2022). However, recent data reveals that the global fisheries and aquaculture production in next years would be increase at the rate of 1.5% up to 184.6 million tonnes per annum**.** Fisheries and aquaculture production in 2020 was projected to have a sale value of 406 billion USD of which 265 billion USD came from aquaculture **(**FAO, 2022**)**. Even though the production of marine and freshwater fish is expanding quickly but freshwater aquaculture still dominates the industry. In addition, 75% of the volume of worldwide edible aquaculture is constituted of freshwater fish (Edwards *et al.,* 2019).

Aquaculture and Fisheries sector in Pakistan

Pakistan is an agricultural country with abundant natural resources of both fresh and marine water for aquaculture sector. There are rivers, lakes, ponds, and other water bodies covering approximately 8,563,820 km^2 (Jarwar, 2008). These water resources are regarded as suitable for aquaculture. With an 1120 km coastal line, there is a lot of opportunity for coastal

fishing, and addition to this Shrimp farming is an important industry in Pakistan. The country has a long coastline along the Arabian Sea, which provides ideal conditions for shrimp farming. In recent years, the shrimp farming industry in Pakistan has grown rapidly (Hassan *et al.,* 2022). The country has become one of the world's leading exporters of farmed shrimp, with a focus on the export of black tiger shrimp. The major species farmed in Pakistan are the black tiger shrimp and the white leg shrimp (Mohsin *et al.,* 2017).

Furthermore, the state owns an open maritime region, known as the Exclusive Economic Zone (EEZ) of approximately 350 nautical miles. Despite these vast resources and opportunities for aquaculture, there is a little advancement in the fisheries and aquaculture sector of Pakistan (Zahra *et al.,* 2022). The less production of aquaculture is due to farmers negligence and awareness about the adoption of advanced extensive and semi-intensive techniques used in aquaculture systems. Coastal and deep-water marine sources, on the other hand, are still not used for aquaculture. As a result, from a marine perspective we are entirely dependent on natural availability. Therefore, the aquaculture farming system production is predicted to be between 179,900-180,000 from fish farming and 600,000 metric tonnes from natural catch (Mohsin *et al.,* 2017).

The future of Pakistan's fisheries sector is dependent on various factors such as sustainable fishing practices, proper management of fish stocks, investment in infrastructure and technology, and effective regulations and policies. Additionally, factors such as environmental conditions, population growth, and economic stability also play a role in shaping the future of the aquaculture sector (Nazir *et al*., 2021). Many countries like China, India Bangladesh and Vietnam have seen significant growth in aquaculture, owing mostly to developments in the technological advancement of fish production to meet the expanding global requirement for fish, as well as the ecological and economic viability of fish farming (Ogunfowora *et al.,* 2021).

Although in Pakistan there are some shortcomings like, lack of infrastructure and technology, limited investment, poor management and regulations, limited market access and lack of knowledge and skills (Shah *et al.,* 2018). In addition, some fish disease outbreak such as White spot syndrome virus (WSSV), Enteric red mouth disease (ERM) and Epizootic ulcerative syndrome (EUS) have a significant adverse effect on aquaculture production in Pakistan (Laghari, 2018). These outbreaks can occur due to poor water quality, overcrowding, and the use of weak and infected seed stock. To prevent and control disease outbreaks in aquaculture, it is important to implement good management practices, such as maintaining

water quality, practicing proper sanitation, and using disease-free seed stock. Additionally, prophylactic measures, effective monitoring, surveillance and disease diagnosis are crucial to detect early signs of disease and implement necessary action write the reference here (Nazir *et al.,* 2016).

Phytotherapy Alternative to Synthetic Drug in Fisheries and Aquaculture

The use of medicinal plants in aquaculture and fisheries is a relatively recent effort to combat the harms of chemical drugs. While the potential of herbs for their multifunctional biological components is encouraging (Immanuel *et al*., 2009). Medicinal plant can be utilized not only as treatments, but also as stress resistance booster, growth promoters and infection preventatives (Tadese *et al.,* 2022) as a result, herbal treatments in disease management are gaining popularity since they are cost-effective, eco-friendly and have less adverse effects on both environment and living organism (Mariappan *et al.,* 2023). For a range of disorders, a considerable segment of the global population, particularly in underdeveloped nations, relies on traditional medicine. Plants are key sources of strong and potent drugs, and several hundred genera are used medicinally. Secondary metabolites of phytochemical components found in plants, such as alkaloids, tannins, and flavonoids, protect against a variety of illnesses (Ravikumar *et al*., 2010).

For many years in China medicinal plants have been utilized as medication to cure various fish diseases because, mostly medicinal plant can act as immunostimulants and activates non-specific defensive systems of fish and improves the overall immune system of fish (Yin *et al*., 2008). Therefore, medicinal plants can be used as an alternative to synthetic drugs for fish health management. These herbs are not only safe for consumption, but they also play an important function in aquaculture. Various research has demonstrated that herbal supplements improve the health of fish and guard against illnesses (Awuchi *et al.,* 2022). The innate immune system of fish is thought to be the initial first line of defense against pathogenic infections. Many plant chemicals have been identified to have non-specific immunomodulatory effects in both humans and animals (Bahi *et al.,* 2023) and over a dozen tested in fish and shrimp (Savaya *et al.,* 2020). Several plant compounds seem to be potential antimicrobial agents against bacteria viruses found in shrimp and fish (Pandey *et al*., 2012). In addition, some medicinal plant also helps to stimulate spermatogenesis in male fish and gonadal maturation and high egg viability in female fish (Vijayakumar *et al.,* 2021). This helps to achieve natural viable spawners especially out of season, improves egg quality and fecundity (Bromage *et al.,*

1992). Aids better hormonal and cellular immune response, increases resistance to specific and non-specific infections, enhances the hepato-pancreas activity Dhas *et al.* (2015) and thereby, the efficient digestion and assimilation of feed nutrients maintains feed intake during inclement weather, natural and safe with no side effects for shrimp and fish (Michael, 1999).

Uses of Medicinal Plant in Aquaculture

In aquaculture sector, some of the plants used that have been found to have an immune-stimulant property are commonly used to improve the health and disease resistance of aquatic animals (Emeka *et al.,* 2014). Garlic (*Allium sativum*) is known for its antibacterial, antiviral, and antiparasitic properties, and it has been found to improve the immune response and disease resistance of fish and shellfish (Lee & Gao, 2012) and Grapeseed (*Vitis vinifera*) extract is rich in antioxidants and has been found to improve the immune response and disease resistance of fish and shrimp (Valenzuela-Gutiérrez *et al.,* 2021). Indian Gooseberry (*Phyllanthus emblica*) is a natural source of antioxidants, which can help protect fish from diseases and improve their overall health (Hoseinifar *et al.,* 2020).

Turmeric (*Curcuma longa*) is known for its anti-inflammatory properties and can be used to help reduce the severity of fish diseases such as *Aeromonas hydrophila* while Neem (*Azadirachta indica*) leaves are traditionally used as an antimicrobial agent, and they can also help to improve fish growth and reduce susceptibility to disease. (Mondal *et al*., 2021). Horny Goat Weed (*Epimedium spp.*) is believed to increase blood flow to the pelvic area, which can improve the health of the reproductive system in fish (Low & Tan, 2007), while Maca Root (*Lepidium meyenii*) boost energy and stamina in fish, which can improve their ability to breed (Gül *et al.,* 2022). Herb such as Saw Palmetto (*Sabal serrulata*) is considered to help with the production of sperm and eggs, which can improve fish fertility (Cronin, 2002). Shatavari herb help to regulate hormones such as testosterone and cortisol in fish, which can improve their ability to breed and medicinal plant like Gokshura (*Tribulus terrestrislinn)* is believed to improve the reproductive systems of fish (Omitoyin *et al.,* 2013).

Ashwagandha (*W. somnifera*) is not typically used in fish nutrition or aquaculture, but there are a few studies that have investigated its potential use as a supplement for fish (Pandey *et al*., 2012) and addition to this ashwagandha root powder could be used as a growth promoter for tilapia fish, increasing their weight and length. Dietary supplementation with ashwagandha (W. *somnifera*) improves the lipid profile and histomorphology of intestinal tissues in Nile tilapia (*Oreochromis niloticus*) and alters the inflammatory response to Bacterial infection (Zahran *et al.,* 2020). Similarly, (Srivastava *et al.,* 2020) also found that ashwagandha

leaf powder could improve the immune response and disease resistance of Rohu and Indian major carp. The crude combination of *W. somnifera* and *Mucuna pruriens* with other herbals significantly influenced the maturation and offspring quality of the spent spawners of *Penaeus monodon* (Citarasu *et al.,* 2002).

Ashwagandha (*Withania somnifera)*

The genus Withania Belonging to the family (Solanaceae) has twenty-three species, the majority of which are found in the Canary Islands, north Africa, Asia, and Southern Europe. According to Tole & Saifu, (2019) *W. somnifera* and *W. coagulans* are the most economically important species that are also widely farmed because of their widespread use in natural medicine. These species are mostly farmed in India's subtropical areas. However, *W. somnifera* is more economically significant (Mir *et al.,* 2011). Although many *Withania spp*. have been utilized in conventional medicine to treat a variety of diseases but *W. somnifera* specie is the most well-known type, not only for its economic value but also due to its medicinal potential, and they are highly commercialized and grown in Pakistan, Iran, India, and Afghanistan (Nayar, 1964).

Plants in the Withania genus are perennial, growing to heights of 0.5- 2.0 m, and can be found in grasslands, shrub suburbs having branching or unbranched stem possessing yellow or green flowers. They have simple, petiolate, ovate, alternating, or unequal pairs of leaves with sharp tips. Fruits are 6 mm in diameter, orange-red when ripe, globous, and surrounded by a green calyx. The seeds are tiny, compressed, yellow, smooth to reticulate, and extremely light (Uddin *et al.,* 2012). In terms of folk medicine, *W. somnifera* sometimes known as "winter cherry" are the most significant plant in the *Withania* genus and has the most well-known therapeutic properties. This plant traditionally has been utilized in Indian medicine, and its roots may be found in over 200 pharmaceutical formulas (Sandhya & Sushil, 1998).

W. somnifera (Ashwagandha, Indian ginseng) is the most well-known species, extensively used as a medicinal herb for a variety of maladies, including boosting the immunological and haematological systems, anti-inflammatory action that aids in skin disorders and osteoarthritis and antioxidative benefits. It is also used to treat diabetes, hypothyroidism, depression, stress and cardiovascular disorders known for its aphrodisiac properties (Bhattacharya *et al.,* 2001).

Chemical examination of several plant components of *W. somnifera* yielded a plethora of chemicals belonging to diverse chemical classes. Medicinal plants have been used to enhance the health of people and societies since ancient times indigenous medicinal plant

products generated from plant components such as leaves, seeds, barks, and stems have been used as a chemotherapeutic agent and have a specific physiological function on the human body. Active ingredients such as flavonoids, tannins, and phenolic chemicals are the most significant natural bioactive elements of plants (Alam *et al*., 2012). The ashwagandha plant is reported to have amino acids, reducing sugar alkaloids, steroids, volatile oil, and glycoside. The root is rich with starch ranging from 6.09-9.46mg/g crude fiber constituents up to 25%, tannins 0.39 to 0.82 mg/g, minerals such as Al, Zn, Na, K, Mn, Ni, Cd, Fe, Cu, Ca, total sugar ranging from 2.52 to 9.52 mg/g, non-reducing sugar ranging 2.37 to 7.62 mg/g, and reducing sugars ranging from 0.15 to 2.10 mg/g (Kaliyadasa *et al.,* 2018).

W. somnifera have some most important with anolides such as withaferin A, Withanolide D, 27-hydroxy withanolide B, 16β-acetoxy-6α, 7α-epoxy-5α-hydroxy-1-oxowitha 2, and steroids such as sitosterol, β-sitosterol, withanolide A, withanolide B. (Rohit & Sumita, 2012). Similarly, chlorinated withanolides 27-deoxy-16-en-withaferin A, 17-hydroxy withaferin A, 2,3-dihydro withanone-3β-O sulfate, 4-deoxyphysalolactone, withanolide A, withaferin A, 24,25-Dihydrowithanolide A, 2,3-dihydro-3β-hydroxy withanone, 27-hydroxy withanone, withanolide C, withanone from leaves of *W. somnifera* (Misra *et al.,* 2012).

Biological Activities

Given the numerous uses of Withania species in traditional medicine for a variety of purposes, research has gradually explored their biological effects. The biological effects of *W. somnifera*, notably its antioxidant capacity and phytochemical contents, differ depending on the process extraction technique used. According to Dhanani *et al.* (2017), *W. somnifera*, on the other hand, has been related to both anti-inflammatory and antioxidant activities, and it has shown good anti-AlCl3-induced neurotoxicity in rats (Elhadidy *et al.,* 2018). Many studies have shown that Ashwagandha has dramatic effects on the healthy development of white blood cells, indicating that it is a potent chemoprotective and immunoregulator drug. In mice research, a powdered root extract of *W. somnifera* was observed to increase total white blood cell count (Kashyap *et al.,* 2020). Furthermore, this extract hinders possible allergic responses such as delayed-type hypersensitivity and increased phagocytic activity of macrophage (Davis *et al.,* 2000) and antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterium strains (Ali *et al.,* 2001). In immature male Wistar rats, aqueous extracts of *C. coccineum* and *W. somnifera* showed a strong influence on testicular maturation and

increase the serum concentration of testosterone and gonadotrophins hormones (Abdel-Magied *et al.,* 2001).

In the current study Nile tilapia, a culturable fish is used for experimental purposes, which is cultured in more than a hundred countries across the globe (Lèveque, 2002). To address domestic and worldwide market needs Tilapia fish farming is presently rapidly expanding over the world to supply a low-cost source of fish protein. Even though other tilapia species are cultivated across the world, Nile tilapia (*O. niloticus) is* the fastest-growing fish and reaches market size in 8 -10 months of duration (Coward & Bromage, 2000). Moreover, Tilapia has become a key priority in fishery and aquaculture due to their capacity to efficiently utilize natural feeds. Tilapia are herbivores in nature, predominantly feed on vegetation and algae, and are frequently reared in canals and manmade lakes to manage the growth of algae and vegetation. Nile Tilapia (*Oreochromis niloticus*) is resistant to illnesses and handling, tolerant to a diverse range of environmental variations, grows quickly, and is easily bred (Mendonca *et al.,* 2012).

Hypothesis

We assumed that Ashwagandha (*W. somnifera*) root and leaves powder enhances immunity and fertility of male Nile tilapia (*O. niloticus*). So, to prove the hypothesis, the present research focuses on the following aim and objectives.

Aim and Objectives

To evaluate the effect of Ashwagandha (*W. somnifera*) plant by feeding leaves and roots supplemental diets to Nile Tilapia (*Oreochromis niloticus*) for 80 days and evaluate its effect on:

- Hematological indices (RBCs, WBCs, HCT, Hb etc.).
- Immunological parameters (Albumin, globulin, respiratory burst activity, lysozyme activity etc.).
- Biochemical Indices including (AST, ALT, LDH).
- Antioxidant enzymes (SOD, POD, CAT) and TBARS of testicular tissues
- Gonadosomatic index (GSI).
- Examination of histology of testicular tissues of Nile tilapia.
- Cortisol and testosterone hormones.

MATERIALS AND METHODS

The current research was conducted at the Fisheries and Aquaculture Research Station, Department of Zoology, Quaid-i- Azam University, Islamabad, Pakistan.

Collection and Transportation of Experimental Fish

About 200 healthy Nile tilapia (*Oreochromis niloticus)* were procured and transported in close oxygenated tight plastics bags from NARC, Islamabad to the Fisheries and aquaculture Research Center Station, QAU Islamabad. After transportation, fish were shifted to concrete raceway for acclimatization. Fish were acclimatized for 14 days and fed at *ad libitum* with a basal feed having 32% crude protein. Prior to shifting, the raceway was thoroughly cleaned and whitewashed with lime to prevent any bacterial disease, and fish were treated with 0.2% $KMNO_4$ solution for disease or parasite attack.

