Antifertility effect of *Ocimum sanctum Linn* in *Oreochromis mossambicus*

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Antifertility effect of *Ocimum sanctum Linn* in *Oreochromis mossambicus*

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By

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2022

"In the Name of ALLAH, the Most Beneficent, the Most Merciful"

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is my original work. I have not previously presented this work elsewhere for any other degree.

Rehmana Aslam

Dedicated to:

My loving parents, siblings, respected supervisor, and my beloved Nieces Zunaira Fatima, Hareem Fatima and Nephew Muhammad Musa Hussain, and dear friend Sana Ahmed.

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LIST OF ABBREVIATIONS

MT	Methyltestosterone
LH	Luteinizing hormone
EDTA	Ethylenediaminetetraacetic acid
CAT	Catalase
SGR	Specific growth rate
FCR	Feed conversion ratio
WG	Weight gain
IBW	Initial body weight
FBW	Final body weight
MCHC	Mean corpuscular hemoglobin concentration
MCH	Mean corpuscular hemoglobin
ELISA	Enzyme-linked Immunoassay
MCV	mean corpuscular volume
FeCl3	Ferric chloride
TS	Transverse section
FeSO4	Ferrous sulphate
GPX	Glutathione Peroxidase
H2O2	Hydrogen peroxide
AST	Aspartate Aminotransferase
ALT	Alanine Aminotransferase
LDH	Lactate Dehydrogenase
kg	Kilogram
mM	Millimolar
MO	Mature oocyte
NADH	Nicotine adenine dinucleotide

YG	Yolk granules
AT	Atresia
SL	Seminiferous lobule
PBS	Phosphate Buffer Saline
IT	Interlobular tissue
POD	Peroxidase
GSI	Gonado-somatic index
GST	Glutathione –S- Transferase
Rpm	Revolution per minute
GSH	Reduced Glutathione
SOD	Super Oxide Dismutase
GSR	Glutathione reductase
GPX	Glutathione peroxidase
TBARS	Thiobarbituric Acid Reactive Substances Assay
ADC	Apparent digestibility coefficient
PCV	Packed cell volume
U/L	Unit per liter
HPTA	Hypothalamic-Pituitary-Testicular Axis
Kg	Kilogram
µm	Micromolar
µl	Microliter
µmol	Micro molar
µmol	Micromole
µg	Microgram
PBS	Phosphate buffer saline
mg	Milligram

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ABSTRACT

After carps, tilapia is the second most culturable fish. However, due to its rapid reproduction, wild tilapia (*Oreochromis mossambicus*) poses a severe threat to the freshwater system, particularly in aquaculture. Approximately 75% of published research shows that introducing tilapia into aquaculture has a negative impact. Similarly, due to the risk of side effects, hormonally administered mono-sex tilapia is not satisfying consumer demand. Therefore, this study aimed at determining the effect of the Tulsi (*Ocimum sanctum*) plant on the fertility of males and females' tilapia (*Oreochromis mossambicus*), by examining the histomorphological alternation of testis and ovaries, estimating the gonadosomatic index, and analyzing the level of plasma testosterone. A 90-day feeding trial in a replicate of three was conducted in fiberglass tanks. Uniform size and active *O. mossambicus*, average body weight 3.4± 0.4g were equally distributed in 12 fiberglass tanks (20 fish/tank) having well-aerated water. The experiment was divided into four groups named control group To, and three treatment groups T1, T2, and T3 groups. Tilapia fingerlings in the To group were fed with 40% crude protein basal diet while the other, three groups were fed 40% crude protein basal diet supplemented with graded level 1%, 2%, and 3% of Tulsi (*O. sanctum*) by weight of the diet. Initially, fish were provided feed at the rate of 7% body weight, three times a day. Afterward, based on the body weight, feeding frequency and ratio were changed. Results indicated a dose-dependent significant effect of *O. sanctum* on the growth performance of *O. mossambicus*. At the end of the study, the T3 group showed the highest weight gain, SGR, and the lowest FCR. The addition of *O. sanctum* to the diet had a considerable impact on the apparent digestibility of nutrients, i.e., an increase in apparent digestibility of protein, fat and dry matter as compared to the control group. In addition to these *O. sanctum* also showed significant ($P>0.05$) dose-dependent positive effects on blood indices i.e., the T3 group showed the highest RBCs and WBCs count, hemoglobin level, HCT%, MCH, MCHC; significantly higher ($P>0.001$) activity of antioxidant enzymes SOD, POD, CAT, GSR, GST, GPX, GSH, and the lowest LPO level. Metabolic enzymes LDH and AST did not show any significant difference ($P<0.05$) among the control and treatment group while ALT showed a dose-dependent lower level in *O. sanctum* fed group. The GSI of both male and female fish was negatively affected by *O. sanctum* supplemented diet in a dose-dependent manner. Both sexes males and females at higher doses showed about 35% and 28% respectively

decrease in GSI as compared to the control group. The histology of testis and ovaries of fish fed *O. sanctum* indicated deformation in seminiferous lobules and interlobular tissues in testis and necrosis, atresia, increase in vacuolation, and fusion of ova in ovaries. Moreover, a significant ($P < 0.001$) increase in plasma testosterone and estradiol level was also observed in *O. mossambicus* fed males and females respectively. Based on results, the *O. sanctum* plant could be recommended for improving the growth, health status, and controlling the reproduction of *O. mossambicus*. Further studies are needed to elucidate the hormonal level impacts and to determine the optimum treatment regime for the induction of 100% infertility.

INTRODUCTION

Due to an ever-growing population, it is imperative to produce good quality protein in huge quantities to deal with the problem of human starvation and malnutrition. Fish is a great source of protein that is high in quality, containing a lot of vitamins minerals, and Omega-3 polyunsaturated fatty acids that have prodigious nutritional potential and are expected to help the poor beat hunger and malnutrition in the coming years (Hussain *et al*., 2018). Due to the recent awareness of healthy foods, fish is increasingly appreciated and accepted around the world (Harris *et al*., 2020). Its palatability (Hawkey *et al*., 2021), its easy digestion (Kitano &Yamamoto, 2020), and its high retention of protein in the consumer's body allow better nutrition compared to other sources of animal protein (Gustavsson, 2020).

Due to the Omega- 3 oils ω−3 fatty acids, fish is believed to have reduced triacylglycerol, hypotension, heart problems, and upgraded the parameters of blood coagulation parameters (Djuricic & Calder, 2021). It has an excellent flavor, high nutritional value, and higher digestibility, 85- 95% (Lu *et al*., 2021). Fish is a great source of important vitamin A & D, minerals, and unsaturated fats (Khalili Tilami & Sampels, 2018). It is predicted that demand for fish will soon outpace all available supplies due to revolutionary changes in people's eating habits around the world and consumers' perception of fishery products as healthy foods (Henchion *et al*., 2017). A relatively little amount of energy is required for fish farming systems than other systems (Lieke *et al.,* 2021).

Worldwide aquaculture is one of the fastest-developing food industries having a great capacity to meet global demand for aquatic food by helping marine and inland to capture fisheries (FAO, 2016; Garlock *et al*., 2020). In 2012 about 158 million tons production of fisheries increased globally (Rodríguez-Luna *et al*., 2021). According to population growth, an extra 40.1 million tons of aquatic food will be required by 2030 to maintain current per capita consumption during the next two decades (Singh & Khanna, 2021). In aquaculture researchers now prefer to use dietary additives instead of synthetic antibiotics, as they extremely downregulate metabolic enzymes, have muscle building, and revival of lethal pathogens in aquatic biota (Ahmadifar *et al.,* 2019; Tkaczyk *et al*., 2020).

In Pakistan, attitudes towards fish farming are being widely developed to produce proteins of animal origin. Traditionally, the culture of Indian large carp and Chinese carp family carps (*Ctenopharyngodon idella* and *Hypophthalmichthys molitrix*) is carried out in the country (Bilal *et al*., 2021). Due to certain

circumstances, the cultivation of these fish did not lead to high fish meat production and profitable fish farming. Pakistan falls under the tropic zone, belonging to a dry and semi-arid climate zone, with sparse and irregular rainfall, and groundwater are affected by high salinity (Fatima *et al*., 2021).

Because of this high salinity, traditionally farmed fish experience stress and cannot function properly. As a result, they have limited growth and minimal harvest characteristics. As a result, there is a great necessity to identify other fish species that may successfully spawn in these salty environments. Hence, they showed restricted growth and low harvested properties. These conditions are responsible for the unprofitable fish farming in the region. Therefore, there is an urgent need to identify other fish species that can breed profitably in these salty areas (Iqbal *et al*., 2021). Because of its strong resistance to salt and other environmental challenges, tilapia probably is the best viable fish cultivated in these places. Tilapia species have many properties and have potential significance, and they are recommended for culture in salt marshes (Syed *et al*., 2021).

Tilapia has many attractive characteristics, i.e., growth rates, captive spawning capacity and acceptance of ordinary and non-natural feeds, efficient feed conversion, adaptableness to numerous environmental conditions, and resistance to the disease under cultured conditions. Due to all these properties, this group of fish is widespread in many different places of the globe (AbuDalo *et al.*, 2021; Chen *et al.,* 2018). *Oreochromis* genus dominates tilapia culture worldwide, specifically, *O. mossambicus, O. aureus, and O. niloticus.* Due to their ability to adapt to a considerable degree of adversarial environmental and management conditions tilapia is dominated. A tilapia fish's core taxonomic categorization is as follows:

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterygii

Order: Perciformes

Family: Cichlidae

Genus: Oreochromis

Species: *Oreochromis mossambicus*

Common name: Mozambique tilapia

The development of early sexual development has a negative impact on tilapia cultures and prolific breeding, which in turn results in overpopulation and consequently stunted growth, production of uneven fish sizes, longer culture periods as well as low marketable sized fish (Varadaraj & Pandian, 1990; Toguyeni *et al*., 2002). Under optimal conditions, certain typesof tilapia mature in early stages and in small sizes. Each mature female can produce hundreds tothousands of offspring every 28 to 45 days (Gu *et al*., 2018). Early breeding activities and successive breeding cycles led to overstock populations in tilapia culture systems. This overpopulation leads to competition for food and stunting, making it difficult to obtain more than 150 grams of fish (Blázquez & Saillant, 2018).

1.1 Culture of Tilapia

Tilapia culture has also increased unusually sharply over the past two decades. As a result, over the past 15 years, tilapia production has increased sixfold, from 383,655 tonnes in 1990 to 4,677,613 tonnes in 2013 (Mugwanya *et al*., 2021). After carp, tilapia is now the world's second-largest cultivated fish. (Solanki and Ujjania, 2021; Gabriel, 2019). It is also the most prevalent of all farmed fish (Hodar *et al.,* 2020). The impressive global aquaculture production is the result of the widespread adoption of intensive production systems, whichare associated with increased yields per unit area due to higher stocking densities (Gabriel, 2019).

Tilapia may be grown in several entities due to their omnivorous feeding habits. They convert feed efficiently and grow at a rate comparable to most other farmed fish (Azaza *et al*., 2020). Because of its outstanding table fish qualities, including as solid white meat and no pebbles between muscles, it is often consumed. Most tropical and subtropical nations raise tilapia. It is an excellent table fish with solid white flesh and no bones between muscles, so they are extensively putative by consumers. Tilapia is cultured in most tropical and subtropical countries. Tilapia farming also received a lot of attention in current years (Miccoli*et al*., 2021).

The female tilapia matures at a very early age (15-30g); this is the major issue faced during tilapia culture (Bardhan *et al*., 2021; Gu *et al.,* 2019), and because of

earlier maturation, they breed proliferating which leads to overcrowding in production systems, subsequently decreasing growth (Maulu *et al*., 2019) which results in stunted populations. Tilapia mature very early. Each mature female can produce hundreds to thousands of offspring within 28 to 45 days (Yongo *et al*., 2018). Early breeding activities and successive breeding cycles led to overstock populations in tilapia culture systems which leads to competition for food and stunting growth, making it difficult to obtain more than 150 grams of fish (Forneck *et al.,* 2021).

Various strategies have been used to limit the uncontrolled production of tilapia and reduce the fluctuation in the size of the collected fishin order to produce cost-effective crops. These approaches include parthenogenesis, sex reversal, cage/tank culture, predator usage, high stocking density, sterilization, intermittent/selective fishing, and the use of slowly growing tilapia species (Khalil *et al*., 2014; Ghosal *et al.,* 2021). Monosex farming, particularly all-male tilapia culture, is the greatest answer to this problem. Male tilapias typically grow faster than females. Farming of all-male tilapia is preferred because it takes advantage of the rapid growth rate while eliminating reproduction (Ashour *et al*., 2020).

In the 1970s due to the hormone sex reversal technique, it is possible to produce the tilapia population, which consisted entirely of male unisexual populations which grew evenly. Since the mid-1980s, the tilapia farming industry has expanded rapidly due to advances in research, market development, and processing of food and growing systems (Bardhan *et al.,* 2021). Wokeh & Orose (2021) described several approaches and techniques that can be utilized to limit tilapia reproduction. Unisexual male populations with relatively fast development rates, mainly causedby androgenic hormone sex reversal, are favored, and commonly employed in nations that produce huge quantities of tilapia, like China (Shrestha *et al*., 2018).

1.1.1 Monosex Tilapia

The concept of monosex culture production of tilapia (preferably male) presents an opportunity to control undesirable spawning, minimizing stunted growth, shortening culture duration, and subsequently improving yield and economic return (Omeje *et al*., 2020). All-male cultures of tilapia are preferred because they exhibit rapid growth (Mlalila *et al*., 2015), as they spend less energy on reproduction (Ngalya *et al.,* 2020) compared to females. Frequent breeding behavior and precocious

maturity of tilapia have enthused the development of various techniques, including hand sorting of sexes (Nunes & Rocha, 2015), hybridization and genetic manipulation (Chen *et al.,* 2018), temperature (Shrestha *et al.,* 2018; Zhu *et al*., 2016; Sule, 2021), and hormonal induction (Curzon *et al*., 2021; Naraballobh *et al.,* 2022; Bardhan *et al*., 2021; Mukherjee *et al*., 2018; Parvez *et al*., 2021), tocontrol spawning to achieve an all-male population with good marketable sizes (Wokeh & Orose, 2021; Chen *et al.,* 2018).

It is thought that all-male tilapia culture can be developed by reversing sex by using hormones (Dussenne *et al*., 2020). In Nile tilapia, *Oreochromis niloticus* the synthetic androgen 17-methyltestosterone (TM) is an excellent technique for producing all-male progeny (Ghosal *et al*., 2021). This hormone is easily excreted by treated animals and has no withdrawal symptoms (Yousefi *et al*., 2022). The effectiveness of therapeutic hormonal reversal depends on the type of hormone, the dose, the time of treatment, and the length of treatment. There have been many published attempts to optimize sexual reversal methods for oral 17α-methyltestosterone (MT) by varying various parameters such as hormone dose, duration of treatment, etc (Chhun *et al.,* 2021).