Preparation of Experimental Feed

A basal feed of 32% crude protein (CP) was formulated by the nutritive requirement of *O. niloticus* as shown in (Table No. 1). Dry feed ingredients including corn gluten, sunflower, wheat flour, fish meal, rice polish soya bean meal, vitamin and minerals premixes were finely grounded. Moreover, the Ashwagandha root and leaves powder supplements were mixed at graded level in the feed ingredients. The whole ingredients were mixed following the formulations and dough was prepared by the addition of water. Then, the dough was passed through a meat grinder for pellets formation. The prepared pellets were placed in the oven for drying at 60℃ for 24 hours and then crushed into small, crumbled pieces according to fish mouth size. Both the experimental and control diet were packed in separate airtight Ziplocs and stored in a cool place to prevent moisture and microbial contamination. The fish were fed twice a day at the rate of 5% of their body weight. Six different feed batches contain graded levels of Ashwagandha roots and leaves powder were prepared i.e., 3 experimental feeds comprised Ashwagandha root while the other 3 feed with Ashwagandha leaves and the control diet comprise of basal diet only.

Experimental Design

After 14 days of acclimatization period, completely randomized 80 days experiment was designed in triplicate. About 168 fish having initial body weight (90.2g ± 2.0) and length

of (12.7cm ± 1.5) were equally distributed in 21 fiber tanks (8 fish/tank) having capacity of 1000L with a stocking density of 1.08g/L. Tank were randomly divided into the following seven groups. The first three tanks were assigned as control group while remaining tanks were assigned as treatment groups i.e., R5, R10, R15, L5, L10 and L15. During the experiment fish were fed with 32% crude protein diet for 80 days at the rate 5% of their body weight, while feeding rate was adjusted fortnightly according to their body mass. Moreover, 32% crude protein diet was formulated with different supplements (Root and Leaves powder) are as follows:

Control group (C): Diet without supplement

L5: Fish diet supplemented with 0.5% of leaves powder

L10: Fish diet supplemented with 1.0 % of leaves powder

L15: Fish diet supplemented with 1.5 % of leaves powder

R5: Fish diet supplemented with 1.0 % of roots powder

R10: Fish diet supplemented with 1.0 % of roots powder

R15: Fish diet supplemented with 1.5 % of roots powder

Similarly, during the experiment temperature, DO level and pH of the water were regularly monitored. Water was partially replaced after every 2 or 3 days by monitoring the water quality and turbidity. Total ammonia concentrations were measured by ammonia kit (API freshwater test kit). Water temperature was fluctuated between 25-28℃ by adding water into fiber glass tanks daily.

Fish Sampling

Prior to the day of sample collection, fish were starved for 24 hours. Following that, fish were instantly anesthetized with MS-222 (30mg/L) (Abdel-Tawwab *et al*., 2020). Fish length and weight were measured, and blood samples were collected by using a 3ml pre heparinized syringe by caudal vein. Heparinized fresh blood sample (n=9) was stored in EDTA tubes for analysis of blood hematology while for serum (n=9), blood sample from fish were stored in serum separation SST tube which was then centrifuged at 5000xg for 20 min at room temperature. The sera of blood were used for biochemical analysis like ALT, AST, LDH, lysozymes activity, cortisol, and testosterone. IgM, globulin, and albumin were calculated from blood plasma. Fish were sacrificed on an ice box. The testicular tissues were sampled (3 fish/tank) and fixed in 10% formalin for microscopy examination and GSI was determined by weighing the testes of each male Nile tilapia. The target organ was then dipped in liquid

nitrogen (-196℃) and placed subsequently in a refrigerator at -20℃ for further analysis of antioxidant enzyme activity.

Growth Performance

Before dissection, each fish length and weight were noted and the total number of fish in each fiber tank were calculated to find the average body weight.

For growth performance the following formula was used.

Weight gain (%) = $\frac{\text{Final body weight}-\text{Initai body weight}}{\text{Final body weight}}$x 100

SGR (%) =$\frac{\ln(\text{Final weight})-\ln(\text{Initial body weight})}{\text{Number of days of experiment}} x100$

(Where *ln*=natural log)

Hematological Parameters

Hematological indices like WBCs, RBCs, Hemoglobin, HCT, MCH, MCHC, and MCV were analyzed from fresh blood by using automatic hematology analyzer (Sysmex hematology analyzer). All the samples were run in duplicate with 15 second interval time and mean values were calculated.

Immunity Parameters

Blood from 3-5 fish of each group was collected in SST (Serum Separation Tubes) tubes for immunological assays. The blood in these tubes was allowed to coagulate at room temperature for 30 minutes. After the following previous step centrifugation at 1000-2000g of these tubes was done at room temperature for 10 minutes. Serum was isolated through a micro pipette in an Eppendorf tube for immunological test and stored in 4℃ till further study.

Total Proteins Determination

The Lowery *et al.* (1951) method was used to quantify the total proteins in the plasma of both control and treatment groups. 1 mg of BSA (bovine serum albumin) was dissolved in 1 ml of D-H_2O in a clean test tube to make Standard proteins BSA (bovine serum albumin1:1) solution. Standards were made by diluting the stock solution with distilled water in such a way that the volume of each solution was 5 ml in each test tube. After the dilution procedure (2000 µl) proteins were transferred through a pipette into a different test tube and then added (2000

µl) of Alkaline $CuSO_4$ reagent, well mixed, and incubated for ten minutes at 25℃. Next 200µl of Folin-Ciocaltaeu reagent was added to each test tube and incubated at 25°C for 30 minutes again and absorption was measured at 660 nm by spectrophotometer. The standard calibration curve of absorbance values was plotted against protein concentration and then total protein concertation in the unknown sample was determined using a spectrophotometer by means of using a standard curve plot.

Plasma Immunoglobulin IgM

A sample of blood plasma was used to evaluate the plasma's immunoglobulin level. Blood samples were drawn by using a syringe and poured into the tubes for plasma separation and centrifuged at 1500 x g for 10 minutes. blood corpuscles were removed and again spun at 2000x g to settle down platelets and the supernatant was pipetted out and considered as plasma.

Anderson & Siwicki, (1995) method was performed for the quantification of immunoglobin in plasma. To precipitate immunoglobulin from plasma, 100 L (0.1 mL) of polyethylene glycol (12%) was mixed with 100 L (0.1 mL) of plasma at 1:1. The resultant solutions were centrifuged at 7000 g for 10 minutes after being incubated at 25℃ for 2 hours with constant shaking on an incubator shaker and at 660 nm, the optical density of the protein content was measured. Proteins from the supernatant were subtracted from the total proteins in the plasma to get the total IgM level.

$$Total\ IgM = Plasma\ protein\ content - Supernatant\ protein\ content$$

Lysozymes Activity

Following Anderson & Siwicki, (1995) method lysozyme activity in blood plasma was determined. 900 µL of *Micrococcus lysideikticus* in phosphate buffer saline (6.2 pH) (Sigma, St Louis, MO, USA) was mixed with 100 µL of serum. Mixing serum with bacteria, changes in the absorbance of the reaction were determined at 450 nm after a 1-minute interval for 10 minutes. Lysozymes activity was determined by the use of standard hen egg white lysozyme (Sigma-Aldrich).

Respiratory Burst Activity

The Anderson & Siwicki (1995) methodology were used to measure respiratory burst activity using freshly heparinized blood. Activity of free oxygen radicals or ROSs produced by leucocytes were determined with the help of this techniques. The nitro blue tetrazolium (NBT,

Sigma, St. Louis, MO, USA) reduces into a dark blue precipitate inside the phagocyte, known as formazan granules, during this reaction process. NBT solution (0.2%) was mixed with 0.1 ml of freshly collected blood from fish and then incubated at 250C for 30 minutes. Following incubation 50 µL of resultant suspension was mixed with 1000 µL/ 1mL of NN-Dimethyl Formamide (DMF, Sigma, St. Louis, MO, USA) in a fresh clean test tube and centrifuged at 3000×g for 5 minutes. OD at 540 nm of Supernatant formed during the centrifugation process was checked and for reference, the range blank sample consisting of all the chemical described above were mixed except the fresh blood sample which was replaced by distilled water and measured at the same OD value.

Albumin, Globulin, and A: G ratio Determination

The albumin content (ALB) was measured following the protocol mentioned by bromocresol methodology using the commercial kit Lab-test Diagnóstica S.A. (Lagoa Santa, Minas Gerais, Brazil). Total globulin from serum was obtained by subtracting total albumin from total protein. The albumin to globulin (A: G) ratio was also determined.

Blood-Biochemical indices

Aspartate Aminotransferase (AST)

Using a protocol of sigma-Aldrich-R active Assay Kit (MAK055) serum AST activity of both the control and treatment groups was measured.

Briefly, each sample and standards were run in duplicate, and a glutamate standard solution was prepared for calorimetric determination (concentration of 10ul of 0.1M glutamate standard diluted with 990ul of AST buffer). A blank sample was prepared, and 1-20µl serum sample was applied to each well of a 96-well plate. AST assay was added to the sample to make a net volume of 50µl. Prepared master mix for all reactions and were added to each sample and absorbance at 450 nm was checked after 2-4 minutes.

Alanine Aminotransferase (ALT)

Serums of both the control and plant (root and leaves powder) fed group were analyzed for ALT activity followed the protocol of sigma-Aldrich Active Assay Kit (MAK052). By following the protocol each sample and standard solution were run in duplicate and then for calorimetric determination, pyruvate standards of 990µl dilution were prepared. For reference, the blank sample was generated and 1-20µl of each sample was added into 96 well plates and

followed by the addition of ALT assay buffer into each sample to make a volume up to 20µl. Then the same step was followed as discussed in the AST Assay by adding master reaction mix and noted absorption at 570nm after 2-3 minutes.

Alkaline Phosphate (ALP)

The serum of the control and treated group for ALP was calculated by using sigma-Aldrich-R alkaline phosphate detection kit (AFP). For analysis of ALP activity automatic automatic analyzer (Cobas C-311, Japan) was used.

Lactate Dehydrogenase (LDH)

Franciscato *et al.* (2011) method was followed for the activity of LDH by the production of NADH. The sample was prepared as mentioned in the protocol and the absorption reading was checked at 500nm.

Antioxidant Enzymes Determination

The testicular tissues were homogenized using a Dounce manual homogenizer (Sigma, Aldrich) in 100 mmol potassium phosphate buffer (PBS) containing 1 mmol Ethylenediaminetetraacetic acid (EDTA) and the centrifuge at 4℃ for 30 minutes at 12000×g. the supernatant was collected in an Eppendorf tube and the pellet was discarded .sample were stored at -20℃ for further analysis.

Superoxide Dismutase (SOD)

Modified Kakkar *et al.* (1984) techniques were used for SOD activity in testicular tissues. 0.3 ml supernatant and 0.1 mL of 186 µM phenazine methosulphate solution mixed with 1.2 mL of 0.052 mM sodium pyro-phosphate buffer (pH=7.0). Next 0.2 mL of 780 µM $NADH^{+}$ was added to start the reaction and then added 1mL of glacial acetic acid was to stop further. colour changes were measured at 560nm wavelength with the help of a spectrophotometer. One unit of SOD is defined as the amount of enzymes/protein in mg that hinder the quercetin oxidation by 50%. The results of SOD activity were presented in moles/min/mg protein using a molar coefficient of 6.22103/M cm.

Catalase (CAT) Assay

The activity of Catalase in testicular tissues was calculated following the Chance & Maehly, (1955) technique 2500µl of 50 mM Pbs buffer (pH=5), 100 µl 0f supernatant, and 400 µl of 5.90 mM H2O2 were mixed and the reaction mixture absorption was checked at 240 nm wavelength via spectrophotometry. The result was expressed as nmol per min per mg protein with a molar coefficient of 43.6/ M cm.

Per-oxidase (POD) Assay

Chance & Maehly, (1955) and Bibi (2012) method was used to quantify POD activity in testicular tissues. For the reaction, 2500µl of 50 mM phosphate buffer solution (pH=5), 300ul of 40 mM hydrogen peroxide, 100ul of 20 mM Guaiacol, and 0.1 ml of supernatant were mixed and after 1-minute absorption was checked at 470 nm. POD activity was measured in nmol per min per mg protein with a molar coefficient (of 2.66×104 M-1).

Lipid Peroxidation (LPO/ TBARS)

Wright *et al.* (1981) method for the determined activity of LPO in testicular tissues of fish was followed.580ul of 0.1 M Pbs (pH 7.4), 200ul supernatant, 200u l ascorbic acid (100 mM) and 20ul Fecl3 (100 mM) were mixed to form 1000ul reaction mixture. The sample was subjected to a water bath solution at 37℃ for 1 hour prior to adding 10% of 1.0 ml of trichloroacetic acid solution to stop the reaction. The tubes were all heated for 20 minutes in a water bath after 1000ul of thio-barbituric acid was added and the sample was cooled in an ice bath and centrifuged for 10 minutes at 25×102g. Absorption was checked at 535 nm wavelength after a 1-minute interval via spectrophotometer.

Testosterone ELISA

For testosterone serum concentration, the calbiotech, Inc. (CBI) Testosterone ELISA Kit(Catalog No. TE373S) is used followed by (Paulson et al., 1977; Mc-carm, 1985; Ekins, 1984).

Reagent Preparation Method

First, prepared enzyme conjugate(20x), followed by 1x wash buffer(1x) preparation, and then stored at 25℃.

Testosterone Assay Procedure Method

The following procedure of the Calbiotech Inc. (CBI) Testosterone ELISA Kit (Catalog No. TE373S), samples were pipetted out from both the control and experimental group into the well and absorbance was measure on ELISA reader calibrated at 450nm.

Cortisol ELISA

Cortisol Eliza Kit (Calboitech Inc., catalog No.C0368s (96tests) sample were used for cortisol assay. Assay was conducted by following Kit recommended procedure.

Reagent Preparation

To make Cortisol-enzyme Conjugate Solution first dilute and then mixed the Cortisol enzyme conjugate with assay diluent at the ratio of 1:21. Then Add 475 ml of ionized H20 into (20x of 25ml) of content in the bottle and store at room temperature for further analysis.

Cortisol Assay Procedure Method

The followed procedure of the Calboitech Inc. (CBI)Cortisol Eliza Kit (catalog No.C0368s (96tests) sample were pipetted out from both the control and experimental group into the well and absorption for optical density was measured through ELISA reader calibrated at 450nm.

Gonadal Somatic Index (GSI)

At the end of the experiment, five fish from each group were weighed and their gonadal mass was measured after the fish was scarified. The following formula was used to calculate the gonadal somatic index.

$$\text{GSI} = \frac{\text{weight of gonads in gram}}{\text{weight of fish in gram}} \times 100$$

Histology of Testicular Tissues

To examine the changes in gonadal tissues of Nile tilapia**,** (3 fish/ group) were sampled from seven groups and was fixed in 10% neutral buffered formalin, dehydrated in alcohol, cleared in xylene, embedded in paraffin and then sectioned in 5um thickness. The serial sectioned were subjected to stanning with hematoxylin and eosin (Spencer *et al.,* 2013).

Gene Expression Analysis

for analysis of gene expression of GnRH hormone brain tissue samples from each group were fixed in RNA later and stored at 4^0C until further analysis.

Isolation of RNA

Gene expression was analyzed by real time PCR. (APPLIED biosystem, foster city, CA, USA). In brief, tissue samples were removed from 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 15sec.the sample was again incubated for 5 min at room temperature before being centrifuged at 12000rpm for 5 min at 4. Afterwards the upper colorless aqueous layer was separated and transferred to a new micro centrifuge. The separated aqueous phase was vortexed briefly with chilled iso prophy alcohol (absolute) prior to centrifugation the samples were again incubated for 10 minutes at room temperature and hen centrifuged at 12000rpm for 10 mins. The pellets were washed in 75 % ethanol (0.5ml) (prepared in DEPC treated water) and liquid phase was discarded. After that air drying of pellets was done and then dissolved in 50µL of nuclease-free water. The isolated RNA was stored at -80 ^{0}C analysis.

Quantification of RNA

Nano drop ND1000 (thermos scientific USA) was used to evaluate the quality and quantity of RNA. The quantification of total RBA concentration in sample was performed by measuring absorbance at 260nmwhile to check the purity of samples the Nd at 260 and 280 was used with estimated values between 1.9 and 2.0.

Synthesis of cDNA

Each isolated RNA was reverse transcribed to c DNA by using the method as reported earlier by (Amir *et al.*,2019) in brief, the reaction mixture of 20µLwas prepared by mixing 8µL RNA,4µL of dNTPs,1µLof MMLV-RT,2.5µL random primer,0.5 µL and 3 µL DEPC water. The reaction mixture was incubated in water bath at 37 degrees for an hour and then in pcr machine (BIO-RADT100TM thermal cycler) at 55^0C for 5 min. Nano drop ND1000 (Thermo scientific, USA) was used evaluate the concentration of synthetic cDNA in each sample. The prepared cDNA was immediately stored at temperature of -20^0C.

Primer Designing

The primers shown in table(A) were self-designed and oligo primer analysis software version1.1.2 was used for primers designing and manufacturing from humanizing genomics macrogen. The nucleotide sequence of corresponding genes of Nile tilapia was obtained from gene bank NCBI (www.ncbi.nlm.nih.gov)quality of cDNA and its compatibility with primer was checked by performing a simple PCR followed by gel electrophoresis of each sample in duplicate. Afterwards, qPCR was performed protocol previously reported by (Ahmad *et al*., 2020). briefly, 20µL reaction mixture was prepared by mixing 0.4µL of reverse and 0.4µL of 4µLwith 7.6µL of syringe water,10µL of SYBER GREEN and 1.6µL of diluted cDNA. The PCR condition was optimized along with cycle numbers (initial denaturation 95^0C or 4 min followed by 40 cycles at 95^0C for 15 seconds and subsequently, 62^0C for 15 sec). The efficacy of PCR reaction for each gene was measured by slope of standard curve using serial dilutions of cDNA of control sample. Then RNA levels of each gene were compared with the expression of beta actin reference gene of Nile tilapia. The relative variation in gene expression were calculated by standard CT**.**

Table A: Target gene and primer sequence

Primer	Forward primer	Reverse primer	MT	Accession
GnRH	GAAGAGGGATCTGGACAACTTC	AGGTGATTCCTCTGCACAAC	55^0C	AY381298

Statistical Analysis

The results were analyzed and as mean±S.E .Two-way ANOVA was used to compare groups fed with Ashwagandha supplemented diets. A post-hoc LSD test was used to determine the difference in mean values between different groups (Control and treated). The data analyzed using SPSS software version 21. The level of significant was set at ($P<0.05$). Graph pad prism version 9.0 was used to plot data on graphs.

RESULTS

Growth Parameters

The comparative effect of graded level of the two different parts (Root and Leaves) of Ashwagandha on growth performance of Nile tilapia is shown in Fig. 1. Statistical analysis done by two-way ANOVA showed a significant effect of different plant parts (Root and Leaves) and dosage levels of Ashwagandha on growth performance, i.e., WG (Plant parts,$F_{1,14}$=90.037, P<0.01; dosage level, $F_{6,14}$=2261.131, P<0.01), SGR (plant parts, $F_{1,14}$=2231.1, P>0.01; dosage level, $F_{6,14}$=204.245, P<0.01), The FCR was also effected by plant parts ($F_{1,14}$=170.667, P>0.01) and dosage level ($F_{6,14}$=4043.111, P<0.01) of Ashwagandha Moreover, a significant interaction between two variable in Table 11 indicated the manner how plant parts and dosage level of Ashwagandha influence the growth performance and FCR of fish. Pairwise comparison between plant parts of Ashwagandha at every dosage level indicated a significantly higher positive effect on every growth parameter. Furthermore, post hoc pairwise comparison between dosage level of two different plant parts indicated that leave powder at 10 g/kg diet showed the highest %WG (128.11) while Root powder showed its maximum effect (112.2%) at 5 g/kg diet. Overall result indicated that maximum growth performance was observed in R15 group i.e., at 15g/kg diet of root powder.

Hematological Parameters

The comparative effect of the graded level of Ashwagandha on the hematological indices of fish is shown in Fig. 02. Statistical analysis done by two-way ANOVA showed overall a significant effect of plant parts (Root and leaves powder) and different dosage level on the hematological indices i.e., WBC (plant parts $F_{1,14}$=2.653, P<0.01; dosage level $F_{6,14}$= 290.5, P<0.01), RBC (plant parts $F_{1,14}$=1.512, P<0.01; dosage level $F_{6,14}$=240.002, P<0.01), PCV (plant parts $F_{1,14}$=15.009, P<0.01; dosage level $F_{6,14}$=255.532, P<0.01), MHC (plant parts $F_{1,14}$=61.753, P<0.01; dosage level $F_{6,14}$=2504.338, P<0. Furthermore, a significant interaction between two variables (Plant parts and dosage level of Ashwagandha) indicated how these two variables affect the hematological indices of Nile Tilapia except the MHCH ($F_{6,14}$=171.433, P=0.012) as shown in Table 12. Furthermore, post hoc pairwise comparison between dosage level among all the group indicate dose dependent, comparatively most significant positive effect was Ashwagandha root comparative to leaves powder. However,

15g/kg root powder showed more significant effect compared to all the treated group as shown in table.

Immunity Parameters

The comparative effect of the graded level of Ashwagandha root and leaves on the nonspecific immunological indices is shown in Fig. 03. Statistical analysis done by two-way ANOVA showed a significant of plant parts and dosage level of Ashwagandha on the nonspecific immunological indices i.e., lysozymes activity (plant parts $F_{1,14}$=0.040, P<0.001; dosage level $F_{6,14}$=30.720, P<0.001), Respiratory burst activity (plant parts $F_{1,14}$=0.899, P<0.001; dosage level $F_{6,14}$=358.702, P<0.001), Albumin (plant parts $F_{1,14}$=0.020, P<0.001; dosage level $F_{6,14}$=6.964, P<0.01), A/G(plant parts $F_{1,14}$=2.194, P<0.01; dosage level $F_{6,14}$=17.131, P<0.01).However no significant effect of plant parts ($F_{1,14}$=0.124, P=0.730) and dosage level ($F_{6,14}$=0.378, P=0.770) were observed in globulin concentrations. Furthermore, no significant interaction between two variables (plant parts and dosage level) indicated how these two variables as shown in Table 13 affect the immunological parameters of Nile Tilapia. Pairwise comparison between different plant parts at every dosage level indicated the most significant effect of root supplement in contrast to leaves powder on the non-immunological indices of fish. Furthermore, post hoc pairwise comparison between dosage level indicated dose dependent, while the most significantly positive effect of root powder at 15g/kg diet were the best among all the groups.

Biochemical Parameters

Two-way ANOVA also indicated the effect of Ashwagandha on various biochemical parameters of Nile Tilapia as shown in Fig. 4. Significant effect of plants parts ($F_{1,14}$=16.333, p<0.01) and dosage level ($F_{6,14}$=312.595, P<0.01) were observed on the serum AST activity of fish. Similarly, the plant parts ($F_{1,14}$=10.840, P<0.01) and dosage level ($F_{6,14}$=44.142, P<0.01) also significantly altered the LDH concentration of Nile Tilapia. In addition to this only the dosage level ($F_{6,14}$=4.706, P<0.01) significantly affect the ALT concentration of fish. Moreover, a non-significant interaction between plant part and dosage level of dietary supplement indicated that these two variables have no effect on the biochemical indices in Table 14. Pair wise comparison at every dosage level indicated that a significantly lower concentration of these biochemicals in response to increase the dosage level were observed as

compared to control group. Furthermore, post hoc pairwise comparison between dosage level indicated that biochemical parameters (AST & LDH) did not change while significant effect was observed in ALT concentration.

Antioxidant Activity

The comparative effect of the graded level of different plant parts (root and leaves) of Ashwagandha on the testicular tissues is shown in Fig. 5. Statistical analysis done by two-way ANOVA showed a significant effect of different plants parts, and dosage level of Ashwagandha on antioxidant enzymes of the testicular tissues i.e., Catalase (Plant parts $F_{1,14}$=0.010, P=0.022; dosage level, $F_{6,14}$=0.319, P=0.012) superoxide dismutase (Plant parts $F_{1,14}$=0.032, P=0.161; dosage level, $F_{6,14}$=0.171, P=0.014) peroxidase (Plant parts $F_{1,14}$=0.52, P<0.001; dosage level, $F_{6,14}$=0.082, P=0.009) but no significant effect were observed in the lipid peroxidase activity (Plant parts $F_{1,14}$=0.004, P=0.0953; dosage level, $F_{6,14}$=0.551, P=0.054) in the testicular tissues of Male Nile Tilapia. Furthermore, significant interaction between two variables (plant parts and dosage level) indicated how these variables affected the antioxidant enzymes activity as shown in Table 15. Pairwise comparison between different plant parts at every dosage level indicated the most significant dietary root supplement of Ashwagandha in comparison to leaves powder. The dietary level of 15g/kg of root supplement has a more profound effect in the testicular antioxidant enzymes.

Cortisol and Testosterone

The comparative effect of the graded level of plant parts (root and leaves) of Ashwagandha on the different hormonal profile of Nile Tilapia is shown in the Fig. 06. Statistical analysis done by two-way ANOVA showed a significant effect of different plant parts, and doses level of Ashwagandha on hormonal activities i.e., Cortisol (Plant parts $F_{1,14}$=14953.390, P<0.01; dosage level $F_{6,14}$=52360.699), testosterone (plant parts $F_{1,14}$=46839.281, P<0.001), dosage level $F_{6,14}$=160896.868, P<0.001) gonadal somatic index (doses level $F_{6,14}$= 10.760, P<0.001). However no significant effect of plant parts ($F_{1,14}$=1.172,0.295) were observe in the gonadal somatic index of male Nile Tilapia. Furthermore, significant interaction between two variables (plants parts and dosage level) indicated how these variables affect the hormonal profile along with gonadal somatic index. Pairwise comparison between different plant parts at every dosage level show that root effect was most significant in comparison to

leaf powder as shown in the table (8,9,10). Furthermore, post hoc pairwise comparison shows that root powder of 15g of Ashwagandha root were the best among all the other group.

Gene Expression

The comparative analysis of gene expression of GnRH in diet having Ashwagandha root and leaves powder is presented in Fig. 7. Statistical analysis showed significant differences ($P<0.05$) in GnRH gene expression among all experimental group of Nile tilapia. Pairwise comparison showed significant upregulation in expression of GnRH was observed in R15 group followed by L15>R10>R5>L10.

Table 01:Formulation of 32% CP diet for *O. niloticus* with Ashwagandha (*W. somnifera*) roots and leaves powder supplement at graded level

Ingredients	Control	L5(0.5%)	L10(1%)	L15(1.5%)	R5(0.5%)	R10(1%)	R15(1.5%)
Fish meal	16	16	16	16	16	16	16
Soybean	15	15	15	15	15	15	15
Rice polish	15	15	15	15	15	15	15
Wheat flour	15.5	15	15	15	15	15	15
Sunflower	15.5	15.5	15	14.5	15.5	15	14.5
Corn Gluten	12	12	12	12	12	12	12
Fish oil	05	05	05	05	05	05	05
Min and Vit premix[a]	02	02	02	02	02	02	02
Vit C	02	02	02	02	02	02	02
CMC	02	02	02	02	02	02	02
Root powder	00	00	00	00	0.5	1.0	1.5
Leaves powder	00	0.5	1.0	1.5	00	00	00
Total	100	100	100	100	100	100	100

[a] Composition of mineral and vitamin premix kg− 1: manganese, zinc, iron, copper, iodine, selenium, cobalt, calcium carbonate, vitamin A, vitamin D3, vitamin E, vitamin K3, vitamin B1, vitamin B2, vitamin B6, vitamin B12, biotin, folic acid, nicotinic and pantothenic acid.

Table 02: Proximate composition of experimental diets

Ingredient %	Control	L5	L10	L15	R5	R10	R15
Moisture	10.23	10.26	10.32	10.54	10.19	10.25	10.27
Crude protein	32.7	32.23	32.25	32.43	32.12	32.42	32.24
Crude lipids	13.43	13.14	13.43	13.42	13.12	13.33	13.32
Crude fiber	7.06	7.04	7.1	7.03	7.03	7.09	7.34
Total ash	14.14	14.21	14.12	14.32	14.21	14.14	14.11
Ashwagandha leaves		5	10	15			
Ashwagandha roots					5	10	15

Table 03: Growth performance of Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder

Parameter	Control	L5	L10	L15	R5	R10	R15	F	P	S.E.
IBW(g)	90.1 ±0.4	89.4 ±0.1	91.3 ±0.5	90.5 ±0.9	89.6 ±0.6	89.6 ±0.1	90.3 ±0.3	0.75	0.616	0.4
FBW(g)	132.4±1.8^{d}	161.4±1.4^{c}	172.4±1.4^{c}	161.0±1.5^{c}	190.2±1.6^{b}	187.4±1.9^{c}	208.2±1.1^{a}	2.801	0.029	1.41
WG	42.2 ±0.01^{e}	71.9 ±0.07^{c}	82.1±0.02^{c}	69.6 ±0.07^{d}	100.6 ±0.08^{b}	97.5 ±0.06^{b}	117.0 ±0.03^{a}	1.977	0.030	0.0
SGR	0.01±0.02^{d}	0.09 ±0.07^{b}	0.14 ±0.06^{c}	0.06 ±0.05^{b}	0.06 ±0.04a^{b}	0.23 ±0.04^{ab}	0.32 ±0.04^{a}	118	0.006	0.05
WG%(g)	46.85±1.8^{d}	80.51±1.4^{b}	90.1±1.1^{c}	76.9±1.6^{d}	112.2±1.9^{b}	107.9±1.4^{ab}	128.17±1.1^{a}	182	0.05	1.32
FCR	2.25 ± 0.2^{a}	2.02 ±0.1^{b}	1.92 ±0.9^{b}	2.14 ±0.8^{b}	1.87 ±0.4^{cd}	1.97 ±0.5^{c}	1.79 ±0.7^{d}	55.7	0.01	0.1

Data is presented as Mean± SE (n=6). One- way ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case superscripts represented a significant difference ($p<0.05$). Control (C) (Ashwagandha free diet), L5 (5-g leaves/kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root/kg diet), R10 (10-g root/kg diet), R15 (15-g root/kg diet).

Table 04: Hematological indices of Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder

Groups	C	L5	L10	L15	R5	R10	R15	F	P	S. E
WBC (10^3/µl)	8.40 ± 0.01^{e}	9.47 ± 0.02^{d}	10.5 ± 0.03^{b}	11.5 ± 0.02^{ab}	9.7 ± 0.01^{c}	10.5 ± 0.02^{b}	11.6 ± 0.04^{a}	240.3	0.01	0.03
RBC (10^6/µl)	4.40 ± 0.14^{f}	5.58 ± 0.13^{e}	6.51 ± 0.18^{c}	7.55 ± 0.14^{b}	5.67 ± 0.13^{e}	6.69 ± 0.14^{c}	6.69 ± 0.12^{a}	22.88	0.01	0.18
HBG (g/dl)	12.74 ± 0.28^{e}	13.87 ± 0.26^{d}	14.83 ± 0.2^{c}	15.91 ± 0.28^{b}	14.86 ± 0.21^{c}	14.82 ± 0.2^{c}	16.79 ± 0.2^{a}	472.4	0.01	0.21
PCV/HCT %	37.48 ± 0.3^{d}	39.09 ± 0.3^{c}	41.15 ± 0.3^{b}	41.73 ± 0.4^{b}	39.65 ± 0.3^{c}	41.61 ± 0.4^{b}	43.82 ± 0.3^{a}	17.98	0.05	0.36
MCV (um^3)	90.7 ± 0.31^{c}	92.7 ± 0.34^{c}	93.8 ± 0.64^{b}	92.6 ± 0.24^{bc}	92.8 ± 0.34^{c}	91.8 ± 0.24^{d}	96.5 ± 0.34^{a}	126	0.01	0.34
MCH (pg)	30.6 ± 0.3^{e}	31.8 ± 0.1^{e}	33.8 ± 0.4^{c}	35.8 ± 0.2^{b}	31.8 ± 0.1^{e}	32.8 ± 0.6^{d}	38.3 ± 0.3^{a}	216	0.01	0.43
MCHC(g/dl)	33.6 ± 1.1^{e}	34.7 ± 1.5^{d}	35.7 ± 1.7^{c}	36.6 ± 1.5^{b}	34.6 ± 1.2^{d}	36.6 ± 1.6^{b}	37.6 ± 1.2^{a}	311.7	0.01	1.3

Data is presented as Mean± SE(n=9). ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case superscripts represented a significant difference ($p<0.05$). Control (C) (Ashwagandha free diet), L5 (5-gram leaves/kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root/kg diet), R10 (10-g root/kg diet), R15 (15-g root/kg diet).