Among the different methods of treating fish sexual reversals, the degree of hormone incorporation by oral administration is more successful than any other method (Suseno *et al*., 2020). Among the different techniques developed to supply male tilapia to crops, namely artificial breeding, hybridization, genetic manipulation, and hormone reversal, the latter being the most common and economically viable (Amer *et al.,* 2021; Hasanuzzaman *et al*., 2021).

Androgens are steroid hormones. Steroids are the group of lipids that affect growth and development. The hormone testosterone has no effect as an androgen when administered orally and has a short interval of action on parenteral administration due to rapid liver metabolism (Torre *et al*., 2021). Synthetic androgens are active substances that have a long-term effect after injection or oral administration. Androgens have two physiological effects: 1) androgenic activity, which promotes the development of male characteristics, and 2) anabolic activity, which triggers protein biosynthesis (Silva *et al.,* 2021). As a result, many countries around the world, such as the United States, the European Union (Golden *et al*., 2021), and Asian countries

(Naylor *et al.,* 2021) have strict requirements for chemical/non-toxic aquatic products. Today, nutritional supplements or components that can increase fish health, growth, feed conversion capacity, and ultimately ensure the safety and quality of aquaculture products are needed to replace antibiotics and other synthetic agents. (Mariappan *et al.,* 2020).

Many scientists have used the synthetic androgen 17α-methyltestosterone (MT) to reverse the sexual behavior of different fish populations at different concentrations and processing times, particularly tilapia species (Srikwan *et al*., 2020). Chávez-García (2020) study, virilization, and feminization are most commonly induced by 17 α-methyltestosterone and 17 β-estradiol, respectively. Srikwan (2020) studied the sexual reversal used in early life in tilapia, 17α methyltestosterone (MT), typically used to produce fish populations with less than 5% of phenotype females. The impact of MT treatment on human food safety was also assessed. The results that allow you to reverse sexual behavior using TM and feed young children will not negatively affect their human safety.

All male populations (100%) were acquired through a few workers who reversed sex by using androgens. Mateen & Ahmed (2015) treated *O. niloticus* fry at the age of 10 and 30 days, for 14 days, with 50 mg of 17-α MT/kg feed accounted for 4%, 5%, and 6% of body weight, respectively. Get 100% men in all combinations by using food that contains containing alpha-methyl testosterone (MT) at 40 mg/kg. Zaki *et al.* (2021), while studying the implications of applying hormone therapy to the closed water system to reverse tilapia sex, he gained a 100% male population using foods containing alpha-methyl testosterone (MT) at 40 mg/kg. They also reported an insignificant difference in fry growth, survival, or feed alteration rate between different treatments.

Zaki *et al*. (2021) reported in a study of sex reversals in Nile tilapia that sex reversals had no significant effect on tilapia growth performance. Some authors believe that any improvement in androgen-treated tilapia is associated with increased growth in males rather than with more traditional anabolic responses associated with better protein synthesis and increased muscle mass. Each of these procedures, including hormonal therapies for sex reversal, has its own set of problems (Rodríguez Montes de Oca & Dabrowski, 2015) and restrictions of present procedures

and methods accepted (Wokeh & Orose 2021) an unconventional method is worth investigating. As part of research into alternative means of controlled reproduction, medicinal herbs that have been successfully used to create sterility in laboratory animals havebeen studied (Zargari *et al*., 2018).

1.2 Application of Pharmaceutical Plant

The alternate sex ratio in fish is controlled by natural reproductive inhibitors found in medicinal herbs. Pawar *et al*. (2020) medicinal plants are defined as "any plant in one or more of its structures that contains substances that can be used for therapeutic purposes or precursors of chemical-pharmaceutical synthesis." Plant parts that contain therapeutically active chemical components, such as seeds, flowers, fruits, stems, bark, rhizomes, and roots, are used in disease prevention and therapy (Benkhnigue *et al*., 2022).

Plant components, often known as phytochemicals, are non-nutritive plant compounds or bioactive elements that defend plants from microbial diseases or insect influxes (Tukur *et al*., 2020). When analyzing fertility regulators of different plants or their extracts following oral therapy in vivo, the ecbolic impact may exhibit anti-implantation, periodic destruction of estrous, anti-luteinizing hormone (LH) action, luteolytic activity, and luteal inhibitory activity(in animal models, e.g., rats) (Boer & Cotingting, 2014). The report showed that antifertility/sterility is induced in laboratory animals through the medicinal plant with much success (Sengupta & Dutta, 2022). This finding prompted researchers to evaluate to decrease tilapia fertility in ponds, several plants have natural reproductive inhibitory compounds. Šeregelj et al. (2022) define phytochemicals as "plant-produced chemicals that can affect health but are not needed by humans as essential nutrients."

Sadeer *et al*. (2019) discovered 577 plant species with bioactive chemicals that have been proved to work as alternative oral fertility regulators in both males and females (i.e., humans). Abridine, rich polysaccharides, forage, embelin, oleanolic acid-3-glucoside, p- Coumaric acid, and vicolide, according to these authors, have 100 percent anti-fertility activity, whereas acacetin, luteolin, momocharins, piperine, plumbagin, sitosterol, yuanhuatine, yuehchukene, and luteaster inhibit fertility. Despite extensive investigation, the researchers believe that the mechanisms by which plant extracts and active components exert anti-fertilization effects are yet unknown.

Many studies have demonstrated that using herbs in fish feed improves growth and disease resistance (Shahsavani *et al*., 2021). Herbs are utilized as an alternative to antibiotics in fish health management because they are inexpensive, environmentally safe, and have fewnegative effects. Many herbal and plant items are used in the aquaculture industry to treat ailments, increase growth, decrease stress, stimulate appetite, boost immunity, and avoid infections in healthy fish production.

Garlic contains allicin, an active chemical that stimulates increased food intake (Shakya, 2017). Malar & Charles (2013) reported that the addition of 50 mg of synthetic kg-1 allicin to the tilapia diet after 45 days of culture increased its body weight by more than 2-3%. Livol (IHF-1000) is an all-plant product that contains a variety of herbal ingredients that increase digestion and thus promote the growth of farmable fish. Ji *et al*. (2009) observed that herbs promote the use of lipids and fatty acids by cells as well as the accumulation of proteins, resulting in better growth performance in the Pagrus large.

Taxonomic classification of *Ocimum sanctum* L.is given below

Kingdom	Plantae
Order	Lamiales
Family	Lamiaceae
Genus	*Ocimum*
Species	*O.tenuiflorum*
Binomial	*Ocimumsanctum L*

A leafy grass, 30–90 cm tall, sometimes with wood at the base. Stems and branches are dressed in soft hair. The inflorescence is slender, 15–20 cm long, bracts to the calyx, broadly oval or cordate- ovate, acuminate, ciliate.

Bioflavonoid compounds are found in super bio-powders® and stimulate the growth of *Cyprinus carpio*. Fenugreek was used to boost growth performance, illness resistance, and immunity in *Labeo rohita* and *Oreochromis mossambicus*, rosemary *Oreochromis niloticus*, and basil *Oreochromis mossambicus*, respectively. Thyme boosts European sea (*Dicentrarchus labrax*) bass development and nutrient usage. The bioactive chemicals thymoquinone, thymol, carvacrol, and nigellon are primarily responsible for *N. sativa* therapeutic benefits. (Iqbal *et al*., 2018). The significantly

increased growth rate and resistance to *N. Sativa* seed oil seedlings fed 2% of *N. sativa* supplements. When 1% *N. sativa* supplemented diet is fed to it Carp fry showed an increased growth rate and an increase in growth and immune performance of young *Oncorhynchus mykiss* animals fed 2.5% of *N. sativa* supplements (Baba *et al.,* 2016; Latif *et al.,* 2021).

1.2.1 Properties of ingredients

Eugenol and isoeugenol are well known to possess antioxidant activity. It's also used for the treatment of gastrointestinal and respiratory complaints (Ulanowska & Olas, 2021). Ursolic acid, main components of tulsi leaf, has been demonstrated to have anti-fertility properties in amphoteric rats and male mice. Ursolic acid inhibits spermatogenesis and lowers sperm count due to its anti-estrogenic properties. Other pharmacological properties linked to ursolic acid include anticancer, hepatoprotective, anti-inflammatory (oral and topical), anti-ulcer, antibacterial, anti-hyperlipidemic, and antiviral action. (Rajan *et al.,* 2020). Studies have shown that UA has multiple pharmacological effects, including anti-inflammatory, hepatoprotective, anti-tumor, cardioprotective, neuroprotective, antibacterial, antihypertensive, antidiabetic, antifungal, antiviral, and trypanosine (Pironi *et al.,* 2018).

Rosemary acid has a variety of biological properties, including antiviral, antibacterial,anti-inflammatory, and antioxidant properties. Rosemary acid is found in medicinal plants, herbs, and spices, and it is helpful to one's health (Sestili *et al.,* 2018). Carvacrol mediates the antimicrobial, antioxidant, anti-inflammatory, and anticancer properties (Ahmad *et al.,*2021). The chief biological properties of linalool are sedatives, anxiolytics, analgesics, anticonvulsants, anti-inflammatory drugs, local anesthetics, and antifungals (Bouyahya *et al.,*2021). It has significant anti-cancer activity, affecting the growth and proliferation of many cancer cells. It also has antibacterial, anti-inflammatory, and antifungal activity (Gu *et al*., 2021).

These chemicals have been shown to increase insulin signaling and reduce hyperglycemia, as well as reduce oxidative stress by upregulating antioxidants and decreasing pro-inflammatory signaling. Oleanolic acid is used for liver treatment (hepatotoxicant) (Mehta *et al*., 2018).

1.3. The Aim and Objectives

The present study aimed to elucidate

i) The effect of *Ocimum sanctum* plant on the fertility of males and females in *Oreochromis mossambicus*, by examining the histomorphological alternation of testis and ovaries

ii) To Calculate the gonadosomatic index

iii) To Analyze the level of plasma testosterone in males and estradiol in female.

iv) To investigate the potential of dietary phytogenics to delay gonadal development andsexual maturation of tilapia (*Oreochromis mossambicus*) and improve production.

MATERIALS AND METHODS

2.1 Experimental Station

A 90-day (3-month) experiment was conducted at the Fisheries and Aquaculture Research Center, Department of Zoology, Quaid-i-Azam University, Islamabad, Pakistan.

2.2 Collection of Fingerling

Healthy and active fingerlings of tilapia, *Oreochromis mossambicus* with no sign of infection, were collected from National Agricultural Research Centre Islamabad (NARC) and transported by adopting the live hauling technique to the Fisheries Research Centre, Quaid-i- Azam University Islamabad, on 14 July 2021. Initially, they were placed in a fiberglass tank and acclimatized for 14 days. During this duration, they were nourished with a 40% crude protein basal diet. To avoid water deterioration fecal material and uneaten feed were removed regularly through siphoning. Moreover, water quality parameters were regularly checked and found within the optimal range of species. i.e., temperature (25 to 26°C), pH (7.3-7.5), dissolved oxygen (6.2 to 6.8mg/l), and ammonia level ≤0.05mg/L). For the initiation of experimental work, 12 rectangular fiberglass tanks (capacity, 250L) were selected and properly sanitized with the salt solution. After sanitization, water was added to the tanks, and aeration was started.

2.3 Feed Preparation

Feed ingredients mentioned in table 1 were purchased locally while *Ocimum sanctum* (local name tulsi, collected from the Qarshi Herb garden at Qarshi Industries (Pvt.) Ltd, Hattar, District. Haripur, Khyber Pakhtunkhwa). Feed was prepared by following the standard method mentioned by Younus *et al*. (2020). Briefly, all dry ingredients including (*Ocimum sanctum*) were finely ground. Following that, basal and *O.sanctum* supplemented diets were manufactured by combining dry items in the appropriate proportions in a locally made mixer. The dough was then prepared by adding water and oil. The dough is passed through a meat grinder and noodles were cut into small pallets. The pallets were dried at room temperature and saved separately in airtight bags and stored in a refrigerator. Fortnightly new batch of diets was prepared.

Table 1: Composition of 40% crude protein basal diet

S.No	Ingredients	%
1	Fish meal	45
2	Wheat bran	4
3	Rice Polish	4
4	Sunflower meal	15
5	Soybean meal	15
6	Gluten	15
7	Vitamins premix	0.5
8	Mineral premix	0.5
9	Carboxy methyl cellulose	1
	Total	**100**

Figure: 1. *O. sanctum L.*

2.4 Experimental Design

A completely randomized experiment in a replicate of three was designed and conducted in semi-control conditions for 90 days. Uniformed size, active and healthy fish were chosen regardless of their sex and evenly distributed in the 12 experimental fiberglass tanks (capacity 250 L) well equipped with an aeration system. Fish were

stocked at a stocking density of 1.5g/L (20 fishes/tank, 60 fish/ treatment)

The experimental fiberglass tanks were randomly designated into 4 groups, i.e., T_0 , T1, T2, and T3 groups based on the following treatment.

Control (T_0): Fish fed a 40% crude protein basal diet without any supplement.

Treatment 1(T1): Fish fed 40% crude basal protein diet supplemented with *Ocimum sanctum* 10g /kg or 1% of the diet.

Treatment 2(T2): Fish fed 40% crude basal protein diet supplemented with *Ocimum sanctum* 20g /kg or 2% of the diet.

Treatment3 (T3): Fish fed 40% crude basal protein diet supplemented with *Ocimum sanctum* 30g /kg or 3% of the diet

Initially, fish were fed 7% of their body weight thrice daily (8.00 am, 1.00 am, and 4.00 pm) for up to 20 days. Afterward feeding frequency and ratio was changed gradually by observing the body weight of fish and it ended with 3% body weight twice a day (8.00 am-4.00 pm). Fish were monitored daily and did not observe any mortality during the feeding trial.

The second experiment was conducted to investigate the Apparent digestibility coefficient%. To find out the apparent digestibility of nutrients, we added 1% chromic oxide in 1g/100g feed. Every day at 8.00 am, the chromic oxide-containing feed was given to the fish, and two hrs later, the remaining feed from each tank was removed; after this, two hrs later, the fecal material was removed from the tank. The remaining feed and fecal material were put in the oven to be dried.