Table 05: **Immunity parameters of Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder**

Groups	C	L5	L10	L15	R5	R10	R15	F	P	S. E
Lysozymes	10.4 ± 0.17^{e}	10.8 ± 0.19^{d}	11.4 ± 0.13^{c}	12.0 ± 0.17^{b}	10.4 ± 0.11^{e}	11.3 ± 0.17^{c}	12.5 ± 0.19^{a}	21.53	0.01	0.14
Respiratory burst	0.27 ± 0.1^{c}	0.39 ± 0.4^{bc}	0.43 ± 0.3^{c}	0.85 ± 0.5^{b}	0.42 ± 0.8^{bc}	0.36 ± 0.5^{c}	0.93 ± 0.4^{a}	16.07	0.01	0.5
IgM	9.1 ± 0.8^{b}	9.4 ± 0.3^{b}	9.8 ± 0.1^{ab}	9.0 ± 0.4^{b}	9.3 ± 0.4^{b}	9.3 ± 0.8^{b}	10.5 ± 0.7^{a}	9.364	0.01	0.1
Albumin	3.8 ± 0.12^{c}	4.0 ± 0.18^{b}	4.0 ± 0.17^{b}	4.8 ± 0.19^{a}	4.0 ± 0.16^{b}	$3.9.0\pm0.14^{b}$	4.9 ± 0.17^{a}	6.204	0.0003	0.12
Globulin	1.43 ± 0.57^{b}	1.25 ± 0.51^{bc}	1.56 ± 0.58^{c}	1.54 ± 0.52^{a}	1.24 ± 0.52^{bc}	$1.23\pm0.^{bc}$	1.48 ± 0.53^{a}	0.6620	0.68	0.56
A/G Ratio	2.71 ± 0.51^{c}	2.51 ± 0.50^{bc}	2.91 ± 0.51^{b}	3.17 ± 0.58^{a}	2.56 ± 0.51^{b}	2.66 ± 0.59^{b}	3.37 ± 0.52^{a}	12.19	0.01	0.59

Data is presented as Mean± SE (n=9). ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case super script represented a significant difference ($p=<0.05$). Control (C) (Ashwagandha free diet), L5 (5-g leaves/kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root/kg diet), R10 (10-g root/kg diet), R15 (15-g root/kg diet).

Table 06: Blood biochemical of Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder

Groups	C	L5	L10	L15	R5	R10	R15	F	P	S.E
AST U/L	69.1±0.31^{a}	70.4±0.37^{a}	69.9±0.39^{a}	65.1±0.39^{b}	69.5±0.32^{a}	66.4±0.42^{b}	64.4±0.49^{b}	178.7	0.06	0.36
LDH U/L	21.3±0.9^{a}	19.5±0.8^{b}	18.6±0.4^{c}	18.1±0.2^{c}	20.0±0.7^{B}	19.7±0.4^{b}	18.6±0.7^{c}	35.52	0.06	0.2
ALT U/L	18.8±0.39^{ab}	19.2±0.31^{b}	20.6±0.33^{a}	20.3±0.36^{a}	17.9±0.37^{c}	18.8±0.32^{ab}	19.7±0.32^{b}	6.207	0.05	0.32

Data is presented as Mean± SE (n=9). ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case super script represented a significant difference (p=<0.05). Control (C) (Ashwagandha free diet), L5 (5-g leaves/kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root/kg diet), R10 (10-g root/kg diet), R15 (15-g root/kg diet).

Table 07: Antioxidant activity of Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder

Parameter	C	L5	L10	L15	R5	R10	R15	F	P	S.E.
CAT	63.1±2.1^{c}	65.2±2.9 ^{b}	67.3±2.2^{ab}	68.5±2.6^{ab}	66.1±2.5 ^{b}	67.4±2.7^{b}	69.4±2.4^{a}	0.009	0.04	2.1
SOD	53.5±1.3 ^{c}	54.6±1.7^{bc}	55.5±1.1^{b}	56.5±1.2^{ab}	55.1±1.9^{b}	56.4±1.7^{b}	57.8±1.6^{a}	0.06	0.03	1.7
POD	65.7±2.0^{c}	66.7±2.0^{b}	67.4±2.01^{b}	68.2±2.04^{ba}	67.1±2.08^{b}	68.7±2.03^{ba}	69.3±2.08^{a}	0.03338	0.001	2.09
LPO	13.1±0.7^{a}	13.7±0.7^{a}	11.9±0.71^{ab}	10.9±0.72^{b}	10.5±0.71^{b}	10.8±0.73^{b}	9.5±0.70^{c}	0.7	0.8370	0.70

Data is presented as Mean± SE (n=9). ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case super script represented a significant difference (p=<0.05). Control (C) (Ashwagandha free diet), L5 (5-g leaves/kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root/kg diet), R10 (10-g root/kg diet), R15 (15-g root/kg diet).

Table 08: **Cortisol concentration in serum of Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder**

Groups	Control	L5	L10	L15	R5	R10	R15	F	P	S. E
Cortisol(ng/ml)	67.2±1.5^{c}	64.8±1.9^{ab}	61.8±1.2^{abc}	60.31.6 ±^{bc}	55.7±1.7^{c}	56.4±1.3^{b}	48.9±1.5^{a}	7.24	0.01	1.2

Data is presented as Mean± SE (n=9). ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case superscripts represented a significant difference ($p<0.05$). Control (C) (Ashwagandha free diet), L5 (5-g leaves/kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root/kg diet), R10 (10-g root/kg diet), R15 (15-g root/kg diet).

Table 09: **Gonadal somatic index of male Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder**

Groups	C	L5	L10	L15	R5	R10	R15	F	P	S. E
Male GSI	0.10 ± 0.02^{d}	0.11 ± 0.01^{c}	0.69 ± 0.02^{b}	0.40 ± 0.04^{bc}	0.52 ± 0.02^{ab}	0.57 ± 0.01^{ab}	0.77 ± 0.02^{a}	1.81	0.0017	0.06

Data is presented as Mean± SE (n=9). ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case superscripts represented a significant difference ($p<0.05$). Control (C) (Ashwagandha free diet), L5 (5-g leaves /kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root /kg diet), R10 (10-g root /kg diet), R15 (15-g root /kg diet).

Table 10: Testosterone concentration in serum of Nile tilapia after 80 days feeding diet supplemented with graded level of Ashwagandha (*W. somnifera*) roots and leaves powder

Groups	C	L5	L10	L15	R5	R10	R15	F	P	S. E
Testosterone (ng/ml)	1.7 ± 0.6^{c}	2.3 ± 0.1^{c}	5.1 ± 0.6^{ab}	8.8 ± 0.2^{ab}	6.3 ± 0.4^{b}	8.8 ± 0.2^{ab}	9.6 ± 0.1^{a}	754	0.01	0.61

Data is presented as Mean± SE (n=9). ANOVA and post-hoc LSD test were used to show pairwise comparison between groups. The lower-case super script represented a significant difference ($p<0.05$). Control (C) (Ashwagandha free diet), L5 (5-g leaves/kg diet), L10 (10-g leaves/kg diet), L15 (5-g leaves/kg diet), R5 (5-g root/kg diet), R10 (10g root/kg diet), R15 (15-g root/kg diet).

Table 11: Summary of two-way ANOVA showing the Ashwagandha effect on growth performance of Nile tilapia.

Growth Performance	SS	df	MS	F-value	P-value
WG					
Plant parts	176.042	1	176.042	90.037	0.000
Dosage	13263.060	3	4421.020	2261.131	0.000
Plant part and Dosage	1854.988	3	618.329	316.245	0.000
SGR					
Plant parts	0.004	1	1321.020	2231.131	0.000
Dosage	0.303	3	518.329	204.245	0.000
Plant part and Dosage	0.028	3	0.009	435.444	0.000
FCR					
Plant parts	0.004	1	0.004	170.667	0.000
Dosage	0.303	3	0.101	4043.111	0.000
Plant part and Dosage	0.028	3	0.009	368.444	0.000

Table 12: Summary of Two-way ANOVA showing the Ashwagandha effect on hematological parameter of Nile tilapia.

Hematological Parameters	SS	df	MS	F-value	P-value
WBC					
Plant parts	0.100	1	0.100	2.653	0.000
Dosage	32.883	3	10.961	290.453	0.000
Plant part and Dosage	0.049	3	0.016	0.434	0.732
RBC					
Plant parts	.067	1	0.067	1.512	0.237
Dosage	31.992	3	10.664	240.002	0.000
Plant part and Dosage	.043	3	0.014	0.322	0.809
PCV					
Plant part	2.356	1	2.356	15.009	0.001
Dosage	106.217	3	35.406	225.532	0.000
Plant part and Dosage	1.598	3	0.533	3.393	0.044
MHC					
Plant Part	1.283	1	1.283	61.753	0.000
Dosage	156.145	3	52.048	2504.338	0.000
Plant part and Dosage	10.689	3	3.563	171.433	0.032
MCHC					
Plant Part	1.283	1	1.283	61.753	0.000
Dosage	156.145	3	52.048	2504.338	0.000
Plant part and dosage	10.689	3	3.563	171.433	0.012

Table 13: Summary of Two-way ANOVA showing the Ashwagandha effect on immunity performance of Nile tilapia.

Immunity Parameters	SS	df	MS	F-value	P-value
Lysozymes					
Plant part	0.007	1	0.007	0.040	0.000
Dosages	16.313	3	5.438	30.720	0.000
Plant part and dosage	0.541	3	0.180	1.019	0.410
Respiratory burst activity					
Plant part	0.002	1	0.002	0.899	0.000
Dosages	2.525	3	0.842	358.702	0.000
Plant part and dosage	0.202	3	0.067	28.737	0.000
Albumin					
Plant part	0.005	1	0.005	0.020	0.000
Dosages	5.236	3	1.745	6.964	0.003
Plant part and dosage	0.024	3	0.008	0.032	0.992
Globulin					
Plant part	0.014	1	0.014	0.124	0.730
Dosages	0.129	3	0.043	0.378	0.770
Plant part and dosage	0.165	3	0.055	0.486	0.697
A/G					
Plant part	0.170	1	0.170	2.194	0.05
Dosages	3.982	3	1.327	17.131	0.000
Plant part and dosage	0.103	3	0.034	0.445	0.724

Table 14: Summary of Two-way ANOVA showing the Ashwagandha effect on biochemical indices of Nile tilapia.

Biochemical indices	SS	df	MS	F-value	P-value
AST/UL					
Plant part	1.638	1	1.638	16.333	0.001
Dosages	94.021	3	31.340	312.595	0.000
Plant part and dosage	0.785	3	0.262	2.611	0.087
LDH/UL					
Plant part	2.509	1	2.509	10.840	0.005
Dosages	30.653	3	10.218	44.142	0.000
Plant part and dosage	1.197	3	0.399	1.724	0.202
ALT/UL					
Plant part	3.872	1	3.872	1.614	0.222
Dosages	33.882	3	11.294	4.706	0.015
Plant part and dosage	2.757	3	0.919	0.383	0.767

Table 15: Summary of Two-way ANOVA showing the Ashwagandha effect on antioxidant activity of testicular tissues of Nile tilapia.

Antioxidant activity	SS	df	MS	F-value	P-value
CAT					
Plant part	1.200	1	1.200	0.010	0.022
Dosages	117.003	3	39.001	0.319	0.012
Plant part and dosage	.939	3	0.313	0.003	0.000
SOD					
Plant part	2.620	1	2.620	0.032	0.161
Dosages	42.602	3	14.201	0.171	0.014
Plant part and dosage	1.392	3	0.464	0.006	0.099
POD					
Plant part	3.599	1	3.599	0.52	0.000
Dosages	29.458	3	9.819	0.082	0.009
Plant part and dosage	1.728	3	0.576	0.005	0.000
LPO					
Plant part	0.039	1	0.039	0.004	0.953
Dosages	18.196	3	6.065	0.551	0.654
Plant part and dosage	15.367	3	5.122	0.466	0.710

Table 16: Summary of Two-way ANOVA showing the Ashwagandha effect on hormonal profile (cortisol, testosterone) and GSI of Nile tilapia.

Hormonal Profile	SS	df	MS	F-value	P-value
Cortisol					
Plant part	0.102	1	0.102	14953.390	0.000
Dosages	1.073	3	0.358	52360.699	0.000
Plant part and dosage	0.104	3	00.035	5052.333	0.000
Testosterone					
Plant part	16.923	1	16.923	46839.281	0.000
Dosages	174.392	3	58.131	160896.868	0.000
Plant part and dosage	13.219	3	4.406	12196.039	0.000
Gonadal somatic index					
Plant part	0.067	1	0.067	1.172	0.295
Dosages	1.854	3	0.618	10.760	0.000
Plant part and dosage	0.298	3	0.099	1.732	0.000

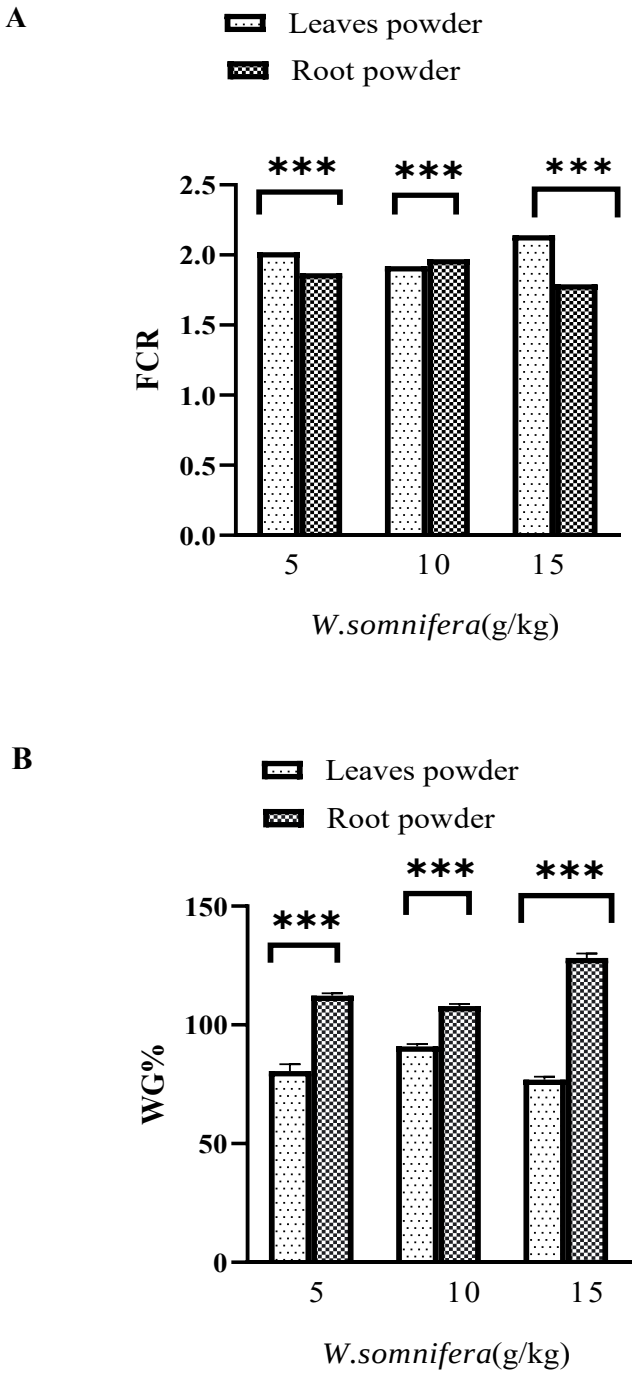


Fig. 1: Graph (A and B) showed student-t test paired wise comparison between same doses of both root and leaves of Ashwagandha inclusion effect on SGR (Specific growth rate), %WG (percent weight gain)