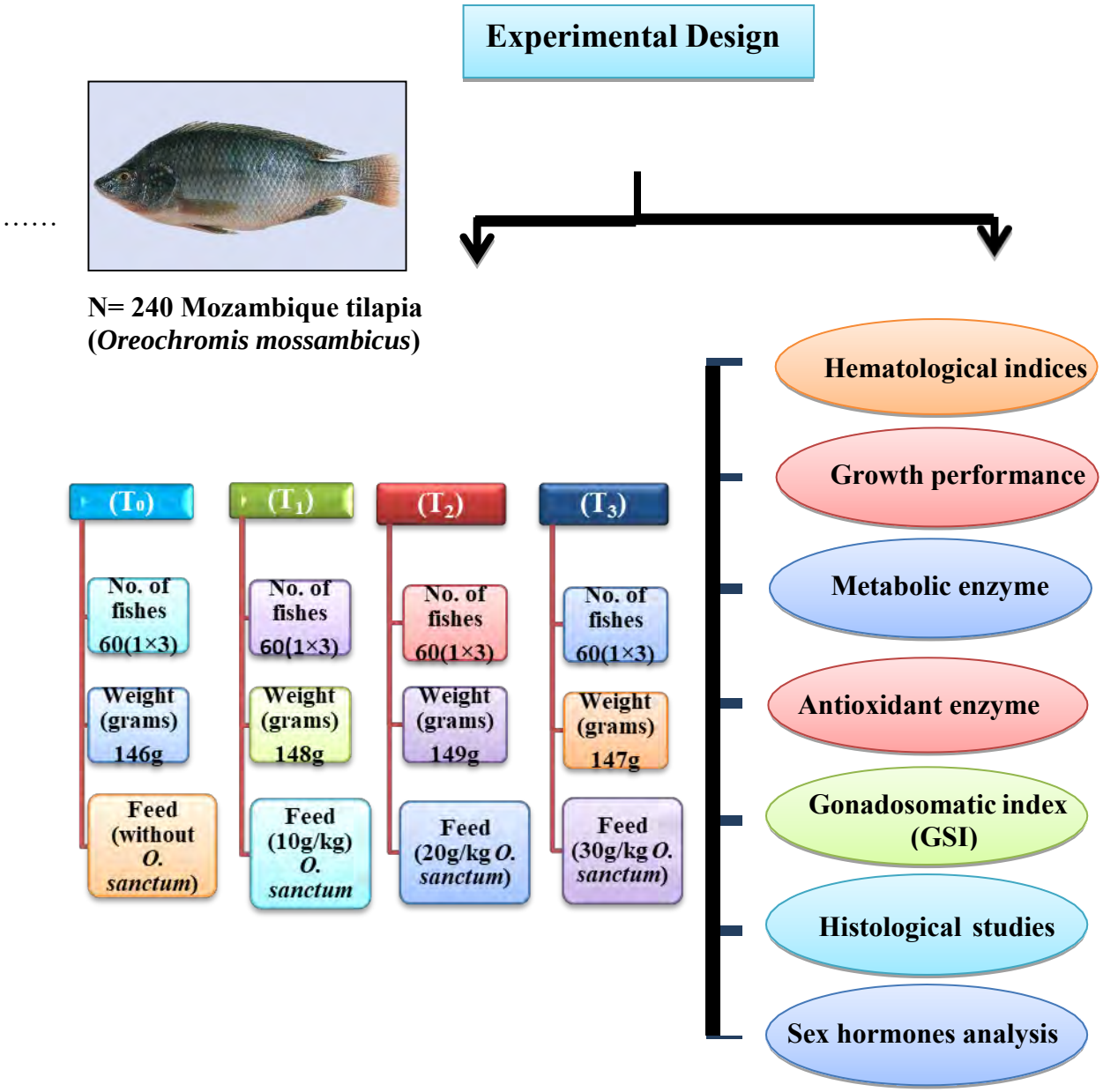


Figure 2. Experimental Design

2.5. Water Quality

During the experiment, all fiberglass tank water was continuously aerated to keep dissolved oxygen levels near saturation, as described by Binalshikh-Abubkr *et al*. (2022). The temperature of the fiberglass tank was periodically recorded and observed between 26-28°C. pH was maintained at an optimum level throughout the experiment. The total amount of ammonia nitrogen, nitrites, and nitrates was determined using the ammonia detection Kit (API freshwater primary Detection Kit) and observed ≤ 0.5mg/L.

2.6 Growth performance

Following the sampling procedure of Suphoronski *et al.* (2019), fish were kept unfed for 24 hrs before sampling. At a sampling day, 15 fish from each tank were taken randomly and collectively weighed for the determination of the growth performance of the fish by using the following formulas.

$WG = W_f - Wi$

$\%WG = [(W_f - W_i)/w_i] \times 100$

$\%SGR = [(\ln W_f - \ln W_i)/\text{No. of days}] \times 100$

FCR= Total feed consumed/total weight of fish

2.7 Blood sampling

After growth measurement, 9 fish/tanks (27 fish/treatment) were taken and anesthetized with sodium bicarbonate buffered MS-222 (0.1g/L). Blood samples were taken following Ahmad *et al*. (2020) procedure. Briefly, the 2-ml heparinized syringe was used to collect blood from the caudal vein. To have enough samples for all assays (hematological parameters, and metabolic enzyme), the blood from three fish in the same tank was pooled into an EDTA tubeand then centrifuge for 15 min at 1500 rpm by centrifugation to separate plasma (Younus *et al*.,2020). Plasma was separated and stored at 4°C till further analysis. For serum, blood from 9 fish/tank (27 fish/treatment) was collected in sterile Eppendorf tubes without anticoagulant and allowed to clot at room temperature; then blood clot was removed with a fine tip, and blood was centrifuged for 10 min at 2000 rpm. The separated serum samples were collected in separate tubes and stored at 4 °C for further hormonal analysis. fish were then dissected inside a cold chamber extracting muscle, gills, intestine, liver, and

braintissues. The gonads were also taken out and weighed and small portions at the tip of posterior and anterior ends and middle were cut and preserved in 10% formalin for histology

2.8 Hematological parameters

For blood parameters like RBCs (10^6/µl) and WBCs (10^3/µl), hemoglobin concentration (g/dl), mean corpuscular hemoglobin concentration (MCHC, g/dl), mean corpuscular hemoglobin (MCH, pg), mean corpuscular volume (MCV, fl), Platelets and differential cell counts (neutrophils%, lymphocytes%, and monocytes%) in all the control and treated groups (n=9) were sent to the Biogene Lab, where they analyzed it by using a well-calibrated top standing hematology analysis analyzer (KENZA ONE Hematology analyzer).

2.9 Metabolic enzymes

2.9.1 Aspartate Aminotransferase (AST)

Serum AST activity of control and experimental groups was measured according to the protocol mentioned in Sigma-Aldrich® Active assay kit (MAK055).

Briefly, all samples and standards were run in duplicates and prepared glutamate standard solution for colorimetric detection (dilute 990µl of AST buffer with 10µl 0.1M of glutamate standard). A blank sample was generated and added1-20µl of serum samples into wells of a 96 well plate. Added AST assay buffer in samples to bring a final volume up to 50µl. Prepared Master Reaction Mix for all samples and standards. Added Master Reaction Mix to each of standard and sample well and mixed it and noted first absorbance reading at 450nm after 2-3 min.

2.9.2 Alanine Aminotransferase (ALT)

The activity of ALT was measured in the serum of control and experimental groups according to Sigma-Aldrich® ALT Active assay kit (MAK052).

The method was as followed, briefly, all samples and standards were run in duplicates and prepared pyruvate standard for colorimetric detection (dilute 990µl of pyruvate standard). Generated blank and added 1-20µl of sample into wells of a 96 well plate. Added ALT assay buffer in samples to bring a final volume up to 20µl. Prepared Master Reaction Mix for all samples and standards. Added Master Reaction Mix to each of standard and sample well and mixed it and noted first absorbance

reading at 570nm after 2-3 min.

2.9.3 Lactate Dehydrogenase (LDH)

Sample..... 25ml

Buffer ..... 200mmol/L (pH=8.2)

Lactate.....260mmol/L

Sodium azide….....7.7mmol/L

The activity of this enzyme (µM pyruvate/mg protein/hr) was determined by NADH synthesis by Venturini *et al.* (2018). The above-mentioned chemicals mixed in an appropriate volume at room temperature. The color reactive was added after this period and incubated for 5 min at room temp. At the end add 200 mmol/L HCl to the tubes, which were then left at room temperature for 5 minutes until the absorbance at 500 nm was observed.

2.10 Apparent digestibility analysis

The apparent digestibility for each nutrient: fats, protein, carbohydrate, and total energy were measured using chromic oxide following the modified protocol of Maas *et al.* (2020). The energy was calculated using a Gallemkamp auto-bomb adiabatic calorimeter. Chromic oxide was quantified via flame atomic absorption after digestion with concentrated nitric and perchloric acid (Varian AA-975 series flame atomic absorbance spectrophotometer) (Adeniyi*et al.,* 2021).

The following formulas were used to calculate apparent digestibility:

Apparent digestibility (%)

= 100- [%Cr_2O_3 in feed÷%Cr_2O_3 in feces] ×[% nutrient in feces÷% nutrient in feed]

2.11 Biochemical Analysis

The tissue homogenate for the estimation of different antioxidant enzymes was carried out byfollowing Yilmaz (2020) method. Briefly, 0.09g of tissues (muscle, and liver) were removed from the refrigerator, thawed, and homogenized in 3ml of KH_2PO4 buffer containing 1 mol EDTA having a PH of 7.4. After that, the mixture was centrifuged for 30 minutes at 12000 rpm at 4°C. Until the antioxidant enzymes were assayed, the supernatant was stored at -20°C (Elbialy *et al.,* 2021).

2.11.1 Antioxidant enzyme extraction

2.11.1.2 Catalase Assay (CAT)

Reagents

Sample….....0.1ml

Phosphate buffer (50 mM pH=5.0).....2.5 ml

H_2O_2(5.9 mM).....0.4 ml

Enzyme extract.....0.1 ml

The CAT activity in the liver and muscles of control and *O.sanctum*-treated fish was evaluated., by the method mentioned by Sanchez *et al.* (2021). The reaction solutions were prepared by mixing above mentions chemicals plus the sample in a cuvette. The spectrophotometer(Agilent, 8453, USA) was adjusted to 240 nm, and the reaction solution's absorbance was measured after 1 minute. Using a molar coefficient of 43.6/M cm, the CAT activity was estimated as nmol/min/mg/protein.

2. 11.1.3 Peroxidase assay (POD)Reagents

Sample.....0.1ml

Phosphate buffer (50 mM pH=5.0).....2.5 ml

Guaiacol (20 Mm).....0.1 ml

H_2O_2(40 mM).....0.3 ml

Enzyme extract.....0.1 ml

POD activity was investigated in the liver and muscles of control and *O.sanctum*-treated fish followed by Ullah *et al*. (2022). Briefly, the reaction solutions were prepared by mixing above mentions chemicals plus the sample in a cuvette. After that, the absorbance of the reaction solution was measured as it changed using a spectrophotometer (Agilent, 8453, USA) set at 470nm after 1 minute. The activity of POD was estimated as nmol/min/mg protein using a molar coefficient of 2.66×10^4 /M cm.

2. 11.1.4 Superoxide dismutase assay (SOD)Reagents

Sample.....0.3ml

phenazine methosulphate..... 0.1 ml (186 μM)

Sodium pyrophosphate buffer.....0.2ml (0.052mM; pH=7.0)

$NADH^{+}$ .....0.2 ml (780 μM)

Glacial acetic acid… 1ml

The superoxide dismutase (SOD) activity in muscles and liver tissue of control and *O.sanctum* treated *O. mossambicus* was analyzed by using the method of Ahmadiefer *et al.* (2021) procedure. Briefly, the reaction solutions were prepared by mixing above mentions chemicals plus the sample in the cuvette. After that, $NADH^{+}$ solution was added to start the reaction. Following that, glacial acetic acid was added to bring the process to a halt after 1 minute. A UV-visible spectrophotometer was used to measure the chromogen's absorbance at 560nm (Agilent, 8453, USA). With a molar coefficient of 6.22 $\times 10^{3}$ /M cm, the results of SOD activity were reported in moles/min/mg/protein.

2. 11.1.5 Lipid Peroxidation Assay (LPO/ TBARS)Reagents

Supernatant .....0.2 ml

Ferric chloride (100 mM)..... 0.02 ml

Trichloroacetic acid (10%) ..... 1.0 ml

Ascorbic acid (100 mM) ..... 0.2 ml

Thiobarbituric acid (0.67%).....1.0 ml

Phosphate buffer (0.1 mM, pH 7.4) ..... 0.58 ml

Following the procedure of Günal et al. (2021), LPO activity in the liver, and muscles tissue of control and *O.sanctum*-treated fish were analyzed. Reaction solutions were prepared by above mentioned volume. Mix phosphate buffer, sample, ascorbic acid, and ferric chloride. Then 1 hour of incubation in a 37°C water bath, the reaction was stopped with trichloroacetic acid. After that, add Thiobarbituric acid and boil all tubes for 20 minutes in a water bath. The tubes were then placed in an ice bath and centrifuged at 25000g for 10 minutes, with the supernatant of each sample collected into clean Eppendorf tubes. To examine a change in the absorbance of the reaction solution, a spectrophotometer (Agilent, 8453, USA) set at 532 nm was employed. At 37°C and a molar extinction coefficient of 1.56 $\times 10^{5}$ /M cm, LPO activity was computed as nM TBARS/min/mg tissue.

2.11.1.6 Glutathione reductase (GSR) activity

By using the procedure of Ibrahim *et al.* (2021), glutathione reductase levels were estimated.

Reagents

Sample.....0.1ml

EDTA (0.5 Mm).....0.1 ml

PBS (0.1M; pH=7.6)..... 1.65 ml

Oxidized Glutathione (1 Mm).....0.05 ml

NADPH (0.1 Mm) .....0.1 ml

To measure GSR levels in the supernatant of liver and muscles tissue reaction solutions were prepared by mixing above mentions chemicals plus a sample in the cuvette. The activity of NADPH was measured at 25°C and a wavelength of 340nm and expressed as nmol of NADPH oxidized/min/mg protein using a coefficient of molar extinction of 6.22×10^3 M^{-1} cm^{-1}

2.11.1.7 Glutathione –S- Transferase Activity (GST)Reagents

PBS ..... 1.475ml (0.1mol; pH=6.5)

Reduced Glutathione…..... 0.2 ml (1 mmol)

2, 4- Dinitrochlorobenzene .....0.025 ml (1mmol)

Homogenate… ..... 0.3 ml

The reaction mixtures of Glutathione–S-Transferase were prepared by mixing above mentions chemicals plus a sample in a cuvette at the appropriate volume. At 340nm, variations in absorbance were recorded, and enzyme activity was estimated as nM CDNB conjugate formed/min/mg protein using a 9.6×10^3 M^{-1} cm^{-1} molar extinction coefficient. (Batool *et al.,* 2018).

2.11.1.8 Reduced Glutathione Activity (GSH)Reagent

Ellman (100 NM).....0.2 ml

Sulfosalicylic acid (4%)..... 1 ml

PBS (pH 7.4)..... 2.7 ml

The reaction mixtures of Reduced Glutathione were prepared according to the above-mentioned volume. After mixing the sample plus sulfosalicylic acid in an appropriate volume. Temp was kept at 4°C for 1 hr and centrifuged for 20 min at 12000g at 4°C. Assay mixture on a Spectrophotometer, the yellow color developed quickly at 412 nm (Abdel-Daim *et al.,* 2020).

2.11.1.9 Glutathione peroxidase activity (GPX)

Glutathione peroxidase activity was observed by the method of Hamid & Al-Sayed (2019).

Sample..... 0.1ml

EDTA (1 mm) ..... 0.1 ml

PBS (0.1 mM pH = 7.4) ……1.49 mL

Reduced Glutathione (1 mm) …..……..0.05 ml

NADPH (0.2 mm) …..……....0.1 ml

Glutathione Reductase (1 mm) ……..0.05 ml

Reduced Glutathione (1 mm) …….……0.05 ml

Sodium azide(1 mm)……0.1 ml

H_2O_2(0.25 mm)……..…...0.01 ml

The reaction mixtures of Glutathione peroxidase were prepared by mixing above mentions chemicals plus a sample in a cuvette at an appropriate volume A spectrophotometer detected the depletion of Nicotinamide adenine dinucleotide phosphate at 340 nm.

2.12 Gonado-somatic index (GSI)

Fish were collected randomly and measured for the weight of fish. Fish was dissected, sexand maturity were identified, and as followed by (Muazu *et al.,* 2020).

2.12.1 Female identification

Ovary from a point and then narrows to the oviduct.The ovary was angular, had a ridge, and granulated

2.12.2 Immature male identification

Testes were thready throughout, smooth and round, with no development or thickness.

2.12.3 Mature male identification

Testes were thick, become white/translucent, smooth, and tapers to the tail. Gonads were removed and weighed. A zero-balance scale was used to measure the weight. Gonadosomatic Index was calculated by Harstad *et al*. (2014).

GSI = Weight of gonads/Weight of fish ×100

2.13 Testosterone ELISA

The Testosterone test kit from Calbiotech, Inc. is a solid phase competitive ELISA. To the streptavidin-coated wells, the serum, testosterone Conjugate, and anti-Testosterone-biotin solution are mixed. Testosterone in the serum competes for binding sites with the Testosterone enzyme (HRP) conjugate. Washing buffer removes unbound Testosterone and testosterone enzyme conjugate. The intensity of the color is inversely proportional to the concentration of Testosterone in the samples after the addition of the substrate. A standard curve linking color intensity to Testosterone concentration is created. (Mulari *et al.,* 2019).

2.13.1 REAGENT PREPARATION

1. Prepare a working solution of 20X enzyme conjugate with assay diluent at a ratio of 1:20.
2. Prepare the wash buffer. Keep at ambient temperature (20-25°C).
3. Before using, bring all reagents to ambient temperature (20-25°C).

ASSAY PROCEDURE

1. Pipette 50 µl of standards, control, or specimen into the designated well.
2. Fill each well with 100 µl of functioning Testosterone-enzyme conjugate reagent.
3. Fill each well with 50 µl Biotin reagent and gently swirl the microplate for 20-30 seconds.
4. Cover the plate and incubate at room temperature for 60 minutes.
5. Drain all liquid from the wells. With 300 l of wash buffer, wash the wells three times.Using absorbent paper towels.

6. Fill each well with 100 l of TMB substrate reagent.
7. Cover the dish and incubate for thirty (30) minutes at room temperature.
8. Fill each well with 50 l of stop solution and gently mix for 15-20 seconds.
9. After darkening the stop solution, read the absorbance on an ELISA Reader at 450nm for each well within 15 minutes.

2.14 Test the principle of estradiol

Competitive binding between E2 and E2 enzyme conjugate for a constant amount of anti-estradiol monoclonal antibody epitops (biotin reagent).

Procedure

1. 25 microliters of control, standards, and specimens were added to wells.

2. The wells were coated with streptavidin for Estradiol concentration determination in the sample.

3. 50 microliters of working solution of Estradiol biotin reagent were added to the wells.

4. 25 microliters of control, standards, and specimens were added to wells.

5. The wells were coated with streptavidin for Estradiol concentration determination in the sample.

6. 50 microliters of working solution of Estradiol biotin reagent were added to the wells.

7. Incubation was done for 45 minutes at 20 to 25 degrees.

8. Add 100µL Estradiol enzyme reagent to the wells.

9. The incubation was done for 45 minutes at 20-25 °C.

10. Decant the liquid from the wells

11. Washing was done three times with 300µL of 1X buffer.

12. 100µL TMB reagent was added to all the wells and incubation was done for 20 mints.

13. 50 µl of stop solution was added to each well to stop the reaction.

14. The absorbance was recorded at 450nm using a microplate reader.

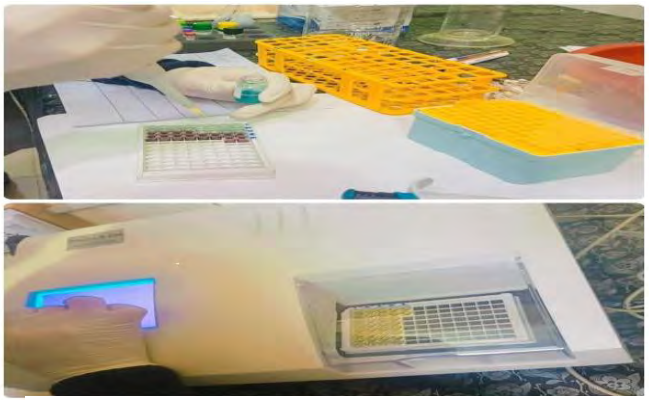


Figure 3 Elisa of testosterone and estradiol

2.15 Histology

Briefly, gonads that were fixed in 10% formalin solution were removed from the refrigerator for anatomy and histology.

2.15.1- Preparation, sectioning, and staining

In the laboratory, all formalin tissues were processed by immersion in serial dilutions of alcohol (i.e., 80 percent, 90 percent, and 100 percent), chloroform, and molten paraffin for 17 hrs in an automated tissue processor (Duplex Processor). The tissues were subsequently embedded in paraffin in an embedding tool at 60°C according to the method (Abdel Rahman *et al.,* 2019).

2.15.2- Dehydration

Following fixation, tissues were dehydrated in different grades of alcohol at room temperature

Ethanol 70%.....2 hrs

Ethanol 80%.....…….2 hrs

Ethanol 90%.....2 hrs

100% Ethanol……3 substitutions (every 2 hrs)

2.15.3- Embedding

After dehydration, tissues were placed in xylene until they were cleared and fixed in paraffinas follows

Xylene I .....60 min Xylene II .....60 min
Paraffin I (58 °C)……..120 min

After embedding, the tissue was transferred into a molten wax boat. After removing the air bubbles, let the wax harden. To cut paraffin sections, a knife or scalpel is used to cut the wax block and place it on a wooden block. Using a microtome, cut 5 µm-8 µm thick sections and tissues were centered on the wax block to form transverse sections (TS). However, due to the size of the organ, some testicular and ovarian tissue was removed at the top. Place at least three pieces of each sample on glass slides in warm water.

2.15.4 - Preparing gonads slides

1. Albumin preparation. Two egg whites were added to 1200 mL of deionized water. Stirring on a magnetic stirrer for 5 minutes. Then, 4 mL concentrated ammonium hydroxide was added. Again, stirred for 5 minutes. Filtering through a low-grade filter (coffee filter). Stored albumin in the dark, in a screw-top glass bottle at 40 °C.

Coating slides

For coating slides, they were separately placed on a slide warmer at a low setting. Using a clean small brush, a thin albumin layer was applied to each slide. The process is repeated at least three times. Slides were dried on the slide warmer. Albumin-coated slides were stored atroom temperature, in the original packaging until required.

Microtomy

Wooden blocks were fixed on a microtome. 2-3 µm thin sections were cut using a microtomein which wooden blocks were placed (Shandon, Finesse 325, UK). The long ribbons of wax having tissues were stretched, following fixation in albumenized glass slides. These slides were kept on Fischer slide warmer at 60 °C. Later, for complete stretching, glass slides were kept in an incubator overnight.

Staining

For staining following steps are carried out

The slides were deparaffinized in xylene I and Xylene II for 3 min respectively.

Hydration

Sections were rehydrated in descending grades of alcohol.

100% alcohol I --- 3 min, 100% alcohol II --- 3 min, 90% alcohol --- 3 min, 70% alcohol --- 3min, Washed with water --- 3 min

Slides were stained in different grades as follows:

Hematoxylin --- 8 min, washed with water --- 2 min, Acidified alcohol --- 1 min, Washed with water --- 2 min, Bluing solution (1mL NH_4OH + 300mL water), Washed with water ---2 min

Dehydration

90% alcohol --- 10 dips, **Eosin ---2 min,** Washed with water --- 2 min 90% alcohol --- 5 min, 100% alcohol I --- 5 min, 100% alcohol II --- 5 min, 100% alcohol III 5 min, Xylene I --- 5 min, Xylene II --- 5 min. After staining, two to three drops of Canada balsam were applied to the slides, which were then covered with coverslips dipped in xylene and kept in an incubator overnight. After this slide is ready for microscopy.

Data Analysis

Experiment data was presented as Mean±S.E. Statistix 8.1 version software was used to analyze the data statistically. Comparison between different groups was done by using one way and two-way analysis of variances (ANOVA) followed by LSD. Moreover, $P<0.05$ level was selected as a significant different.

Results

3.1 Growth performance

Growth performances of both sexes of *Oreochromis mossambicus* after feeding a graded level of *Ocimum sanctum Linn* are summarized in Table 3; All treatment groups readily accepted the *O. sanctum* in feed and were found healthy and active. The body weight of fish among all treatments improved during the 90-day experiment.

One-way ANOVA indicated the significant differences in the final body weight of all groups. Dose-dependent effect was observed that was significantly highest ($p<0.01$) in T3 (30g/kg) followed by T2 (20g/kg) followed by T1 (10g/kg) and the lowest was observed in control. Fish groups fed on high concentrations of *O. sanctum* had higher final body weight, weight gain, and SGR than other groups. Moreover, a significant ($p<0.01$) decrease in the feed conversion ratio (FCR) was recorded. All pairwise comparisons indicated significantly higher SGR in T3 followed by control, and FCR is significantly lower in T3 followed by control. Table No 3 Summarizes the growth parameters of *O. mossambicus* after 90 days of culture.

Results in Table 3 show that FCR decreased significantly ($P<0.01$) in both sexes more decrease in males than in females as compared to the control group of both sexes. FCR decreases with the increase of *O. sanctum* in the experimental diet. The highest FCR value was found in the control followed by the T1 group (10g/kg *O. sanctum*), while the lowest was in the T3 group. FBW, WG, and SGR values were highest in T3 (30g/kg *O. sanctum*) followed by T2 (20g/kg *O. sanctum*) and T1 (10g/kg *O. sanctum*); respectively, all treated groups showed higher statistical value as compared to control group. Male fish show more growth as compared to females. FBW, WG, and SGR values were highest in males compared to females, and FCR decreased in males compared to females (Table 3).

To investigate two combination factors, the effects of different doses of *O. sanctum* and sex on growth parameters, a two-way ANOVA was used. Table 2 shows the results of two-way ANOVA indicating the effects of dietary treatment, sex, and their interaction on the growth performance of tilapia. The results indicated that

independently of the dietary treatments, higher FBW, WG, SGR, Biomass and lower FCR were observed in males compared to females (Figure 2) *O. sanctum* dietary treatment resulted in significantly higher FBW, WG, SGR, Biomass and lower FCR, which increased with increasing the *O. sanctum,* independent of sex. Based on two-way ANOVA, there was no significant interaction of dietary *O. sanctum* and sex on growth performance.

3.2 Hematological Studies

In our study three groups (T1, T2, T3) were exposed to *O.sanctum* lant. In our hematological parameters such as total count of RBCs (10^6/µl) and WBCs (10^3/µl), hemoglobin concentration (g/dl), mean corpuscular hemoglobin concentration (MCHC, g/dl), meancorpuscular hemoglobin (MCH, pg), mean corpuscular volume (MCV, fl), Platelets and differential cell counts (neutrophils%, lymphocytes%, and monocytes%) were analyzed in fish from different treatment groups (n = 9). Hematological parameters like mean hemoglobin content, WBC, PCV, MCH, MCHC, Platelets, and differential cell counts were significantly different ($p<0.05$) with the *O. sanctum* treatment group followed by the control group (Table 4). There was no significant difference ($p>0.05$) in red blood cells between the treatment and controlgroups. Mean corpuscular volume (MCV, fl) was significantly decreased in the treatment group. The fish fed with 10, 20, and 30g/kg *O. sanctum* in the diet showed significantly higher values than the control, which fed the diet without *O. sanctum* (Table 4).

3.3 Blood Biochemical Indices

Serum analysis of metabolic enzyme Serum Alanine transaminase (ALT), aspartate transaminase (AST), and Lactate dehydrogenase (LDH) of tilapia fed with *O. sanctum* in the dietshowed no significant differences ($p > 0.05$) between control and experimental groups in Table

5. The results obtained in the present study revealed that the dietary supplementation of *O. sanctum* at 1%, 2%, and 3% levels were not significantly decreased metabolic enzyme ALT, AST, and LDH levels when compared to the control group (fed diet without *O. sanctum*) (Table 5).

3.4 Apparent digestibility coefficient (ADC %)

The apparent digestibility of all groups after mixing the graded level of *O. sanctum* in the diet for *O. mossambicus* is summarized in table 6. Our results showed that the experimental groups had significantly ($p<0.05$) increased apparent protein digestibility (APD), apparent fat digestibility (AFD), and apparent dry matter digestibility (ADD) followed by the control group. Moreover, a significant ($p<0.05$) significant increase in apparent digestibility was recorded in all treatment groups. Statistically, the value in table 6 shows apparent digestibility increases with theincrease of *O. sanctum* concentration. The treatment group which fed 30g/kg *O. sanctum* in diet, had a higher value of digestibility followed by the T2 group, which fed 20g/kg *O. sanctum* while there was no significant difference ($p>0.05$) in apparent protein and fat digestibility between the control and T1 group which fed 10g/kg *O. sanctum* in table 6.

3.5 Antioxidant enzyme

Table 5 shows the effect of different *O. sanctum* concentrations in the diet on theantioxidant enzymes SOD, POD, CAT, and LPO. SOD, POD, and CAT antioxidant enzyme activity were enhanced in all of the examined tissue (liver and muscle) of tilapia fed with *O.sanctum* diet as compared to control (diet without *O. sanctum*) in table 7. The highest antioxidant activity was found in the T3 group followed by other treatment groups. When compared to the control group, the treatment groups exhibited a significant change ($p< 0.01$).In contrast, the LPO activity showed the opposite trend. As the *O. sanctum* concentration was increased in the diet SOD, POD, and CAT antioxidant enzyme activity were also significantly increased ($p <0.05$), but it decreased the level of lipid peroxidation (LPO) in all examined tissue (liver and muscles). The highest LPO activity was found in the control and the lowest in the T3 group. There was a significant difference ($p < 0.01$) in the LPO value shown in Table 7.

3.6 Glutathione antioxidant

The effect of different concentrations of *O. sanctum* in the diet on the enzymatic antioxidants Glutathione reductase (GSR), Glutathione –S- Transferase Activity (GST), Glutathione peroxidase assay (GSH-px), and non-enzymatic antioxidants Reduced Glutathione Activity (GSH) is shown in table 7. GSR, GST,

GSH-px, and GSH antioxidant activity was enhanced in all of the examined tissue (liver and muscle) of tilapia fed with *O. sanctum* diet

followed by control (diet without *O. sanctum*) in table 7. The highest antioxidant activity was found in the T3 group as compared to other treatment groups. Treatment groups showed a significant difference ($p < 0.01$) as compared to the control group. As the *O. sanctum* concentration was increased in the diet, GSR, GST, GSH-px, and GSH antioxidant activity was also significantly increased ($p < 0.05$) in all examined tissue (liver and muscles). The highest activity was found in T3 and the lowest in the control group. There was a significant difference($p < 0.01$) in the value shown in Table 7.