Ns= non-significant difference; *= P<0.01; **=P<.001; ***=P<0.001

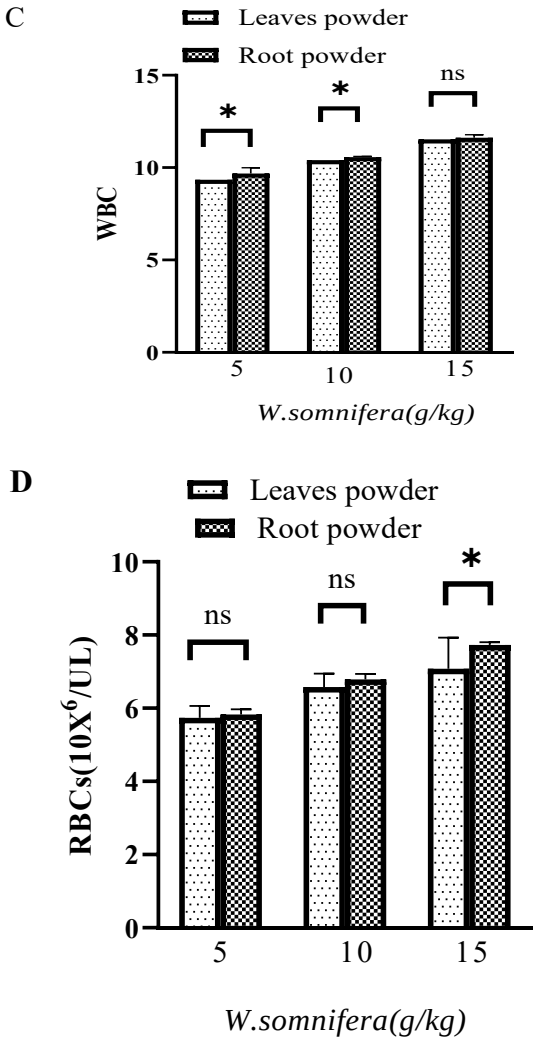


Fig. 2: Graph (C and D) showed student-t test paired wise comparison between same doses of both root and leaves of Ashwagandha inclusion effect on WBC (white blood cell), RBC (red blood cell)

Ns= non-significant difference; *= P<0.01; **=P<.001; ***=P<0.001

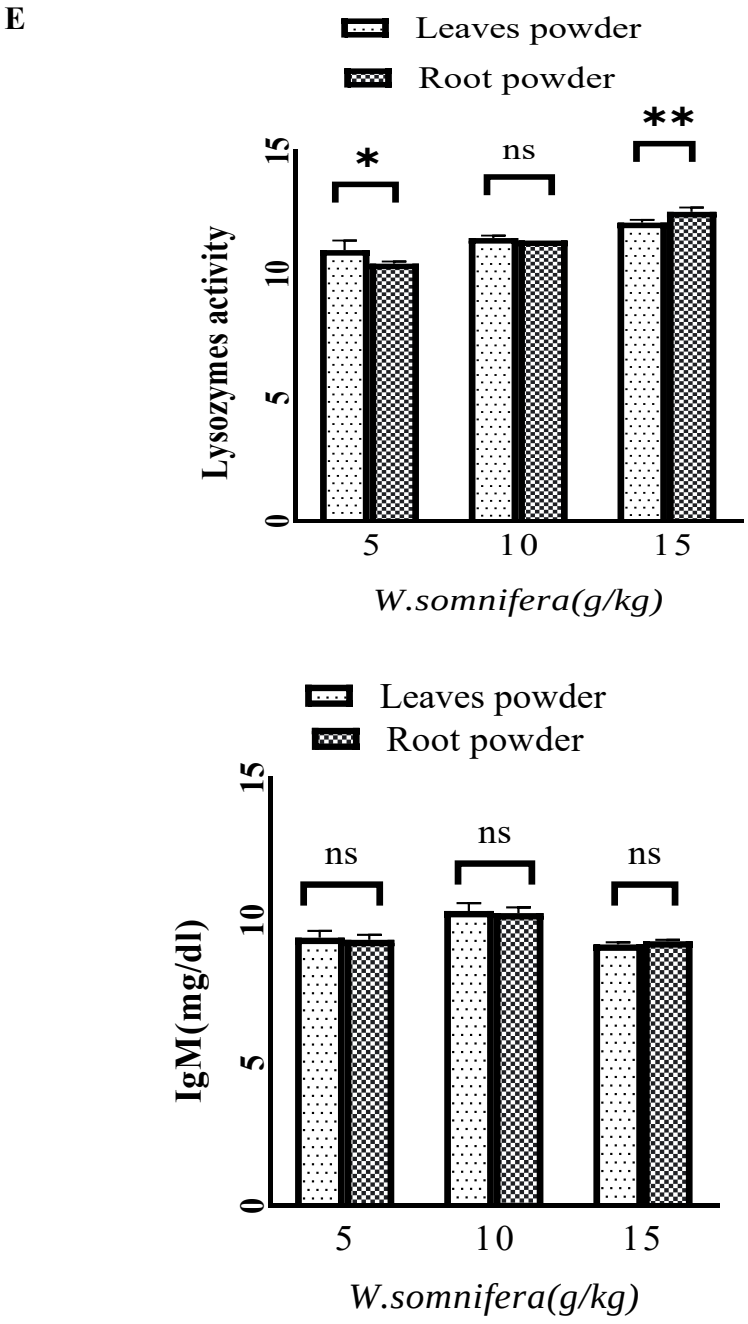
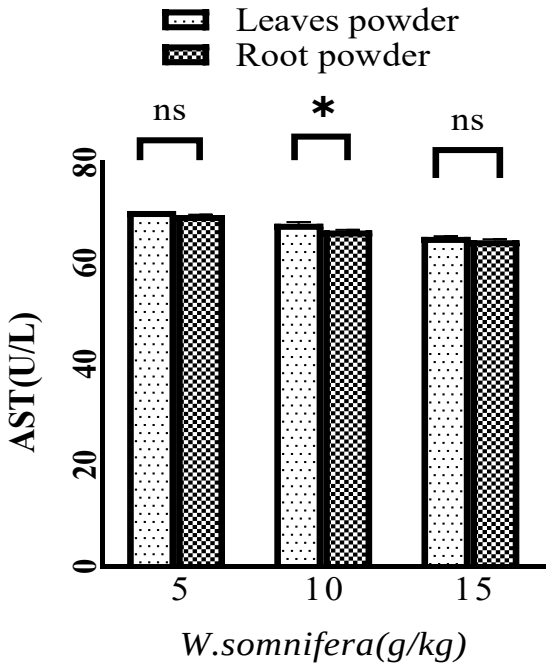


Fig. 3: Graph (E and F) showed student-t test paired wise comparison between same doses of both root and leaves of Ashwagandha inclusion effect on lysozymes activity and Immunoglobulin.

Ns= non-significant difference; *= P<0.01; **=P<.001; ***=P<0.001

G



H

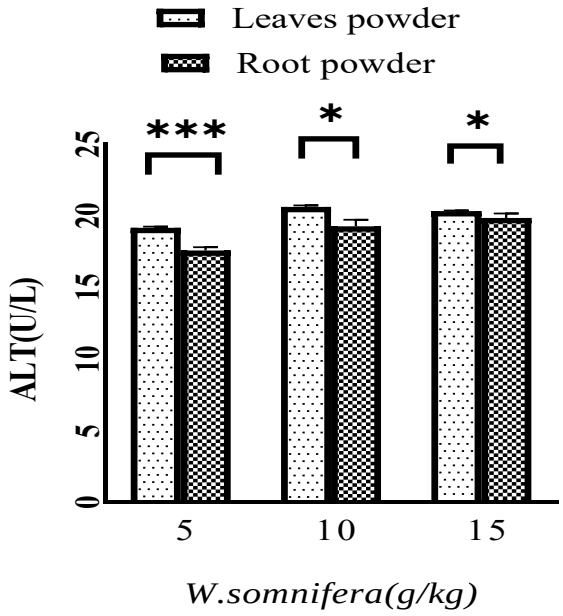


Fig. 4: Graph (G and H) showed student-t test paired wise comparison between same doses of both root and leaves of Ashwagandha inclusion effect on AST (alanine aminotransferase) ALT (alanine transaminase)

Ns= non-significant difference; *= P<0.01; **=P<.001; ***=P<0.001

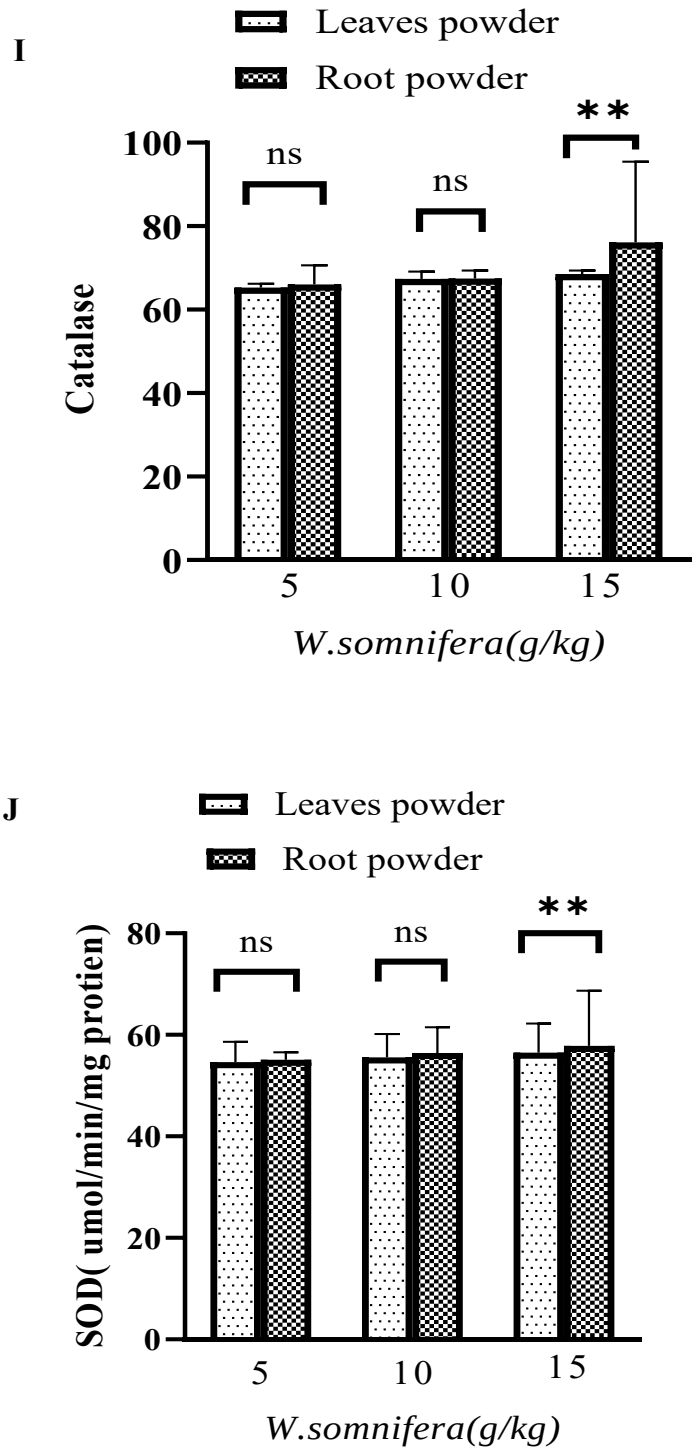


Fig. 5: Graph (I and J) showed student-t test paired wise comparison between same doses of both root and leaves of Ashwagandha inclusion effect on CAT (catalase) and SOD (superoxide dismutase) index

Ns= non-significant difference; *= P<0.01; **=P<.001; ***=P<0.001

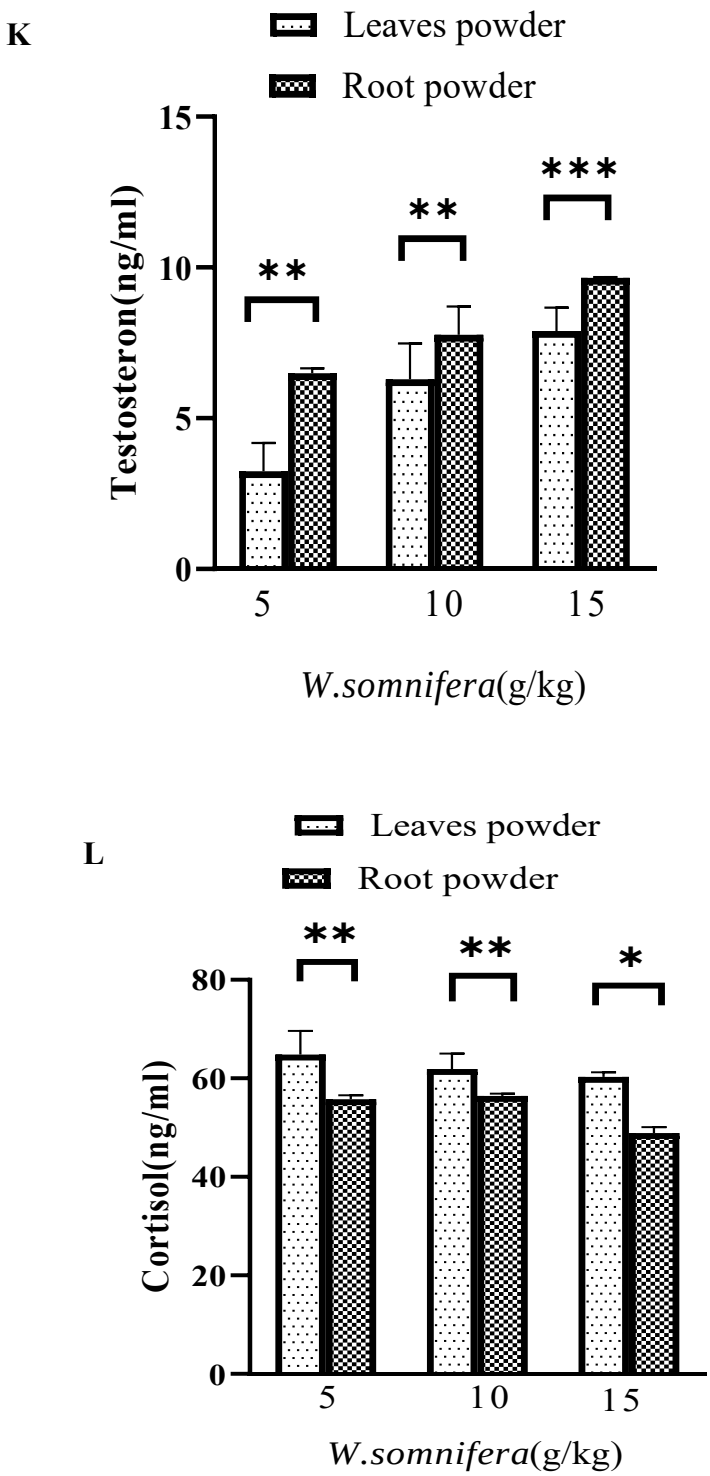


Fig. 6: Graph (K and L) showed student-t test paired wise comparison between same doses of both root and leaves of Ashwagandha inclusion effect on Testosterone and cortisol

Ns= non-significant difference; *= P<0.01; **=P<.001; ***=P<0.001

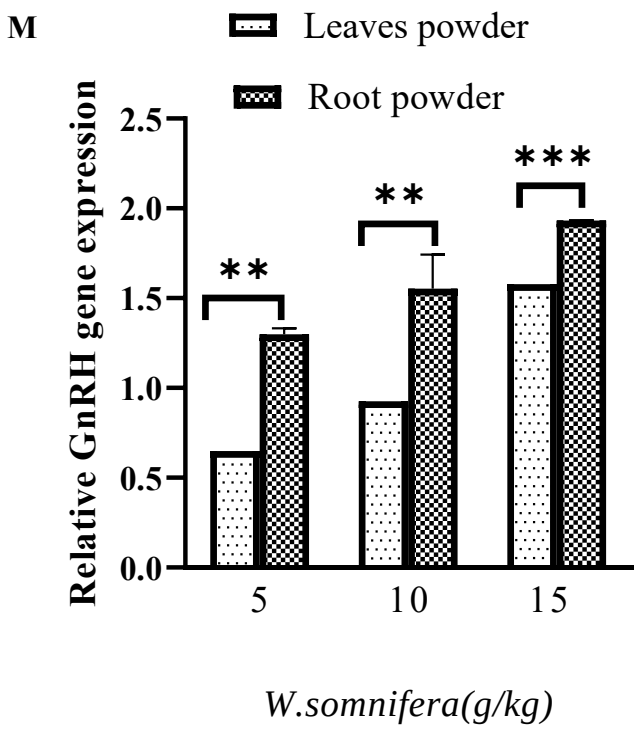


Fig. 7: Graph (M) showed student-t test paired wise comparison between same doses of both root and leaves of Ashwagandha inclusion effect on GnRH gene expression.