3.7 Gonado-somatic index (GSI)

Table 8 summarises the results of the Gonado-somatic index (GSI). After 90 days of treatment, there were significant changes ($P \leq 0.01$) between the experimental groups. The GSI values in the treated groups were lower. However, as the dose of *O. sanctum* was increased, the GSI values in the treated groups declined steadily, reaching their lowest point in T3 (high dose). In a comparison of male and female tilapia, male tilapia group T3 which fed 30g *O. sanctum* per kg GSI value, decreased 35% followed by control while the female tilapia group T3 GSI value decreased to 28% followed by control. Male tilapia group T2, which fed 20g *O. sanctum* per kg GSI value, decreased 27% followed by control, while the female tilapia group T2 GSI value decreased to 21% followed by control. The lowest GSI value change in the TI group, which fed 10g O. sanctum per kg in group T1 GSI value, decreased 15% compared to control while the female tilapia group T1 GSI value decreased to 27% compared to control.

3.8 Analysis of hormone

In the present study, a significant ($P < 0.001$) increase in serum testosterone and estradiol level was observed in *O. sanctum* treated *O. mossambicus* as compared to control. *O. sanctum* is well- known for its testosterone-boosting properties. It is, however, also utilised as an anti-fertility drug. Despite the fact that *O. sanctum* tulsi raises testosterone and estradiol levels, it also lowers the HPTA (Hypothalamic-Pituitary-Testicular Axis).

Blood sexual hormones for males (testosterone, T) and female (estradiol) of *O. mossambicus* showed highly significant differences (P≤0.001) after 3-month treatment (Table 9 and 10). As the *O. sanctum* concentration was increased in the diet, the testosterone and estradiollevel were also increased. The highest value was observed in the T3 group which fed 30g *O.*

sanctum per kg followed by T2 and T1, which fed 20 and 10 g *O. sanctum* per kg, respectively,as compared to the control. There was a significant difference ($p < 0.001$) in the value shown in Table 9 and 10.

3.9 Histology Examination

In the present study, the normal structure was noticed after 3 months of treatment in the ovary of the control group fish (T_0) (Fig.14a). In T1, the ovary of fish fed a low dose of *O. sanctum* (10g/kg) showed separation of the follicular layer (Fig.14b). Necrosis, atresia (Fig.14c), and ova fusion (Fig.14d) were found in medium and high dose ovaries (T2 and T3) treated fish fed O. sanctum (20g/kg and 30g/kg).

After three months of treatment, an examination of the testis showed a normal structure in the control group (Fig.14a). However, testis fed 10g/kg *O. sanctum* in treatment 1 (T1) showed damaged interlobular structures (Fig.14b). Treatments 2 and 3 showed distortion in the seminiferous lobule (Fig.14c) and interlobular tissue (Fig.14 d) (T2 and T3), respectively.

Table 2: Growth performance of *O. mosambicus* fed diets supplemented with graded level of *O. sanctum* powder

Parameter	Sex	T_0	T1	T2	T3	F value	P Value
IBW(g)	Male	3.43±0.03^{ab}	3.33±0.044^{b}	3.51±0.03^{a}	3.42±0.06^{ab}	3.46	0.072
	Female	3.43±0.03^{ab}	3.33 ±0.04^{b}	3.51±0.03^{a}	3.42±0.06^{ab}	3.46	0.072
FBW(g)	Male	15.20±0.17^{d}	17.69±0.24^{c}	19.82±0.19 ^{b}	22.24±0.54^{a}	86.6	0.000
	Female	14.14±0.12^{c}	16.33±1.27^{bc}	18.09±0.83^{b}	20.38±0.45^{a}	15.3	0.001
WG(g)	Male	11.78±0.18^{d}	14.36±0.28^{c}	16.30±0.19^{b}	18.82±0.58^{a}	73.8	0.000
	Female	10.72 ±0.10^{c}	13.01±0.65^{b}	14.57±0.99^{b}	16.96±0.65^{a}	15.1	0.002
WG%	Male	344.35 ±6.81^{c}	432.80 ±13.97^{b}	464.41 ±6.79^{b}	551.88 ±24.61 ^{3}	32.9	0.001
	Female	313.13±3.11^{c}	392.12±23.23^{b}	415.44±30.51^{b}	496.52±13.94^{a}	13.6	0.001
SGR (%BW d^{-1})	Male	1.35±0.01^{c}	1.53±0.03^{b}	1.59±0.02^{b}	1.73±0.04^{a}	37.5	0.000
	Female	1.28±0.08^{c}	1.46±0.05^{b}	1.50±0.06^{b}	1. 65±0.03^{a}	13.4	0.001
FCR	Male	2.24±0.021^{a}	2.03±0.03^{b}	1.79±0.21^{c}	1.57±0.04^{d}	96.6	0.000
	Female	2.41±0.02^{a}	2.20±0.09^{ab}	1.98±0.11^{b}	1.72±0.06^{c}	14.5	0.001
Biomass	Male	136.83±1.66^{d}	159.20±2.15^{c}	178.33±1.71^{b}	200.13±4.87^{a}	86.6	0.000
	Female	127.23±1.04^{c}	147.00±5.60^{bc}	162.77±8.76^{b}	183.40±6.22^{a}	15.3	0.001

Data is presented as Mean ± SE (n=3). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Different lowercase letters on mean values within a row show a considerable difference ($P < 0.05$). IBW: initial body weight, FBW: final body weight, WG: weight gain, WG%; Percentage weight gain, SGR=specific growth rate (%/day), FCR: feed conversion.

Table 3. Two-way analysis of variance (ANOVA) of growth parameter of *O. mosambicus* fed diets supplemented with gradedlevel of *O. sanctum* Powder

	df	F value	P value
FBW			
Dose	3	56.77	0.000
sex	1	16.11	0.001
Interaction	3	0.23	0.87
WG			
Dose	3	54.33	0.000
Sex	1	15.59	0.001
Interaction	3	0.23	0.88
SGR			
Dose	3	40.28	0.000
Sex	1	11.92	0.003
Interaction	3	0.037	0.99
FCR			
Dose	3	49.43	0.000
Sex	1	16.41	0.000
Interaction	3	0.04	0.99
WG%			
Dose	3	40.44	0.000
Sex	1	12.09	0.003
Interaction	3	0.17	0.92
Biomass			
Dose	3	56.88	0.000
Sex	1	16.12	0.001
Interaction	3	0.23	0.87

Table 4: Hematological parameter of *O. mossambicus* fed diets supplemented with graded level of *O. sanctum* powder

Parameter	T_0	T1	T2	T3	F value	P Value
WBCs(10^3/µL)	16.31±0.15^{b}	16.67±0.14^{ab}	16.94±0.16^{a}	17.01±0.13^{a}	4.73	0.007
RBCs(10^6/µL)	3.49±0.05^{b}	3.74±0.118^{ab}	3.84±0.18^{a}	3.92±0.07^{a}	2.60	0.069
Hb(g/dL)	9.95 ±0.19^{c}	10.37±0.13^{bc}	10.63±0.18^{ab}	11.08±0.22^{a}	6.83	0.001
PCV%	34.23±0.19^{b}	35.15±0.31^{b}	36.75±0.45^{a}	37.28±0.77^{a}	8.69	0.001
MCV (fL)	108.57± 0.81^{a}	105.69±1.01^{b}	103.22 ±1.24^{bc}	101.73 ± 0.51^{c}	10.4	0.000
MCH(Pg)	47.17±0.12^{b}	46.86±0.11^{c}	47.56±0.56^{a}	47.40±0.08^{ab}	10.7	0.000
MCHC(g/dL)	32.68±0.06^{c}	32.89±0.06^{b}	33.15±0.11^{a}	33.05±0.03^{ab}	8.44	0.001
Platelets(µL)	14.90±0.19^{c}	15.63±0.11^{ab}	15.26±0.16^{bc}	15.80±0.17^{a}	6.25	0.002
Neutrophils%	4.18±0.22^{c}	4.72±0.16^{ab}	4.48±0.15^{bc}	5.14±0.33^{a}	6.21	0.002
Lymphocytes%	73.29±0.82^{c}	74.38±0.53^{bc}	75.56±0.22^{ab}	76.29±0.28^{a}	6.46	0.002
Monocytes%	21.96±0.38^{b}	23.12±0.46^{ab}	23.90±0.34^{a}	22.59±0.43^{b}	4.17	0.013

Data is presented as Mean ± SE (n=9). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Different lowercase letters on mean values within rows show considerable differences ($P < 0.05$). RBCs (106/µl); Red blood cells, WBCs (103/µl), White blood cells, (MCHC, g/dl); Mean corpuscular hemoglobin concentration, (MCH, pg), Mean corpuscular hemoglobin, (MCV, fl), Mean corpuscular volume

Table 5: Effect of feed supplemented with graded level of *O. sanctum* powder on the metabolic enzyme of *O. mossambicus*

Metabolic Enzyme	T_0	T1	T2	T3	F value	P value
LDH(IU/L)	105.01±0.351[a]	104.31±1.75[a]	103.69±0.946[a]	103.21±0.730[a]	0.53	0.67
AST(U/L)	116.16 ±2.71[a]	115.26±3.826[a]	114.79 ±3.44[a]	112.02 ±2.31[a]	0.32	0.81
ALT(U/L)	10.49 ± 0.88[a]	9.75±0.71[ab]	8.95±0.23[ab]	8.39±0.28[b]	2.43	0.08

Data is presented as Mean ± SE (n=9). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Same lowercase letters on mean values within a row are showing non-significantly different ($P > 0.05$). LDH; Lactate dehydrogenase, AST; Aspartate transaminase, ALT; Alanine transaminase.

Table 6: Apparent digestibility coefficient (%) of *O. mossambicus* fed diets supplemented with graded level of *O. sanctum* powder

ADC%	T_0	T1	T2	T3	F value	P value
ADCp%	84.36 ± 0.07^{c}	84.83 ± 0.20^{b}	85.29 ± 0.08^{a}	85.47 ± 0.12^{a}	15.78	0.001
ADCf %	74.15 ± 0.22^{b}	74.24 ± 0.08^{b}	75.90 ± 0.16^{a}	76.30 ± 0.82^{a}	6.64	0.014
ADCd%	77.93 ± 0.13^{c}	78.53 ± 0.35^{bc}	78.99 ± 0.21^{b}	80.16 ± 0.26^{a}	14.2	0.001

Data is presented as Mean ± SE (n=9). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Different lowercase letters on mean values within the row show a considerable difference ($P < 0.05$). ADCp: Apparent digestibility coefficient of protein, ADCf: Apparent digestibility coefficient of fat, ADCd: Apparent digestibility coefficient of dry matter.

Table 7: Antioxidant activity in muscle (M) and liver (L) of *O. mossambicus* after 90-days feeding with graded level of *O. sanctum* powder

Antioxidant	Tissue	T_0	T1	T2	T3	F Value	P Value
POD (µmol/min/mg)	M	69.10±1.23 ^{c}	71.95±1.98 ^{b}	74.45±1.93^{a}	78.19±1.61^{a}	16.1	0.000
	L	92.78±1.155 ^{c}	96.45±1.852^{b}	99.37±1.20^{a}	101.7±1.57 ^{a}	24.4	0.000
CAT (µmol/min/mg)	M	72.64 ±1.18^{b}	75.09 ±1.94^{b}	79.85± 1.87^{a}	81.58± 2.28^{a}	22.0	0.000
	L	117.56±1.42^{c}	121.21± 2.33^{b}	125.13± 2.31^{a}	127.12±2.93^{a}	25.2	0.000
SOD (µmol/min/mg)	M	61.32± 1.53^{c}	63.54±1.96 ^{b}	66.43±2.34^{a}	68.28±2.55^{a}	23.5	0.000
	L	103.91±1.69^{d}	107.33± 1.79^{c}	110.08± 1.50^{b}	113.95±2.02^{a}	53.2	.0000
LPO (µmol/min/mg)	L	79.25± 1.74^{a}	77.41±1.755^{b}	74.50 ± 3.033^{c}	72.38± 3.39^{d}	29.3	0.000
	M	57.71± 1.49^{a}	54.25± 2.81^{ab}	51.12 ±2.45^{bc}	49.08±1.32^{c}	11.6	0.003
GSH(U/mol/mg)	L	55.74±1.51^{d}	59.87±1.07^{c}	63.19±2.31^{b}	67.69±3.20^{a}	13.1	0.002
	M	19.56 ±1.39^{c}	21.88 ±1.71^{b}	22.85±1.32^{ab}	24.35±1.49^{a}	16.1	0.000
GSR(U/mol/mg)	L	27.57 ±1.21^{c}	31.82±1.74^{b}	33.06±1.79^{b}	35.82 ±1.83^{a}	24.6	0.000
	M	9.305±1.77^{c}	12.12±1.55^{b}	14.13±1.67^{ab}	15.75±1.83^{a}	15.2	0.001
GST(U/mol/mg)	L	12.06±0.86^{c}	14.3±0.89^{bc}	15.41±1.01^{ab}	17.62±0.93^{a}	6.28	0.017
	M	6.21±0.60^{b}	7.61±0.89^{ab}	8.81±0.46^{a}	9.94±0.99^{a}	4.37	0.042
GPX(U/mol/mg)	L	60.79±0.85^{d}	64.93±1.35^{c}	69.21±1.39^{b}	74.71±1.55^{a}	29.5	.0001
	M	22.29±0.54^{c}	24.79±0.59^{b}	27.26±0.18^{a}	28.99±1.53^{a}	28.1	.0001

Data are presented as Mean ± SE (n=9). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different *O. mossambicus* groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Different lowercase letters on mean values within rows are showing considerable difference ($P < 0.01$). CAT; Catalase, POD; Peroxidase, SOD; Superoxide Dismutase, LPO/ TBARS; Lipid Peroxidation, GSR; Glutathione reductase, GST; Glutathione –S- Transferase, GSH; Reduced Glutathione, Gpx; Glutathione peroxidase

Table 8: Effect of feed supplemented with graded level *O. sanctum* powder on GSI of *O.mossambicus.*

Groups	GSI Male	% Of difference from T_0	GSI Female	% Of difference From T_0
T_0	1.42±0.13[a]		2.44±0.12[a]	
T1	1.20±0.122[ab]	15%	2.02±0.07[b]	17%
T2	1.04±0.018[b]	27%	1.92±0.10[b]	21%
T3	0.92±0.0156[b]	35%	1.74±0.09[b]	28%
P value	0.019		0.005	
F value	6.04		9.42	

Data is presented as Mean ± SE (n=9). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Different lowercase letters on mean values within rows are showing considerable differences ($P < 0.01$). GSI; Gonado-somatic index

Table 9: Effect of diet supplemented with graded level of *O. sanctum* on blood sex hormones (testosterone) in *O. mossambicus* after 3-month treatment

Groups	Testosterone Hormone(ng/ml)
T_0	4.38 ± 0.12^{d}
T1	5.45 ± 0.12^{c}
T2	6.23 ± 0.06^{b}
T3	8.04 ± 0.06^{a}
F value	188
P value	0.00

Data is presented as Mean ± SE (n=9). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Different lowercase letters on mean values within a row are showing considerable difference ($P < 0.01$).

Table 10: Effect of diet supplemented with graded level of *O. sanctum* on blood sex hormones (estradiol) in *O. mossambicus* after 3-month treatment

Groups	Estradiol Hormone(ng/ml)
T_0	8.54 ± 0.22^{d}
T1	10.53 ± 0.24^{c}
T2	14.12 ± 0.29^{b}
T3	17.28 ± 0.23^{a}
F value	248
P value	0.00

Data is presented as Mean ± SE (n=9). One-way ANOVA followed by LSD post Hoc test shows a pairwise comparison between different groups. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Different lowercase letters on mean values within a row are showing considerable difference ($P < 0.01$).

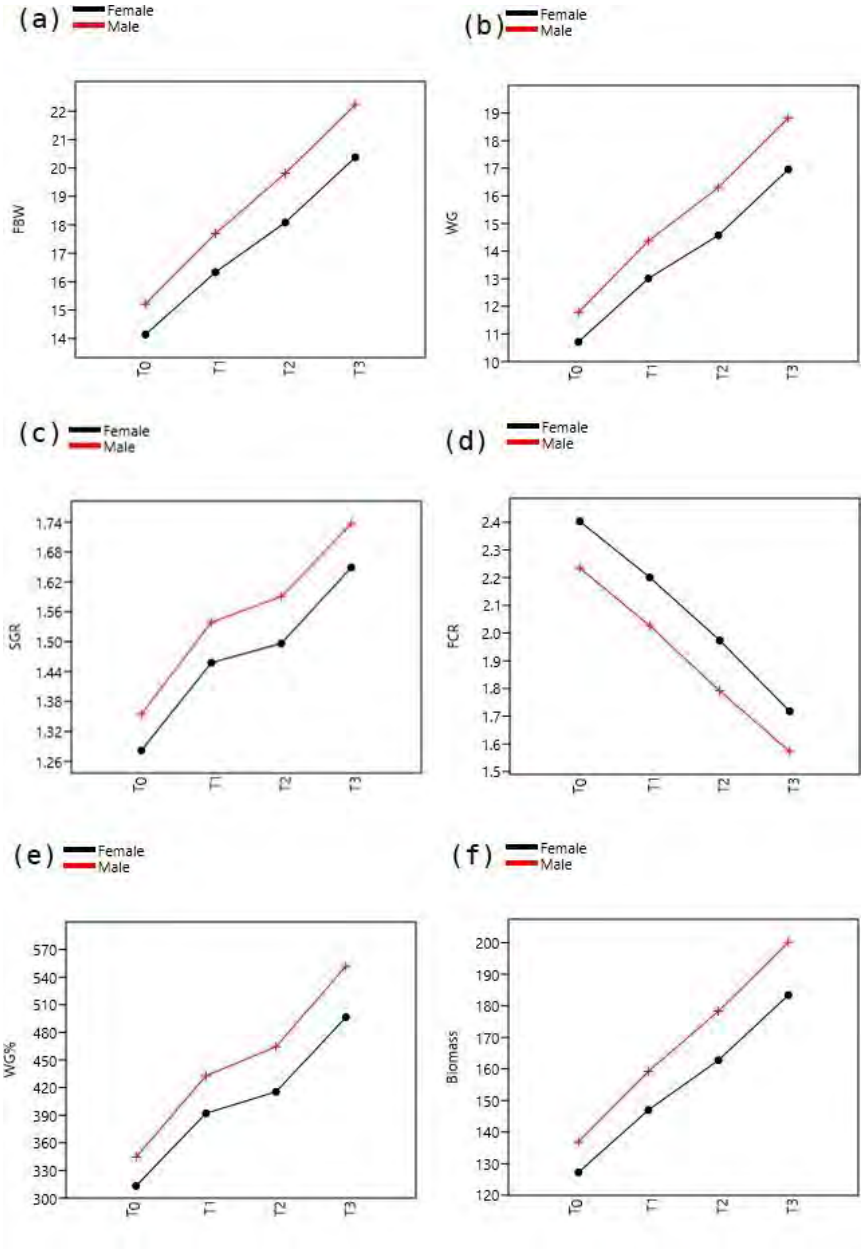


Figure 4: Mean values of a: FBW (final body weight, g), b: WG (weight gain, g), c: SGR (specific growth rate %/day), d: FCR (feed conversion ratio), e: WG% (Percentage weight gain), and f: (Biomass, g) for male and female tilapia. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*).

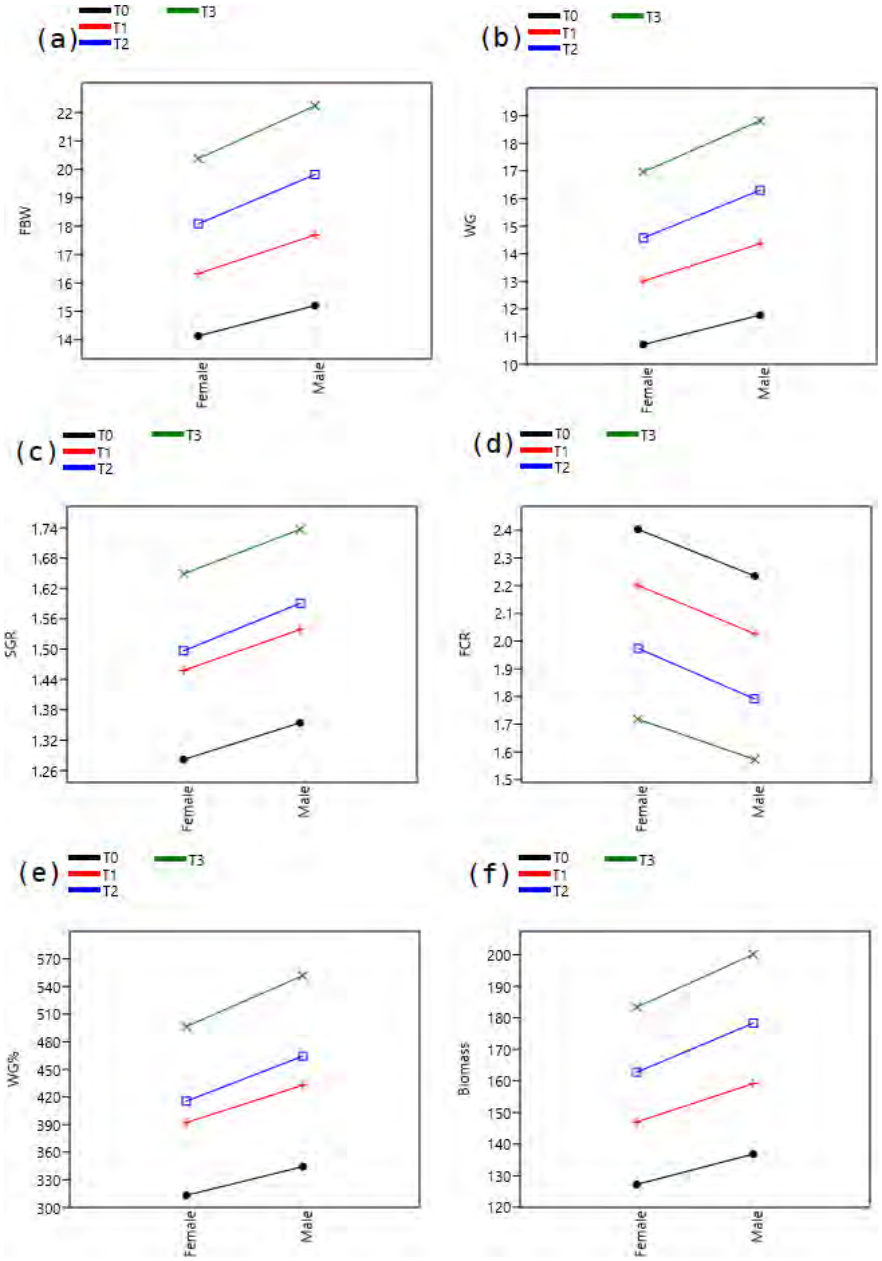


Figure 5: Comparison of female and male tilapia for mean values of a: FBW (final body weight, g), b: WG (weight gain, g), c: SGR (specific growth rate %/day), d: FCR (feed conversion ratio), e: WG% (Percentage weight gain), and f: (Biomass, g). T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*).

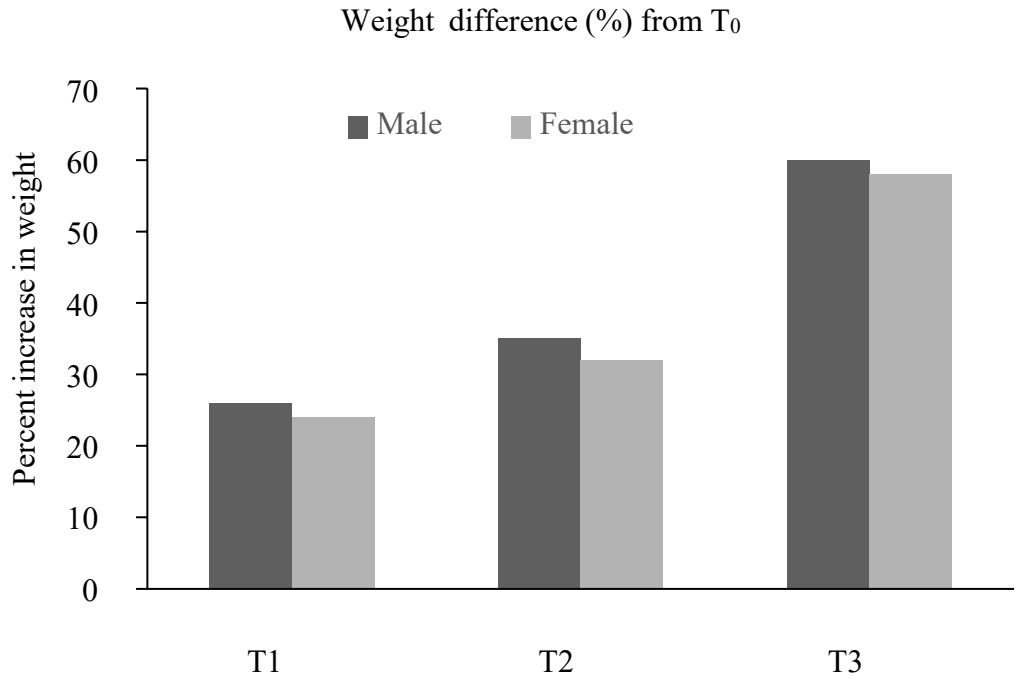


Figure 6: Percentage increase of male and female *Oreochromis mossambicus* weight fed withgraded level of O. *sanctum* supplemented diets compared to the control group (T_0)

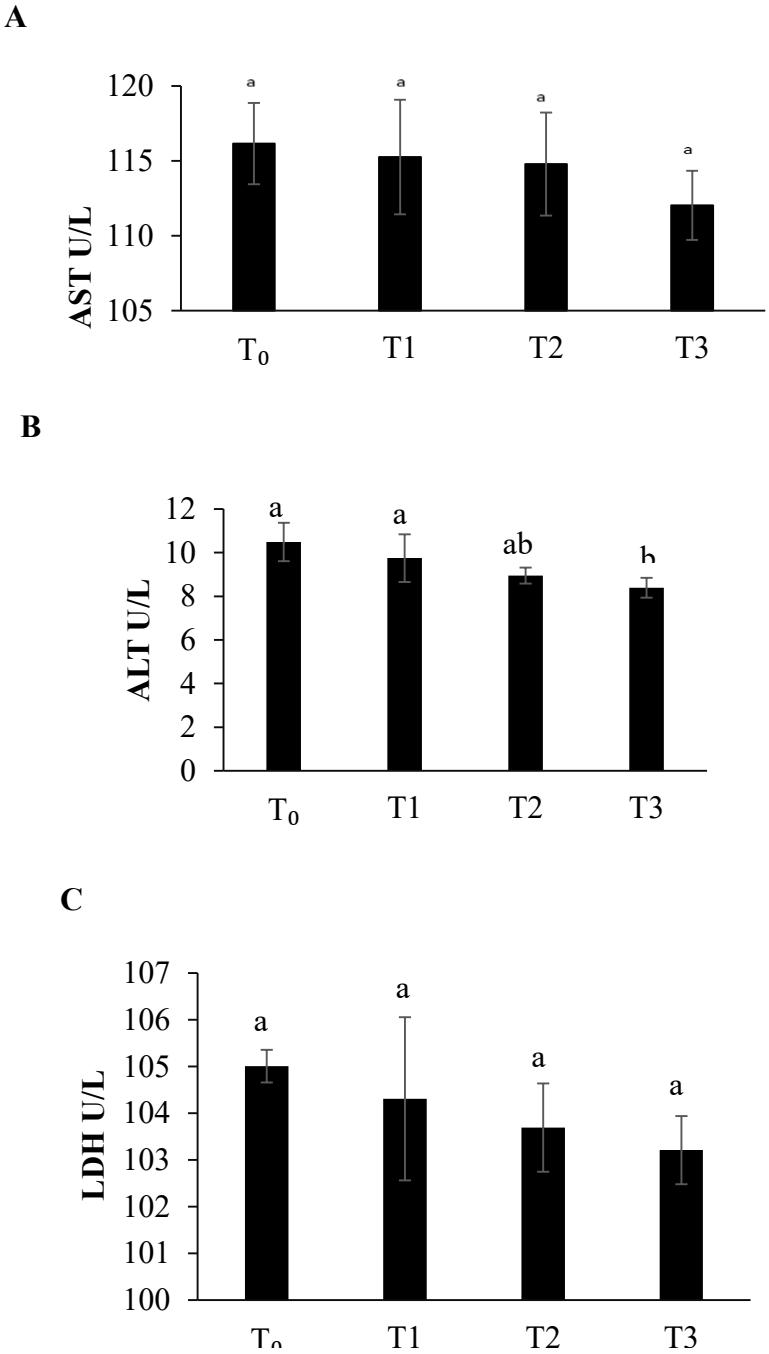


Figure 7. Graphs A, B, and C represent the effect of diet supplemented with graded level *O. sanctum* plant extracts on Metabolic enzymes in *O. mossambicus.* The graph represents the metabolic enzyme in *O. mossambicus* when fed with a diet which contains *O. sanctum* in a different concentration. T_0 (diet without *O.sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Each bar represents Mean ± S.E(n=9). Mean with thedifferent alphabets were significantly different ($p<0.05$).