Ns= non-significant difference; *= P<0.01; **=P<.001; ***=P<0.001

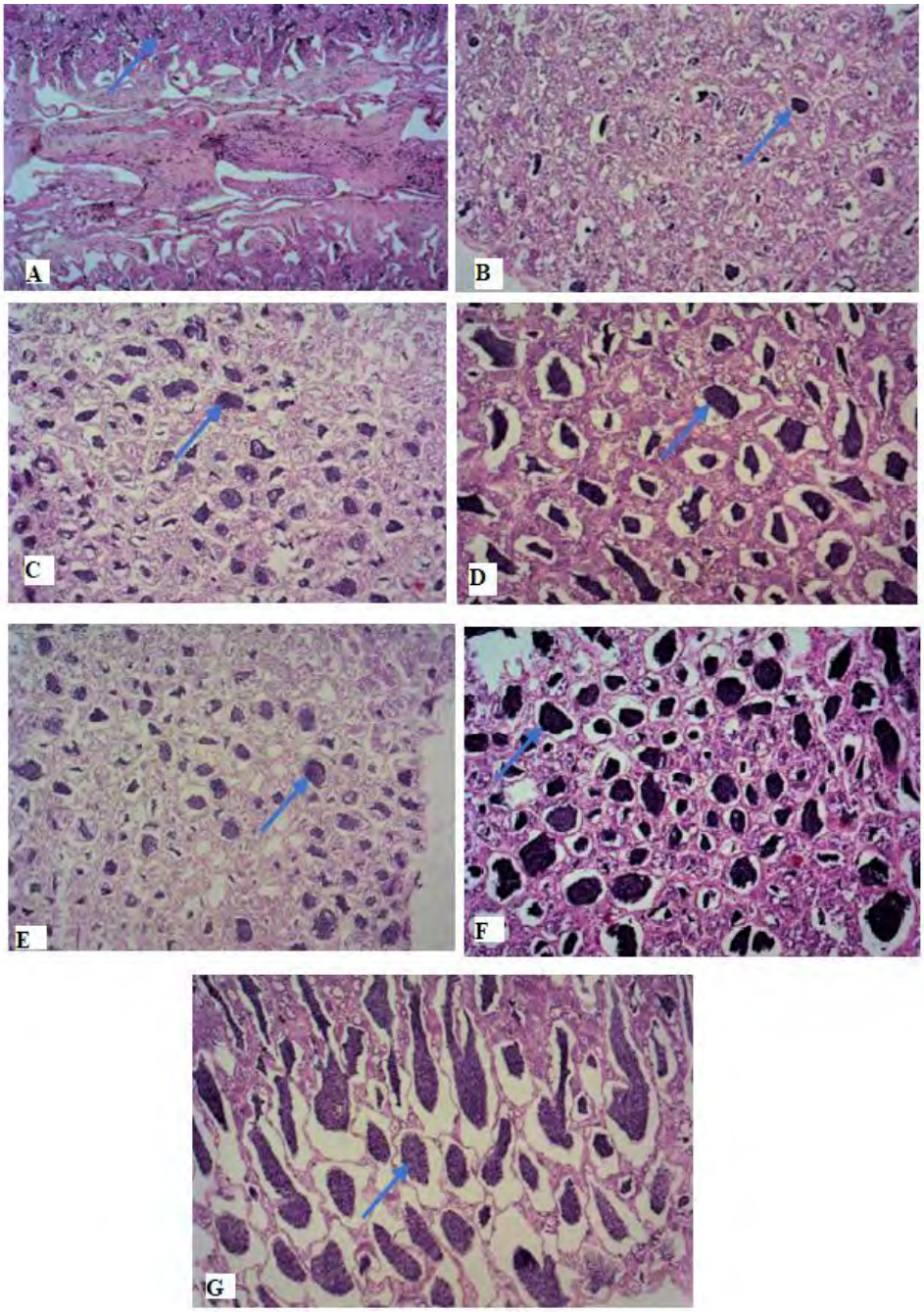


Fig. 8: Histological changes in male Nile tilapia testes. (G) Testis of male Nile tilapia (R15) show highest number of mature sperms (mature sperms turns into black spots after staining) in the ST.(F)Testis of male Nile tilapia (R10) show large number of mature sperms in ST lumen. (D) Testicular tissues of male tilapia(L15) show more sperm count compared to (E) and (C) and (B) i.e., R5, L10 and L5 respectively while (A) gonads of male Nile tilapia (CG) shows few sperm in the lumen of seminiferous tubules. The ↑show the sperm in seminiferous tubules.

DISCUSSION

Although medicinal plants have been utilized for many centuries as antimicrobial capability, anti-stress activity, stimulating appetite, immunostimulants, antioxidants, growth promotors and for fertility treatment. This activity of medicinal herbs has been examined most extensively in human, chicken and mice cell lines (Van Hai, 2015). Medicinal herbs are also used as chemotherapeutics drugs and feed supplements in aquaculture (Chang, 2000) which include components like quinone, lectin, polyphenolic, phenolic, alkaloid, terpenoid, and polypeptide many of which are excellent substitutes for chemicals, antibiotics, vaccines and other synthetic drugs (Harikrishnan et *al.,* 2011). The medicinal plant can be used in aquaculture by various means such as through injection (intraperitoneal, intramuscular) oral administration., immersion and baths (Wu *et al.,* 2010). The frequent use of synthetic pharmaceuticals has several negative consequences for the environment and human health. Antibiotic usage has led to accumulation in the muscle of fish and livestock that are used for meat purposes (Cabello, 2006).

W. somnifera effect on immunology, antioxidant of testicular tissues, hormonal profile (cortisol, testosterone) and hematological effect in Nile Tilapia (*Oreochromis niloticus)* were assessed in this study. Much research was conducted to examine the ashwagandha effect in humans and mice to analyze the immunity, antioxidant activity and fertility factor but few studies are available in the case of its effect on fish. These studies, however, are limited to species other than Nile tilapia.

Medicinal plants are frequently used in aquaculture as a growth promotor and immune stimulant in fish farming. In fact, when incorporated into the diet, they have been found to be useful in boosting the immune system and increasing growth performance (De Vico *et al.,* 2018). In research study Ashwagandha extract supplementation shows significant increase in growth rate (FCR and SGR) compared to controlled growth when fed to *L. vannamei.* (Rao *et al.,* 2020). Moreover, (Kim *et al.,* 1998) found that Shatavari and Ashwagandha incorporated diet increase body weight in *Channa punctatus*.

In our study both roots and leave powder of *W. somnifera* showed significant effect on weight gain (FCR and SGR) but comparison wise the root enrich diet of 15-gram fed have more prominent effect on growth parameter. The growth promoting ability of this plant can

involve several factors which may be responsible for the increase of digestive enzymes (Rao *et al.,* 2020).

Ashwagandha has been demonstrated to boost both red blood cells (RBCs) and hemoglobin levels. The elevation of RBC and hemoglobin enhances capacity of the blood to deliver oxygen into muscles tissues and improves aerobic capacity which suggests ergogenic effect of *W. somnifera* root extract (Choudhary *et al., 2015)*. Hematological indices in current study such as Hct, MCH, Hb, and RBC were significantly high in the R15 (15 gram of root per kilogram diet) which contained more root supplement compared to control group. During these 80 days of feeding trail ashwagandha enrich diet of both root and leaves that contained higher amount of additive in feed show significant effect on these parameters.

In 60-day trials, hematological parameters were examined to determine total (RBC count hemoglobin, Hct, MCHC, MCV, total WBC count,). In another study when *Labeo rohita* fingerlings were fed with *W. somnifera* root extract and dietary l-ascorbic acid, there was a substantial rise in total WBC count, Hct, total red blood cell and hemoglobin in treated compared to control group (Laltlanmawia *et al.,* 2019). The inclusion of Ashwagandha when mixed with molasses in milk enhances hemoglobin level in iron deficient patients.

Serum enzyme activity has been widely utilized to give simple and precise indicators of organ damage in animals and has recently attracted more attention from toxicologists (Mayer *et al.,* 2018). Lactate dehydrogenase (LDH), alanine amino transferase (ALT) and aspartate amino transferase (AST)act as link enzymes between carbohydrate and protein metabolism, but they serve as markers of changing physiological and stress conditions. The activity of the AST and ALT in serum is widely used to reveal tissue injury in fish. (Sampath *et al.*, 2002)

In the present study finding show decrease level of ALT, LDH, and AST by increasing ashwagandha -enrich diet in both root and leaves supplement compared to control group According to Harikrishnan *et al.* (2008) investigation the hepatoprotective effect of ashwagandha root supplement on the, HP (hydroperoxides), and liver marker enzymes such ALP, AST, ALT in induced chronic hyperammonemia toxicity in albino Wistar rats. These liver biomarkers were greatly reduced in rats administered with Ashwagandha root inclusion.

Similarly, Shalaby *et al.* (2006) reported that same result when 3% and 4% Garlic (*Allium sativum*) feed supplementation, show decrease level of AST and ALT in serum

compared to control group in Nile Tilapia (*Oreochromis niloticus*) in 90 days experiment. In the case of our trial, a decline in AST, ALT, and LDH levels indicates that *W. somnifera* has hepatoprotective action, which has radical scavenging activity and can be used to release stress to prevent liver damage. Moreover, the sampling was done in early winter (T 18C) which may be responsible for higher level of these biochemical indices in control group compared to Ashwagandha feed supplement since optimum temperature for Tilapia growth is 27 to 30C.

There have been reports of withaferin A, 1-oxo-5, 6-epoxy-witha-2-ene-27-etnoxy-olide, 1-oxo-5, and other active chemicals in *W. somnifera*. Besides this, it has been investigated that the root extract of Ashwagandha could repair damaged cells, prevent lipid peroxidation and possess antioxidant activity and involved in removal of urea related substances. However, the exact process is still unknown, and needs further study (Harikrishnan *et al.,* 2008).

Another study revealed Ashwagandha contain flavonoids , steroidal lactones (triterpenoids) and phenolic acid which decrease ammonia concentration in serum, and also decrease the level of MDA, and iNOS and upregulate GSH, SOD, POD ,CAT and downregulated p38 mRNA expressions in hepatic tissues (Khalil *et al.,* 2021).

Fish respond to infectious agents in both specific and non-specific mechanisms in the same way as other animals, although they depend primarily on nonspecific mechanisms. Herbal immunostimulants have the capacity to boost fish specific and non-specific defence mechanisms, hence increasing resistance to both bacterial and viral infection (Logambal *et al.,* 2000). Fish (*Labeo rohita*) fed with *W. somnifera* show low mortality and high Immunological (NBT level, Phagocytic activity, total immunoglobulin, and lysozyme activity) after post infection with *A. hydrophila* compared to controlled group (Sharma *et al.,* 2010).

In the present research study, it is found that there was high level of lysozyme and respiratory burst activity in R15 group fed with Ashwagandha root supplement compared to untreated group.

Compared to cellular defense systems, lysozyme activity serves as the principal defense of antibody-mediated immunity. Lysozyme is a natural opponent to unwanted intruders including parasites, bacteria, and viruses and abilities to destroy cell wall of different pathogens (Gatlin, 2007). Moreover, opsonin a type of lysozymes activated phagocytes and complement system and occurs in lymphoid tissues, and plasma of animals (Panase *et al.,* 2017). In the

present study elevation of lysozyme activity in treated group with ashwagandha indicate activation of non-specific defense system.

The immunoglobulins (also known as antibodies), serve as the main components of adaptive immunity and are produced by B cells to combat infections, and identify various types of pathogens. IgM is the primary antibody found in teleost and protects the fish against viruses and bacteria (Srivastava & Pandey, 2015) and consequently it reflects the immunological condition of the fish. The presence of fish IgM is not confined to the serum and secreted by fish epithelial mucus (Lobb & Clem, 1981).

In current trail fish treated with 10g/kg or1% ashwagandha root in feed show increase IgM level compared to 1.5%, and 0.5% of both root and leaves powder and controlled group. saponins containing an additional acyl group (sitoindoside VII and VIII), which is bioactive components of *W. somnifera* activate gene expression via receptor to produced innate immune response that stimulate antipathogenic molecules. A similar experiment performed by Yousefi *et al.* (2020) demonstrated that (*Lavandula angustifolia*) supplemental diet improved lysozymes and total IgM of *Cyprinus carpio* in 60 days feeding trial.

Protein synthesis may also be required to meet the need for tissue repair and to enhance immunological response. Moreover, globulin and albumin are two of the most common proteins in animals (Javed & Usmani, 2015). The liver produces albumin, while globulin is produced by multiple parts of the animal body. albumin function to maintain osmotic pressure across blood and interstitial fluid (Mazeaud *et al.,* 1977). The ratio of albumin to globulin (A:G) is used to detect liver and renal problems. Furthermore, increasing protein levels may not always represent better health status rather, their A/G ratio provides clear pictures. (Javed & Usmani, 2015).

In this regard the current experiment show that A/G ratio of serum in Nile Tilapia were significantly high in R15 among all treatment group as well as compared to control group. Babaheydar *et al.* (2015) observed the same result when different concentration of the wood betony (WB) extract added in feed show significant increase in albumin to Globulin ratio in 8% and 4% supplemental diet compared to 2% and untreated group. Current study demonstrated that Ashwagandha has some positive effect in Nile Tilapia as observed in other fishes.

A significant effect following *W. somnifera* administration, there was high A/G ratio, and improved bactericidal activity compared to the non-treated group as a control. This implies that *W. somnifera* at 0.2% be utilized as a supplement to promote *L. rohita* fingerling development, immunity response, and resistance against *A. hydrophila* (Sharma *et al.*, 2017).

Increased intracellular levels of highly reactive oxygen species (ROS) associated with oxidative stress and can have a negative impact on lipids, DNA, and proteins, during physiological activities in the body (Martínez-Álvarez *et al.,* 2005) and imbalance between ROS generation and antioxidant defense, known as oxidative stress, may result DNA hydroxylation, protein degradation, lipid peroxidation, induce apoptosis, and as a result cell death (Schieber & Chandel, 2014).The antioxidant system includes enzymes like superoxide dismutase, lipid peroxidase, glutathione reductase (GR), catalase (CAT) and glutathione and non-oxidative system like vitamin C and E (Mishra *et al.*, 2015).

The experimental trail in our studies demonstrated that antioxidant system (SOD, POD and CAT) in testicular tissues in Nile Tilapia was significantly increased in higher dose (15g of both and root) as compared to control group. However no significant changes were recorded in LPO in both experimental and control group.

Cadmium (Cd) a transition metal has multiple oxidation state, considered highly toxic, and found in the waste product that are disposed by the industries into water bodies, damage different tissue including testis (Sharma *et al.,* 2015). A similar outcome was studied when the gonadal tissue of albino rats revealed a large decrease in GSI, GSH, LPO SOD, CAT, GST GPX, and CAT, as well as Vitamin C. However, these enzymes were substantial increase when treated with both Ashwagandha and Cd (Prithiviraj *et al.,* 2013).

Several research on *W. somnifera* studied the concentrations of metal ions such as (Kumar *et al.,* 2015) Au, Zn, Fe, and Cu in testicular tissues and the mechanism involved to regulate these ions by *W. somnifera* therapy. SOD requires Zn and Cu as cofactors, while CAT and GPx require Fe and Se, respectively (Shukla *et al.,* 2011). Therefore, logically it can be concluded that *W. somnifera* root extract can exhibit antioxidant activity by supplementing cofactors (Fe, Cu, Zn, & other metal) essential for the appropriate function of different antioxidant enzymes (Sengupta et *al.,* 2018). Another possible mechanism, *W. somnifera* antioxidant capacity is rich with polyphenolic compound which have oxidation-reduction capabilities and neutralize free radicle oxygen into molecular oxygen. (Sengupta *et*

al., 2018) confirmed the extract of fruit, leaves and roots have ascorbic acid along with anthocyanin concentrations. However, the shoot (leaves) of *W. somnifera* contain the best antioxidant activity, in addition to the highest anthocyanin and vitamin content.