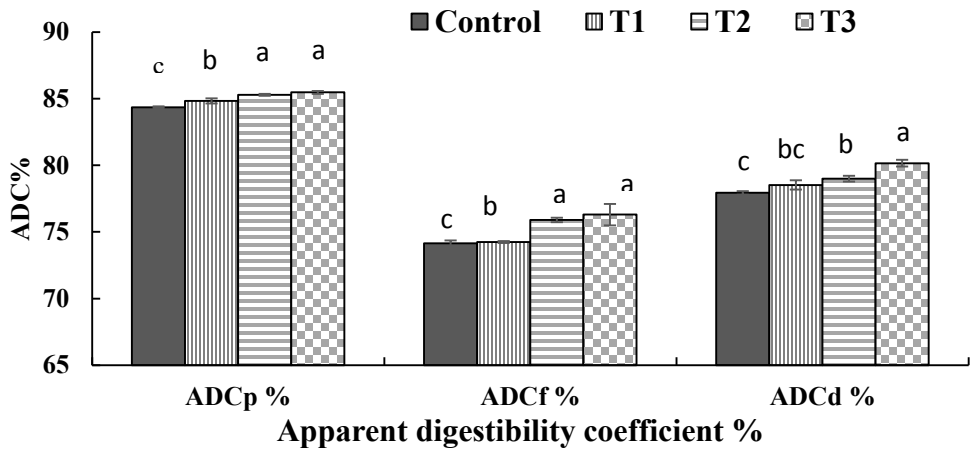


Figure 8. Effect of diet supplemented with graded level *O. sanctum* plant extracts on Apparent digestibility coefficient % in *O. mossambicus.* The graph represents the apparent digestibility coefficient % in fish *O. mossambicus* when fed with the diet which contains *O. sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O.sanctum*). Each bar represents Mean ± S.E(n=9). Mean with the different alphabets were significantly different ($p<0.05$).

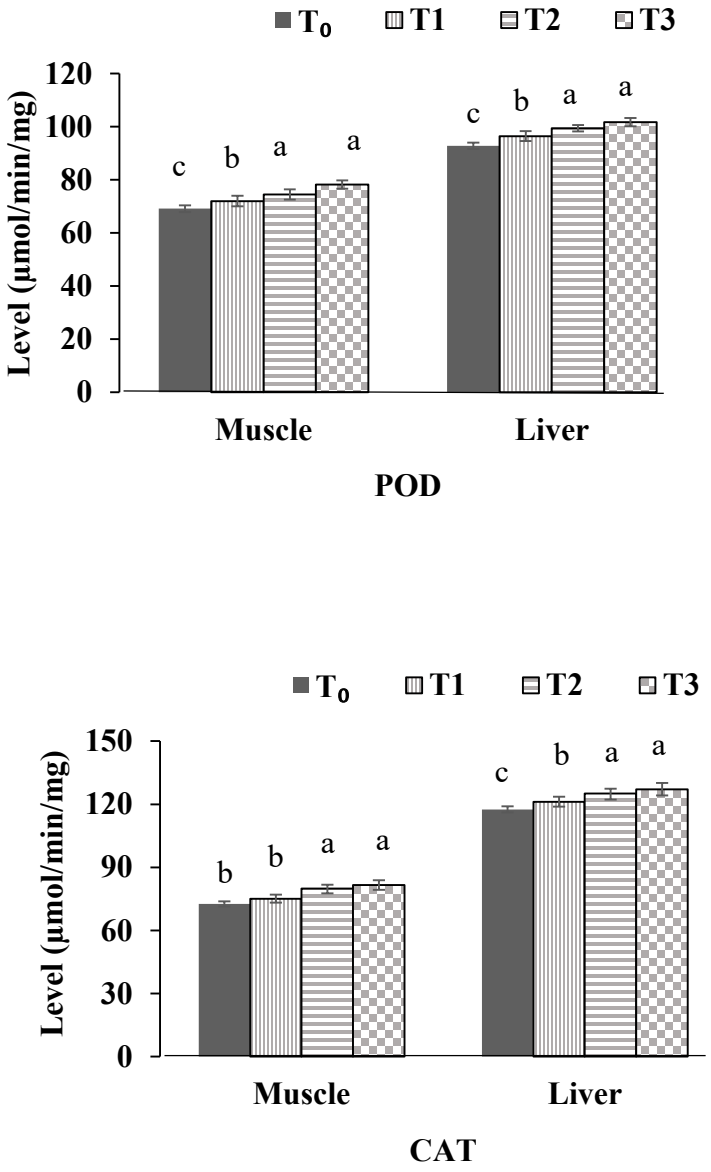


Figure 9. Both graphs represent the effect of diet supplemented with graded level *O. sanctum* plant extract on an antioxidant enzyme (POD and CAT) in *O. mossambicus.* The graph represents the effect of antioxidant enzymes in *O. mossambicus* when fed with a diet that contains *O. sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Eachbar represents Mean ± S.E(n=9). Mean with the different alphabets were significantly different ($p<0.05$).

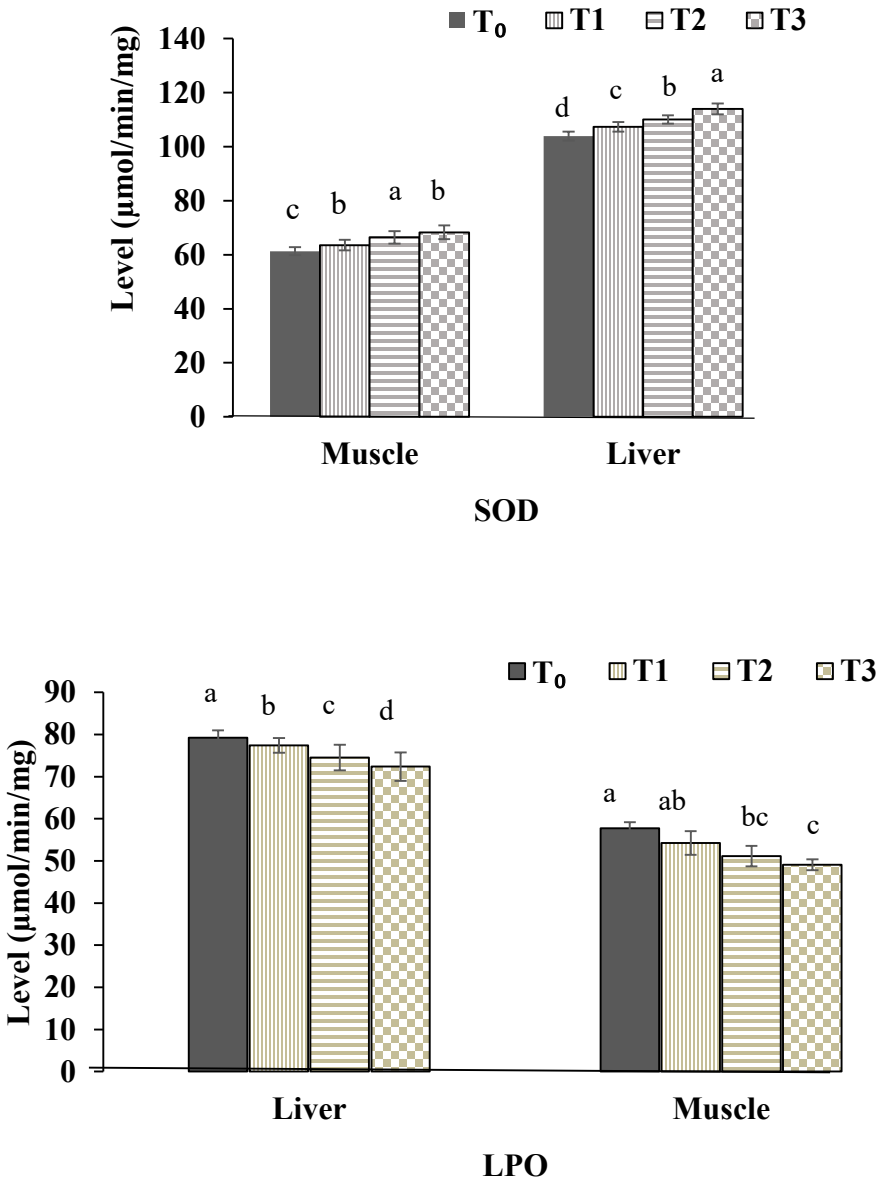


Figure 10. Both graphs represent the effect of diet supplemented with graded level *O. sanctum* plant extract on an antioxidant enzyme (SOD and LPO) in *O. mossambicus.* The graph represents the effect of antioxidant enzymes in *O. mossambicus* when fed with a diet that contains *O. sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Each bar represents Mean ± S.E (n=9). Mean with the different alphabets were significantly different ($p<0.05$).

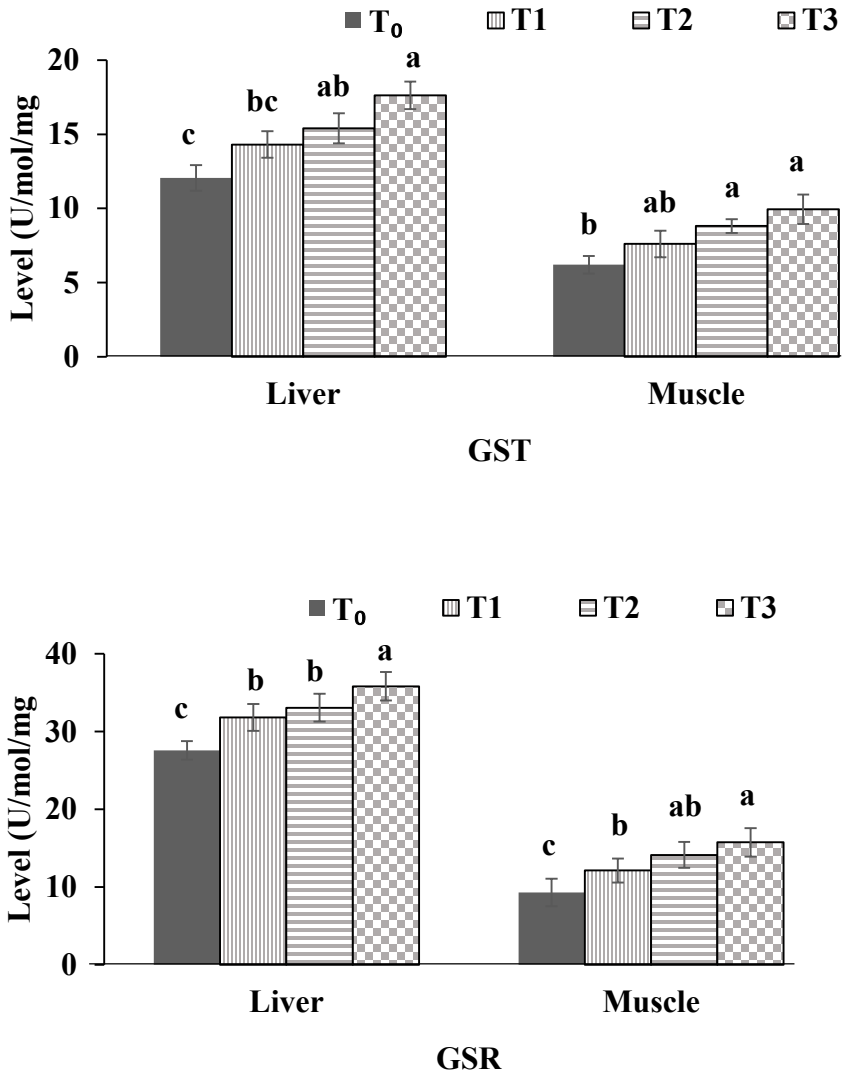
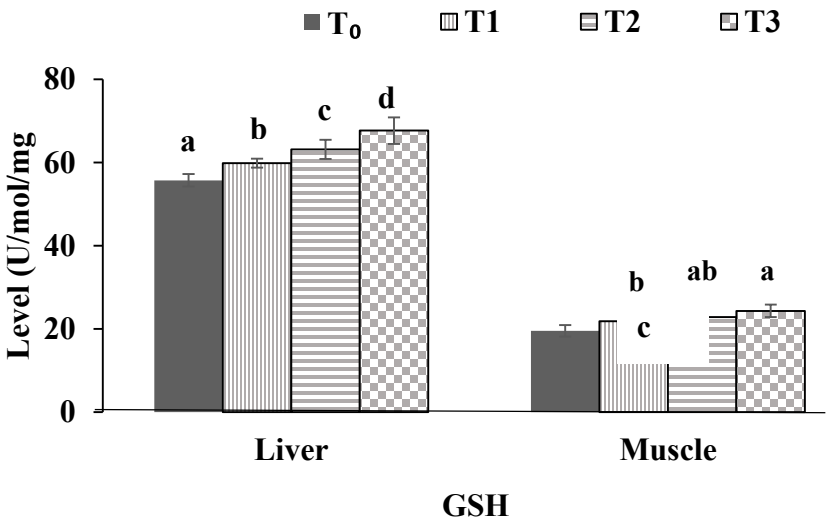


Figure 11. Both graphs represent the effect of diet supplemented with graded level *O. sanctum* plant extract on an antioxidant enzyme in *O. mossambicus*. The graph represents the effect of the glutathione antioxidant enzyme in *O. mossambicus* when fed with the diet which contains *O. sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Each bar represents Mean ± S.E(n=9). Mean with the different alphabets were significantly different ($p<0.05$).



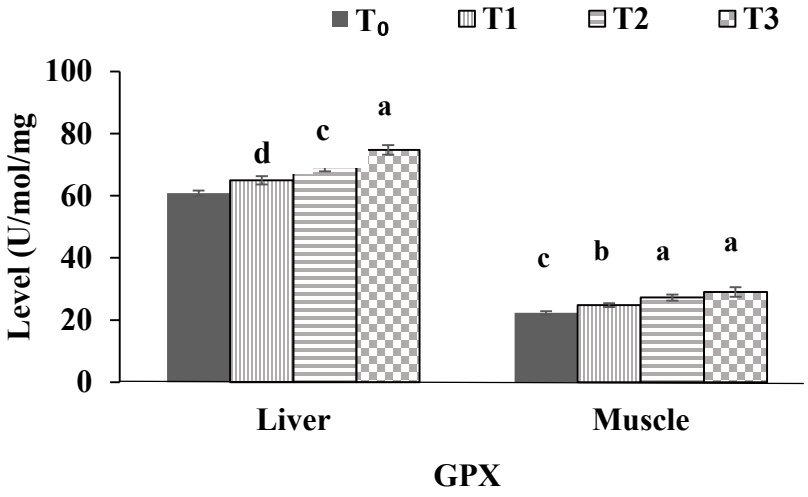


Figure 12. Both graphs represent the effect of diet supplemented with graded level *O. sanctum* plant extract on an antioxidant enzyme in *O. mossambicus*. The graph represents the effect of the glutathione antioxidant enzyme in *O. mossambicus* when fed with the diet which contains *O. sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Each bar represents Mean ± S.E(n=9). Mean with the different alphabets were significantly different($p<0.05$).