In reproductive hormones (testosterone, progesterone, and 17-estradiol) play critical roles in sexual development and maturation (Hosseinzadeh Sahafi *et al.,* 2020) Check this reference. The release of gonadotrophic hormones (LH, FSH) from gonadotrophs in fish species can be controlled by the synchronization of environmental and brain cues, that allow for effective reproduction (Butts *et al.,* 2012). Several studies conducted that ashwagandha root extract boost male sexual function by increasing serum testosterone and improving different sexual domains (Chauhan *et al.,* 2020).

In our study the Ashwagandha plant powder of both root and leaves in R15 group species show prominent serum testosterone level and GnRH gene expression in brain tissues compared to other treated groups (L5, R5, L10 & R10) as well as in non-treated groups. As compared to obtained results, Chauhan *et al.* (2022) evaluated the aphrodisiac effect of a *W. somnifera* root inclusion in adult men in an 8-week, double-blind, placebo-controlled trial. Fifty men with poor sexual arousal were assigned randomly to ingest either 300 mg of root extract or placebo two time in a day show Supplementation with ashwagandha root extract was related with a substantial rise in serum testosterone levels in comparison to placebo.

The effects of *Tribulus terrestris* (TT) and 17-methyl testosterone (MT) supplementation on growth parameter and fertility of male Nile tilapia, *Oreochromis niloticus* were studied in 45 days experiment. The fish fed with TT showed reproductive and growth performance (Net weight gain, FCR, testes weight,) and serum testosterone compared to 17-methyl testosterone (MT) feeding group & controlled group (excluded both MT and TT supplement) and were dose dependent. (Hassona *et al.,* 2020). Therefore, it can be assumed that Ashwagandha works by regulating GnRH neurons in the brain and stimulating their release, hence inducing the synthesis of hormones including luteinizing, Follicle stimulating hormones as well as testosterone. The responsible bioactive compounds that act as Gabba mimetic activity are mostly with none (Dar *et al.,* 2017) and Withaferin A (Ray & Jha, 2001).

In addition to oxidative stress, infertility is also linked to hormonal disturbances caused by psychological, physiological factor. The hypothalamic–pituitary–gonadal (HPG) axis and, consequently, spermatogenesis are negatively impacted by stress-inducing hormones,

particularly glucocorticoids (Chandra *et al.,* 2012). The corticosteroid produced by teleost is found in the renal tissue embedded within the anterior part of the kidney. Concentrations of corticosteroids are depended on fish species, reproductive status and sex (Milla *et al.,* 2009). Gonadotrophin-releasing hormone (GnRH) from the hypothalamus stimulates the anterior pituitary to release FSH and LH. They both subsequently act on the gonads, regulating spermatogenesis and testosterone production. Therefore, when the HPG axis is disrupted by hormones, such as gonadotrophin-inhibiting hormone, prolactin (PRL) and cortisol, spermatogenesis is negatively affected (Nargund, 2015). *W. somnifera* inhibits prolactin which influenced sperm production and avoids the stress-induced cortisol-mediated reduction in testosterone levels (Sengupta et *al.,* 2018).

In our study, findings showed significant decline in cortisol level and high in R15 group followed by R5 group. The effect of cortisol level may be due the present its withaferin A previously isolated (Saour, 1980) which have been reported to show adrenal inhibiting activity without effecting the body and spleen (Nargund, 2015).

Many studies have shown that *W. somnifera* could be useful in the treatment of male infertility (Ambiye *et al.,* 2013). In current histological examination of the testicular tissue reveals large number of mature sperm were observed in R15 and, R10 followed by L15 in fish supplemented with lower doses and non-treated group.

W. somnifera treatment induced testicular development and spermatogenesis in immature Wistar rats by directly affecting the seminiferous tubules and increased testicular sperm production and serum testosterone levels (Ng *et al*., 2020).

Conclusion

This study found that Ashwagandha enhances hematological and biochemical parameters, immunity, and fertility in male Nile tilapia. It has been concluded that *W. somnifera* is an immunomodulator and increases fertility-related factors such as testosterone, cortisol, antioxidant enzymes of testicular tissues, and gonadal weight. Aside from that, both the root and leaves of this plant increase the above parameters, but the root of this plant has a greater beneficial impact, particularly in a dose-dependent manner.

REFERENCES

Abdel-Magied, E. M., Abdel-Rahman, H. A., & Harraz, F. M. (2001). The effect of aqueous extracts of *Cynomorium coccineum* and *Withania somnifera* on testicular development in immature Wistar rats. *Journal of Ethnopharmacology*. *75*(1); 1-4.

Alam, N., Hossain, M., Mottalib, M. A., Sulaiman, S. A., Gan, S. H., & Khalil, M. I. (2012). Methanolic extracts of *Withania somnifera* leaves, fruits and roots possess antioxidant properties and antibacterial activities. *BMC complementary and alternative medicine*. *12*(1); 1-8.

Ali, N. A., Jülich, W. D., Kusnick, C., & Lindequist, U. (2001). Screening of Yemeni medicinal plants for antibacterial and cytotoxic activities. *Journal of ethnopharmacology*. *74*(2); 173-179.

Ambiye, V. R., Langade, D., Dongre, S., Aptikar, P., Kulkarni, M., & Dongre, A. (2013). Clinical evaluation of the spermatogenic activity of the root extract of Ashwagandha *(Withania somnifera)* in oligospermic males: a pilot study. *Evidence-Based Complementary and Alternative Medicine*, *2013*.

Awuchi, C.G., Chukwu, C.N., Iyiola, A.O., Noreen, S., Morya, S., Adeleye, A.O., Twinomuhwezi, H., Leicht, K., Mitaki, N.B. and Okpala, C.O.R. (2022). Bioactive Compounds and Therapeutics from Fish: Revisiting Their Suitability in Functional Foods to Enhance Human Wellbeing. *BioMed Research International*, *2022*.

Bahi, A., Ramos-Vega, A., Angulo, C., Monreal-Escalante, E., & Guardiola, F. A. (2023). Microalgae with immunomodulatory effects on fish. *Reviews in Aquaculture*.*94*(4); 142-145.

Bahrami Babaheydari, S., Paykan Heyrati, F., Dorafshan, S., Mahboobi Soofiani, N., & Vahabi, M. R. (2015). Effect of dietary wood betony, *Stachys lavandulifolia* extract on growth performance, haematological and biochemical parameters of Common carp, *Cyprinus carpio. Iranian Journal of Fisheries Sciences*. *14*(4); 805-817.

Bhattacharya, A., Ghosal, S., & Bhattacharya, S. K. (2001). Anti-oxidant effect of *Withania somnifera* glycowithanolides in chronic footshock stress-induced perturbations of oxidative

free radical scavenging enzymes and lipid peroxidation in rat frontal cortex and striatum. *Journal of ethnopharmacology*.*74*(1);1-6.

Boyd, C. E., McNevin, A. A., & Davis, R. P. (2022). The contribution of fisheries and aquaculture to the global protein supply. *Food security*. *14*(3); 805-827.

Bromage, N., Jones, J., Randall, C., Thrush, M., Davies, B., Springate, J., Duston, J. & Barker, G. (1992). Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*. *100*(1-3);141-166.

Bromage, N., Jones, J., Randall, C., Thrush, M., Davies, B., Springate, J., Duston, J. & Barker, G. (1992). Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (*Oncorhynchus mykiss)*. *Aquaculture*. *100*(1-3); 141-166.

Butts, I. A., Love, O. P., Farwell, M., & Pitcher, T. E. (2012). Primary and secondary sexual characters in alternative reproductive tactics of Chinook salmon: associations with androgens and the maturation-inducing steroid. *General and comparative endocrinology*; *175*(3). 449-456.

Cabello, F.C. (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology*. 8; 1137–1144.

Cai, J., Leung, P.S. (2017). Short-Term Projection of Global Fish Demand and Supply Gaps. FAO Fisheries and Aquaculture Technical Paper No. 607.

Chang, J. (2000). Medicinal herbs: drugs or dietary supplements?. *Biochemical pharmacology*. *59* (3); 211-219.

Chauhan, S., Srivastava, M. K., & Pathak, A. K. (2022). Effect of standardized root extract of ashwagandha (*Withania somnifera)* on well-being and sexual performance in adult males: A randomized controlled trial. *Health Science Reports*. *5*(4); e741.

Chauhan, S., Srivastava, M. K., & Pathak, A. K. (2022). Effect of standardized root extract of ashwagandha (*Withania somnifera*) on well-being and sexual performance in adult males: A randomized controlled trial. *Health Science Reports*. *5*(4); e741.

Choudhary, B., Shetty, A., & Langade, D. G. (2015). Efficacy of Ashwagandha *(Withania somnifera* [L.] Dunal) in improving cardiorespiratory endurance in healthy athletic adults. *AYU (An international quarterly journal of research in Ayurveda)*. *36*(1); 63.

Citarasu, T., Babu, M. M., Sekar, R. R. J., & Petermarian, M. (2002). Developing Artemia enriched herbal diet for producing quality larvae in Penaeus monodon, Fabricius. *Asian Fisheries Science. 15(1); 21-32.*

Cochrane, K., De Young, C., Soto, D., & Bahri, T. (2009). Climate change implications for fisheries and aquaculture. *FAO Fisheries and aquaculture technical paper*. *530*; 212

Coward, K., & Bromage, N. R. (2000). Reproductive physiology of female tilapia broodstock. *Reviews in Fish Biology and Fisheries*. *10*(1); 1-25.

Cronin, J. R. (2002). The potential of astaxanthin: A new multipurpose, high-power antioxidant. *Alternative & Complementary Therapies*. *8*(1); 44-49.

Dar, N. J., Bhat, J. A., Satti, N. K., Sharma, P. R., Hamid, A., & Ahmad, M. (2017). Withanone, an active constituent from *Withania somnifera*, affords protection against NMDA-induced excitotoxicity in neuron-like cells. *Molecular Neurobiology*. *54*(7);5061-5073.

Davis, L., & Kuttan, G. (2000). Immunomodulatory activity of *Withania somnifera*. *Journal of ethnopharmacology*. *71*(1-2); 193-200.

De Vico, G., Guida, V., & Carella, F. (2018). *Urtica dioica* (Stinging Nettle): A neglected plant with emerging growth promoter/immunostimulant properties for farmed fish. *Frontiers in Physiology*. *9*; 285.

Dhanani, T., Shah, S., Gajbhiye, N. A., & Kumar, S. (2017). Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. *Arabian journal of chemistry*. *10*; S1193-S1199.

Dhas, S. A., Selvaraj, T., Citarasu, T., Punitha, S. M. J., & Babu, M. M. (2015). Effect of supplemented diet with maturation plant extract on reproductive performance of *Etroplus suratansis*. *Aquaculture Reports*. *2*; 58-62.

Edwards, P., Zhang, W., Belton, B., & Little, D. C. (2019). Misunderstandings, myths and mantras in aquaculture: its contribution to world food supplies has been systematically over reported. *Marine Policy*. *106*; 103547.

Elhadidy, M. E., Sawie, H. G., Meguid, N. A., & Khadrawy, Y. A. (2018). Protective effect of ashwagandha (*Withania somnifera*) against neurotoxicity induced by aluminum chloride in rats. *Asian Pacific Journal of Tropical Biomedicine*. *8*(1); 59.

Emeka, U., Iloegbunam, N. G., Gbekele-Oluwa, A. R., & Bola, M. (2014). Natural products and aquaculture development. *Journal of Pharmaceutical and Biological Sciences*. *9*(2); 70-82.

FAO, Rome, pp. 607. Camacho-Valdez, V., Ruiz-Luna, A., Ghermandi, A., Berlanga-Robles, C.A., Nunes, P.A.L.D.(2014). Effects of land use changes on the ecosystem service values of coastal wetlands. Environ. Manage. 54 (4); 852–864.

Froehlich, H. E., Gentry, R. R., & Halpern, B. S. (2017). Conservation aquaculture: Shifting the narrative and paradigm of aquaculture's role in resource management. *Biological Conservation*. *215*; 162-168.

Gatlin, D. M. (2007). *Dietary supplements for the health and quality of cultured fish*. Cabi.

Ghosal, S., Lal, J., Srivastava, R., Bhattacharya, S. K., Upadhyay, S. N., Jaiswal, A. K., & Chattopadhyay, U. (1989). Immunomodulatory and CNS effects of sitoindosides IX and X, two new glycowithanolides from *Withania somnifera*. *Phytotherapy Research*. *3*(5); 201-206.

Gül, E. T., Olgun, O., Yıldız, A., Tüzün, A. E., & Sarmiento-García, A. (2022). Use of maca powder *(Lepidium meyenii*) as feed additive in diets of laying quails at different ages: Its effect on performance, eggshell quality, serum, ileum, and bone properties. *Veterinary Sciences. 9(8); 418.*

Harikrishnan, B., Subramanian, P., & Subash, S. (2008). Effect of *Withania somnifera* root powder on the levels of circulatory lipid peroxidation and liver marker enzymes in chronic hyperammonemia. *E-Journal of Chemistry*. *5*(4); 872-877.

Harikrishnan, R., Balasundaram, C., & Heo, M. S. (2011). Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture*. *317*(1-4); 1-15.

Hassan, H. U., Siddique, M. A. M., Abdel-Aziz, M. F. A., Gabo, K., Ahmed, A. E., & Parvez, M. S. (2022). Assessment of growth characteristics and the survival rate of pacific

white shrimp *litopenaeus vannamei* (boone, 1931), reared with different stocking densities in the coastal ponds, sindh, pakistan. *JAPS: Journal of Animal & Plant Sciences*. *32*(6); 1754-1762.

Hassona, N. N., Zayed, M. M., Eltras, W. F., & Mohamed, R. A. (2020). Dietary supplementation of *Tribulus terrestris extract* improves growth and reproductive performances of the male Nile tilapia (*Oreochromis niloticus*). *Aquaculture Research*. *51*(10); 4245-4254.

Hoseinifar, S. H., Sun, Y. Z., Zhou, Z., Van Doan, H., Davies, S. J., & Harikrishnan, R. (2020). Boosting immune function and disease bio-control through environment-friendly and sustainable approaches in finfish aquaculture: herbal therapy scenarios. *Reviews in Fisheries Science & Aquaculture*. *28*(3); 303-321.

Hosseinzadeh Sahafi, H., Dehghan Madiseh, S., Koohilai, S., Hamidinejad, M., & Velayatzadeh, M. (2020). Plasma levels of sex steroids (testosterone, progesterone and 17β-estradiol) in Rohu Carp *Labeo rohita* broodstock from Khuzestan, Iran. *Iranian Journal of Fisheries Sciences*. *19*(6); 3063-3074.

Immanuel, G., Uma, R. P., Iyapparaj, P., Citarasu, T., Punitha Peter, S. M., Michael Babu, M., & Palavesam, A. (2009). Dietary medicinal plant extracts improve growth, immune activity and survival of tilapia *Oreochromis mossambicus*. *Journal of fish biology*. *74*(7); 1462-1475.

Jarwar, A. A. (2008). A status overview of fisheries and aquaculture development in Pakistan with context to other Asian countries. *Aquaculture Asia*. *13*; 13-18.

Javed, M., & Usmani, N. (2015). Stress response of biomolecules (carbohydrate, protein and lipid profiles) in fish *Channa punctatus* inhabiting river polluted by Thermal Power Plant effluent. *Saudi Journal of Biological Sciences*. *22*(2); 237-242.

K Chandra, A., Sengupta, P., Goswami, H., & Sarkar, M. (2012). Excessive dietary calcium in the disruption of structural and functional status of adult male reproductive system in rat with possible mechanism. *Molecular and cellular biochemistry*. *364*(1); 181-191.

Kaliyadasa, E., Jayasinghe, L., & Peiris, S. (2018). Yield and Phytochemical Attributes of Ashwagandha (*Withania somnifera* Dunal) Tubers as Affected by Source of Nutrients. *Journal of Advanced Agricultural Technologies Vol*. *5*(2); 624-630

Kashyap, V. K., Peasah-Darkwah, G., Dhasmana, A., Jaggi, M., Yallapu, M. M., & Chauhan, S. C. (2022). *Withania somnifera*: Progress towards a Pharmaceutical Agent for Immunomodulation and Cancer Therapeutics. *Pharmaceutics*. *14*(3); 611.

Khalil, H. M., Eliwa, H. A., El-Shiekh, R. A., Al-Mokaddem, A. K., Hassan, M., Tawfek, A. M., & El-Maadawy, W. H. (2021). Ashwagandha (*Withania somnifera*) root extract attenuates hepatic and cognitive deficits in thioacetamide-induced rat model of hepatic encephalopathy via induction of Nrf2/HO-1 and mitigation of NF-κB/MAPK signaling pathways. *Journal of Ethnopharmacology*. *277*; 114141.

Kim, D. S. (1998). Effect of Obosan supplemented diet on growth, feed conversion ratio and body composition of Nile tilapia, *Oreochromis niloticus*. *Journal of Aquaculture*. *11*; 83-90.

Kumar, A., Kumar, R., Rahman, M. S., Iqubal, M. A., Anand, G., Niraj, P. K., & Ali, M. (2015). Phytoremedial effect of *Withania somnifera* against arsenic-induced testicular toxicity in Charles Foster rats. *Avicenna Journal of Phytomedicine*. *5*(4); 355.

Laghari, M. Y. (2018). Aquaculture in Pakistan: Challenges and opportunities. *International Journal of Fisheries and Aquatic Studies*. *6*(2); 56-59.

Laltlanmawia, C., Saha, R. K., Saha, H., & Biswas, P. (2019). Ameliorating effects of dietary mixture of Withania somnifera root extract and vitamin C in *Labeo rohita* against low pH and waterborne iron stresses. *Fish & shellfish immunology*. *88*; 170-178.

Lee, J. Y., & Gao, Y. (2012). Review of the application of garlic, *Allium sativum*, in aquaculture. *Journal of the World Aquaculture Society*. *43*(4); 447-458.

Lèveque, C. (2002). Out of Africa: The Success Story of Tilapias. *Environmental Biology of Fishes*. *64*(4);

Lobb, C. J., & Clem, L. W. (1981). The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus, and bile. *The Journal of Immunology*. *127*(4); 1525-1529.

Logambal, S. M., Venkatalakshmi, S., & Dinakaran Michael, R. (2000). Immunostimulatory effect of leaf extract of *Ocimum sanctum* Linn. in *Oreochromis mossambicus* (Peters). *Hydrobiologia*. *430*(1); 113-120.

Low, W. Y., & Tan, H. M. (2007). Asian traditional medicine for erectile dysfunction. *Journal of Men's Health and Gender*. *4*(3); 245-250.

Mariappan, B., Kaliyamurthi, V., & Binesh, A. (2023). Medicinal plants or plant derived compounds used in aquaculture. In *Recent Advances in Aquaculture Microbial Technology* 153-207. Academic Press.

Martínez-Álvarez, R. M., Morales, A. E., & Sanz, A. (2005). Antioxidant defenses in fish: biotic and abiotic factors. *Reviews in Fish Biology and fisheries*. *15*(1); 75-88.

Mayer, F. L., Versteeg, D. J., McKee, M. J., Folmar, L. C., Graney, R. L., McCume, D. C., & Rattner, B. A. (2018). Physiological and nonspecific biomarkers. In *Biomarkers* 5-86. CRC Press.

Mazeaud, M. M., Mazeaud, F., & Donaldson, E. M. (1977). Primary and secondary effects of stress in fish: some new data with a general review. *Transactions of the American Fisheries Society*. *106*(3); 201-212.

Mendonca, B. S., Ribas, B. P., Shinohara, A. I., Vicente, I. S. T., Botelho, R. M., Genovez, L. W., & Pezzato, L. E. (2012). The soy protein as food for tropical fish grown. In *Proceedings of the 9th Meeting of Animal Science*.20-24

Michael Babu, M. (1999). Developing bio-encapsulated ayurvedic products for maturation and quality larval production in *Penaeus monodon* (Doctoral dissertation, Manonmaniam Sundaranar University).

Milla, S., Wang, N., Mandiki, S. N. M., & Kestemont, P. (2009). Corticosteroids: friends or foes of teleost fish reproduction? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. *153*(3); 242-251.

Mir, B. A., Koul, S., Kumar, A., Kaul, M. K., Soodan, A. S., & Raina, S. N. (2011). Assessment and characterization of genetic diversity in *Withania somnifera* (L.) Dunal using RAPD and AFLP markers. *African Journal of Biotechnology*. *10*(66); 14746-14756.

Mishra, V., Shah, C., Mokashe, N., Chavan, R., Yadav, H., & Prajapati, J. (2015). Probiotics as potential antioxidants: a systematic review. *Journal of agricultural and food chemistry*. *63*(14);3615-3626.

Misra, L., Mishra, P., Pandey, A., Sangwan, R. S., & Sangwan, N. S. (2012). 1, 4-Dioxane and ergosterol derivatives from *Withania somnifera* roots. *Journal of Asian natural products research*. *14*(1); 39-45.

Mohsin, M., Mu, Y., Mehak, A., Memon, A. M., Noman, M., & Nazir, K. (2017). Aquaculture in Pakistan: Status, opportunities and challenges. *46*(09); 1872-1875.

Mohsin, M., Mu, Y., Memon, A. M., Kalhoro, M. T., & Shah, S. B. H. (2017). Fishery stock assessment of Kiddi shrimp (*Parapenaeopsis stylifera*) in the Northern Arabian Sea Coast of Pakistan by using surplus production models. *Chinese Journal of Oceanology and Limnology*. *35*; 936-946.

Mondal, P., Garai, P., Chatterjee, A., & Saha, N. C. (2021). Toxicological and therapeutic effects of neem (Azadirachta indica) leaf powder in hole-in-the-head (HITH) disease of fish Anabas testudineus. *Aquaculture Research.* 52(2); 715-723.

Nargund, V. H. (2015). Effects of psychological stress on male fertility. *Nature Reviews Urology*. *12*(7); 373-382

Nayar, S. L. (1964). Medicinal plants of commercial importance found wild in Uttar Pradesh and their distribution. *Journal of the Bombay Natural History Society. 61*; 651-661.

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D.C., Lubchenco, J., Shumway, S.E. & Troell, M. (2021). A 20-year retrospective review of global aquaculture. *Nature*. *591*(7851); 551-563.

Nazir, A., & Khan, M. A. (2021). Using otoliths for fish stock discrimination: status and challenges. *Acta Ichthyologica et Piscatoria*. *51*; 199.

Nazir, K., Yongtong, M., Hussain, K., Kalhoro, M. A., Kartika, S., & Mohsin, M. (2016). A study on the assessment of fisheries resources in Pakistan and its potential to support marine economy.

Ng, Q. X., Loke, W., Foo, N. X., Tan, W. J., Chan, H. W., Lim, D. Y., & Yeo, W. S. (2020). A systematic review of the clinical use of *Withania somnifera* (Ashwagandha) to ameliorate cognitive dysfunction. *Phytotherapy research*. *34*(3); 583-590.

Ogunfowora, L. A., Iwuozor, K. O., Ighalo, J. O., & Igwegbe, C. A. (2021). Trends in the treatment of aquaculture effluents using nanotechnology. *Cleaner Materials*. *2*; 100024

Omitoyin, B. O., Ajani, E. K., & Sadiq, H. O. (2013). Preliminary investigation of Tribulus terrestris (Linn., 1753) extracts as natural sex reversal agent in *Oreochromis niloticus* (Linn., 1758) larvae. *International Journal of Aquaculture. 3*;

Panase, P., Saenphet, S., & Saenphet, K. (2017). Visceral and serum lysozyme activities in some freshwater fish (three catfish and two carps). *Comparative Clinical Pathology*. *26*(1); 169-173.

Pandey, G., Madhuri, S., & Mandloi, A. K. (2012). Medicinal plants useful in fish diseases. *Plant Archives*. *12*(1); 1-4.

Prithiviraj, E., Suresh, S., Lakshmi, N. V., Karthik, M., Ganesh, L., & Prakash, S. (2013). Protective effect of *Withania somnifera* (Linn.) on cadmium-induced oxidative injury in rat testis. *Phytopharmacology*. *4*; 269-90.

Rao, A.R., Reddy, D.R.K., Ramana, T.V., Rao, A.C., Dhanapal, K., Suguna, T., Panigrahi, A., Das, R.R., Ganesh, G. and Pamanna, D. (2020). Effect of dietary supplementation of Aswagandha (*Withania somnifera*) root extract on growth performance, digestive enzymes activities of white leg shrimp *Litopenaeus vannamei*. *Journal of Entomology and Zoology Studies 2020*. *8*(4);2206-2210.

Ravikumar, B., Sarkar, S., Davies, J.E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z.W., Jimenez-Sanchez, M., Korolchuk, V.I., Lichtenberg, M., Luo, S. & Massey, D.C. (2010).Regulation of mammalian autophagy in physiology and pathophysiology. *Physiological reviews*. *90*(4); 1383-1435.

Ray, S., & Jha, S. (2001). Production of withaferin A in shoot cultures of *Withania somnifera. Planta medica*. *67*(05); 432-436.

Rohit, J., & Sumita, K. (2012). Phytochemistry, pharmacology, and biotechnology of *Withania somnifera* and *Withania coagulans*: A review. *Journal of Medicinal Plants Research*. *6*(41); 5388-5399.

Sampath, H., Ramesh, M., & Ramanujam, M. (2002). Responses of plasma transaminase activity in *Cyprinus carpio* var. communis to mercury toxicity.

Sandhya, S., & Sushil, K. (1998). *Withania somnifera: the Indian ginseng ashwagandha*. Central Institute of Medicinal and Aromatic Plants.

Saour, K. Y. (1980). ., Al-Khafaji, S. H., & Abdul-Nabi, M. H. (1992). Anti-granuloma activity of Iraqi *Withania somnifera*. *Journal of ethnopharmacology*. *37*(2); 113-116.

Saour, K. Y. (1980). *Phytochemical Investigation of Withania somnifera Dun. Grown in Iraq* (Doctoral dissertation, M. Sc. Thesis. College of Pharmacy. University of Baghdad).

Savaya, A., Glassner, H., Livne-Luzon, S., Chishinski, R., Molcho, J., Aflalo, E.D., Zilberg, D. & Sagi, A. (2020). Prawn monosex populations as biocontrol agents for snail vectors of fish parasites. *Aquaculture*. *520*;735016.

Schieber, M., & Chandel, N. S. (2014). ROS function in redox signalling and oxidative stress. *Current biology*. *24*(10); R453-R462.

Sengupta, P., Agarwal, A., Pogrebetskaya, M., Roychoudhury, S., Durairajanayagam, D., & Henkel, R. (2018). Role of *Withania somnifera* (Ashwagandha) in the management of male infertility. *Reproductive biomedicine online*. *36*(3); 311-326.

Shalaby, A.M., Khattab, Y.A. and Abdel Rahman, A.M., (2006). Effects of Garlic (*Allium sativum*) and chloramphenicol on growth performance, physiological parameters and survival of Nile tilapia (*Oreochromis niloticus*). *Journal of Venomous Animals and Toxins including Tropical Diseases*. 12; 172-201.

Sharma, A., Chanu, T. I., & Deo, A. D. (2017). Dietary ashwagandha, *Withania somnifera* (L. dunal) potentiates growth, haemato-biochemical response and disease resistance of *Labeo rohita* (Hamilton, 1822) against *Aeromonas hydrophila* infection. *Journal of Entomology and Zoology Studies*. *5*(5); 1113-1119.

Sharma, A., Deo, A. D., Riteshkumar, S. T., Chanu, T. I., & Das, A. (2010). Effect of *Withania somnifera* (L. Dunal) root as a feed additive on immunological parameters and disease resistance to *Aeromonas hydrophila* in *Labeo rohita* (Hamilton) fingerlings. *Fish & Shellfish Immunology*. *29*(3); 508-512.

Sharma, H., Rawal, N., & Mathew, B. B. (2015). The characteristics, toxicity and effects of cadmium. *International journal of nanotechnology and nanoscience*. *3*(10); 112-114

Shukla, K. K., Mahdi, A. A., Mishra, V., Rajender, S., Sankhwar, S. N., Patel, D., & Das, M. (2011). *Withania somnifera* improves semen quality by combating oxidative stress and

cell death and improving essential metal concentrations. *Reproductive BioMedicine Online*. *22*(5); 421-427.

Srivastava, A., Ansal, M. D., & Khairnar, S. O. (2020). Effect of ashwagandha (*Withania somnifera*) root powder supplementation on survival, growth and flesh quality of an indian major carp, *Labeo rohita* (Ham.) fingerlings. *Animal Nutrition and Feed Technology*. *20*(3); 515-524.

Srivastava, P. K., & Pandey, A. K. (2015). Role of immunostimulants in immune responses of fish and shellfish. *Biochemical and Cellular Archives*. *15*(1); 47-73.

Tadese, D.A., Song, C., Sun, C., Liu, B., Liu, B., Zhou, Q., Xu, P., Ge, X., Liu, M., Xu, X. and Tamiru, M. (2022). The role of currently used medicinal plants in aquaculture and their action mechanisms: A review. *Reviews in Aquaculture*. *14*(2); 816-847.

Tole, T. T., & Saifu, Y. T. (2019). Phytochemical investigation of *Withania somnifera* and *Commiphora kataf*. *Journal of Pharmacognosy and Phytochemistry*. *8*(3); 2958-2964.

Tsakiridis, A., O'Donoghue, C., Hynes, S., & Kilcline, K. (2020). A comparison of environmental and economic sustainability across seafood and livestock product value chains. *Marine Policy*. *117*; 103968.

Uddin, Q., Samiulla, L., Singh, V. K., & Jamil, S. S. (2012). Phytochemical and pharmacological profile of *Withania somnifera* Dunal: a review. *Journal of Applied Pharmaceutical Science*. (Issue), 170-175.

Valenzuela-Gutiérrez, R., Lago-Lestón, A., Vargas-Albores, F., Cicala, F., & Martínez-Porchas, M. (2021). Exploring the garlic *(Allium sativum)* properties for fish aquaculture. *Fish Physiology and Biochemistry. 47(4); 1179-1198.*

Van Hai, N. (2015). The use of medicinal plants as immunostimulants in aquaculture: A review. *Aquaculture*. *446*; 88-96.

Vijayakumar, P., Dhas, T. S., Mani, R., & Raguraman, V. (2021). Antiviral activity of sulfated polysaccharides from *Sargassum ilicifolium* against fish Betanodavirus infection. *Aquaculture International*. *29*; 1049-1067.

Wu, Y.R., Gong, Q.F., Fang, H., Liang, W.W., Chen, M. and He, R.J. (2013) Effect of Sophora flavescens on non-specific immune response of tilapia (GIFT *Oreochromis*

niloticus) and disease resistance against *Streptococcus agalactiae*. *Fish and Shellfish Immunology*. 34;

Yin, G. U. O. J. U. N., Ardo, L. Á. S. Z. L. Ó., Jeney, Z. S. I. G. M. O. N. D., Xu, P., & Jeney, G. A. L. I. N. A. (2008). Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) enhance non-specific immune response of tilapia, *Oreochromis niloticus*, and protection against *Aeromonas hydrophila*. *Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines*, 269-282.

Yousefi, M., Vatnikov, Y. A., Kulikov, E. V., Plushikov, V. G., Drukovsky, S. G., Hoseinifar, S. H., & Van Doan, H. (2020). The protective effects of dietary garlic on common carp (*Cyprinus carpio*) exposed to ambient ammonia toxicity. *Aquaculture*. *526*; 735400.

Zahra, S., Shah, S. A. R., & Badeeb, R. A. (2022). A sustainable green–blue revolution in Pakistan: a new perspective from quantile regression analysis. *Environment, Development and Sustainability*, 1-28.

Zahran, E., El Sebaei, M. G., Awadin, W., Elbahnaswy, S., Risha, E., & Elseady, Y. (2020). *Withania somnifera* dietary supplementation improves lipid profile, intestinal histomorphology in healthy Nile tilapia (*Oreochromis niloticus*), and modulates cytokines response to Streptococcus infection. *Fish & Shellfish Immunology*. *106*; 133-141.