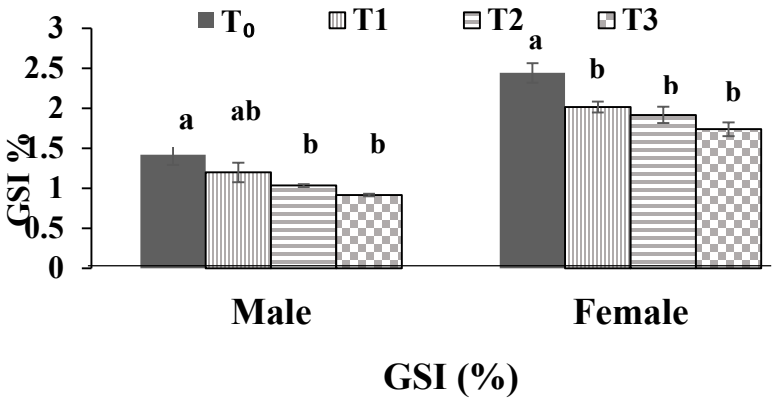


Figure 13. Effect of diet supplemented with graded level *O. sanctum* plant extract on Gonado- somatic index (GSI) in *O. mossambicus.* The graph represents the Gonado-somatic index (GSI) in *O. mossambicus* when fed with the diet which contains *O.sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg*O. sanctum*). Each bar represents Mean ± S.E(n=9). Mean with the different alphabets were significantly different ($p<0.05$).

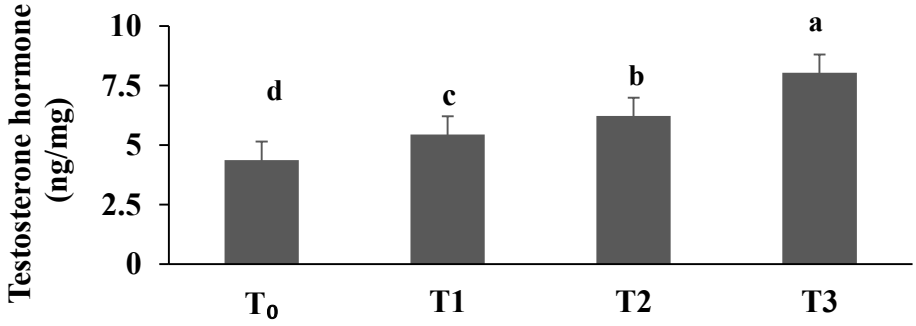


Figure 14. Effect of diet supplemented with graded level *O. sanctum* plant extract on blood sexual hormones (testosterone) in *O. mossambicus.* The graph represents the effect on blood sexual hormones (testosterone) in *O. mossambicus* when fed with the diet which contains *O.sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Each bar represents Mean ± S.E. Mean with the different alphabets was significantly different ($p<0.05$).

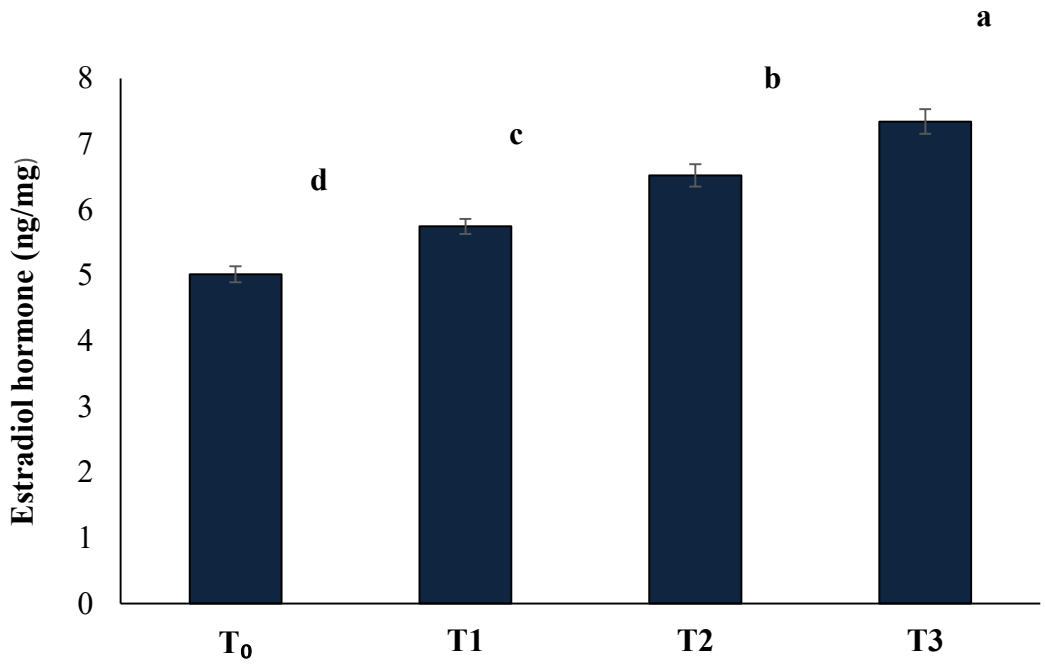


Figure 15. Effect of diet supplemented with graded level *O. sanctum* plant extract on blood sexual hormones (estradiol) in *O. mossambicus.* The graph represents the effect on blood sexual hormones (estradiol) in *O. mossambicus* when fed with the diet which contains *O.sanctum* in a different concentration. T_0 (diet without *O. sanctum*), T1 (10g/kg *O. sanctum*), T2 (20g/kg *O. sanctum*), and T3 (30g/kg *O. sanctum*). Each bar represents Mean ± S.E. Mean with the different alphabets was significantly different ($p<0.05$).

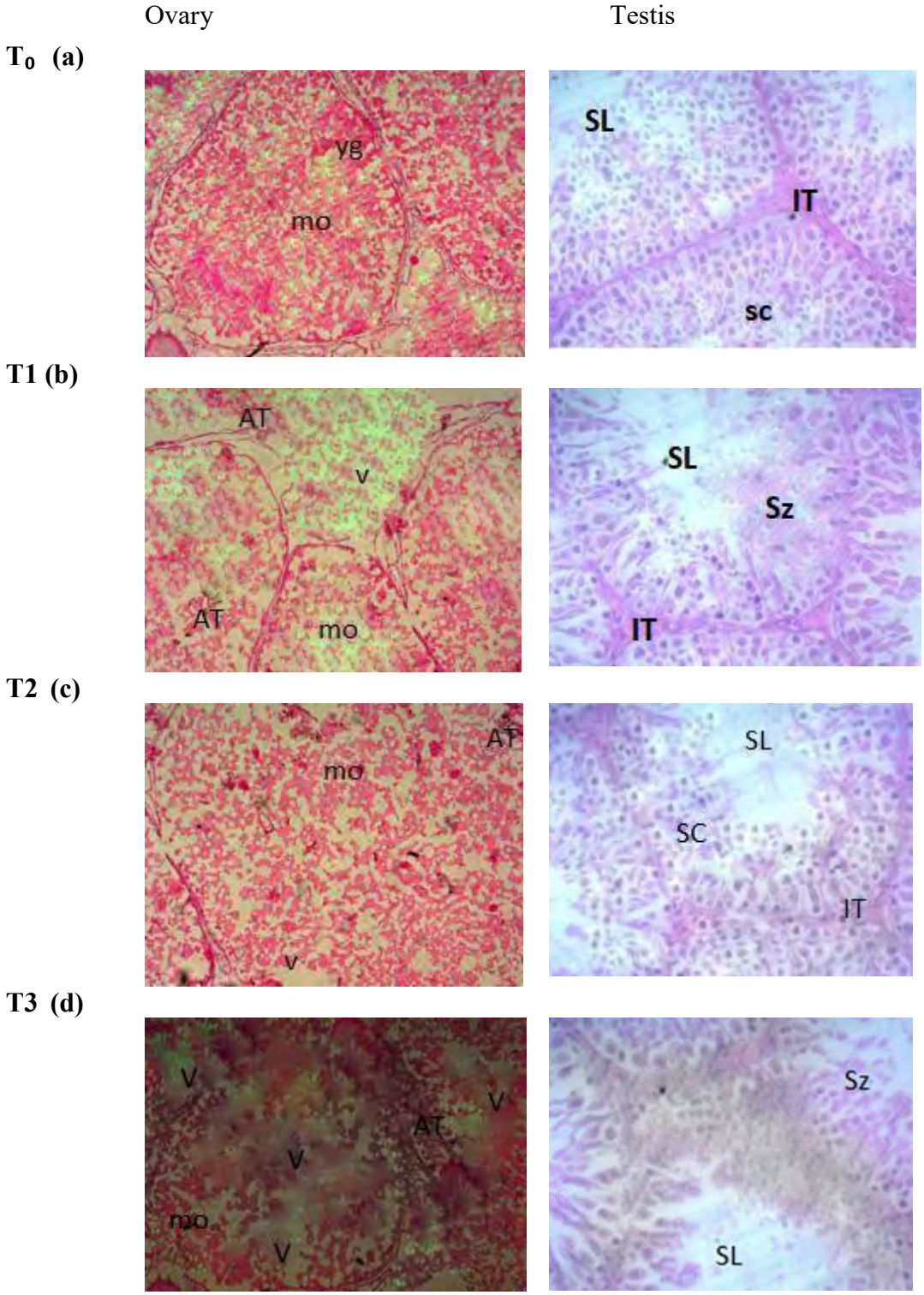


Figure 16. Cross-section of gonads; Mature oocyte (MO) which contains yolk granules (YG) atresia (AT) mature oocyte (MO), vacuolated oocyte stage (V) seminiferous lobule (SL), and the interlobular tissue (IT).

DISCUSSION

Worldwide aquaculture is one of the fastest-developing industries (Garlock *et al*., 2020) havinga great capacity to meet the global demand for aquatic food (Edwards *et al*., 2019). One of the limitations of Tilapia cultures is its nature of prolific breeding, which results in overpopulation and consequently stunted growth (Varadaraj & Pandian 1990; Toguyeni *et al*., 2002). Onepossible solution is an all-male culture of tilapia (Ashour *et al*., 2020) using Methy testosterone which makes tilapia reach market size fish (Bardhan *et al*., 2021). However, the use of MT have several negative environmental concerns (Velazquez & Alter, 2004) to cope with this limitation here an approach was made to use natural plant extract as the possible source to reduceor inhibit gametogenesis.

The present study reports the growth, antioxidant, apparent digestibility coefficient, gonadal somatic index (GSI), histology, and hormonal analysis of tilapia *O. mossambicus* fed on a diet mixed with plant extracts of *Ocimum sanctum.* A dose-dependent effect was observed in the present experiment. The result of our present study revealed that the *O. sanctum* plant had a positive effect on the growth performance of both males and females as indicated by the higher weight gain in the experimental group of our study. This increase in growth may be a result of the anabolic effect of *O. sanctum*. Ursolic acid, an active ingredient in *O. sanctum* has been reported to skyrocket testosterone levels in mice (Mankapure *et al.,* 2013, Srinivasulu & Changamma, 2017) may result in its anabolic effect. Earlier studies have reported that 17a- methyltestosterone treatment showed an increase in the individual growth of tilapia because ofits anabolic effects (Dan & Little, 2000, Khalil *et al.,* 2011, El-Greisy & El-Gamal, 2012). Sikotariya & Yusufzai. (2019) also reported similar results when they fed *O. sanctum* supplemented diets to the *Cirrhinus mrigala* a significant increase in weight gain was observed than the control diet (without *O. sanctum*). Panprommin *et al.* (2015) also observed similar results in *Oreochromis niloticus*, gowth was increased as the *O. sanctum* increased in the diet. Immanuel *et al.* (2009) and Naiel *et al.* (2020) found the same results when medicinal plant extract supplementation was administered to *Oreochromis mossambicus* fish. The herb, *Basella alba, Tribulus terrestris, Ivlucuna, Asparagus racernosus* have been shown to promote sex reversal in fish, as well as increase growth (Cek *et al.,*2007, Moundipa *et*

*al.,*2005). Other research suggested that the significantly greater mean weights were due to improved food conversion efficiency in *Oreochromis niloticus* sex-reversed fry (Chakraborty & Banerjee,2010).

In the present experiment, FCR and SGR were significantly improved as the *O. sanctum* supplementation in the diets was increased. It is in line with the previous studies. According to Gobi *et al.* (2016) *Oreochromis mossambicus* fed *Psidium guajava* leaf extract in the diet exhibited a significant increase in SGR when compared to fish fed a control diet. The same resultwas observed by Panprommin *et al.* (2015) when they fed tilapia with *O. sanctum* supplemented diets. Sikotariya & Yusufzai (2019) reported similar results in *Cirrhinus mrigala* fed with *O. sanctum* supplemented diets. Similarly, Sivaram *et al.* (2004) found a lower FCR value When *E. tauvina* was fed a meal with *O. sanctum* supplementation at a rate of 100-200mg/kg, which was aconsiderably lower concentration than the current studies. In our study, the fish fed with *O. sanctum* incorporated diet. This improvement in FCR may be due to better Apparent Digestibility Coefficients (ADC) of *O. sanctum* supplemented diet.

The Apparent Digestibility Coefficients (ADC) measure the percentage of nutrients in an ingredient that is available to the fish; researchers need ADCs to formulate feeds (Reveco *et al.,*2012) accurately. In our study, the addition of *O. sanctum* in the diets in different concentrations was well accepted by all of the treatment groups. According to our finding, *O.sanctum* in the diet significantly increased the digestibility of lipid, crude protein, and dry matter. In our study, ADC of protein, ADC of lipid, and ADC of dry matter were also significantly higher in T3 and T2 than in the control group. Similar to our study, El- Dakar *et al.* (2007) reported hybrid tilapia*,* fingerlings fed with *O. sanctum* leaves result in a significant increase in AD. ADC was increased for dry matter, ADC for crude protein, and ADC for energy as increased DBL levels in diets. Mohamed *et al.* (2019) reported that the use of chamomile flower and *O. sanctum* by-products inclusion in sheep also significantly increase the apparent digestibility for crude protein, dry matter, and energy.

Hematological parameters reveal the nutritional and health status of the fish and

can thus be used to evaluate the fish's condition (Martin & Król, 2017; Reverter *et al.,*2014). In our study white blood cells significantly increase as the *O. sanctum* concentration increase in the feed but no significant effect on red blood cells. Hb, PCV, MCV, MCH, MCHC, Platelets, Neutrophils, Monocytes, and lymphocytes were significantly increased as the *O.sanctum* concentration increased in the feed. All these parameters significantly increased in the T3 group, which fed 30g/kg *O.sanctum* followed by the other treatment and T_0 group (without *O. sanctum*). Similar tothe present results Shah and Barai (2016) reported significant changes in Hb content, RBC, WBC count, PCV, MCV, MCHC, and MCH values in male albino rats fed on *O. sanctum* administration. The *O. sanctum* is known to influence physiological balance (Cohen, 2014). The addition of *O. sanctum* in the diet might possibly serve as a prophylactic purpose and increase fish immunity as described by (Galina *et al.*, 2009). Our results are linked with Kirubakaran *etal.* (2013) and Farook *et al.* (2019), who reported similar results when fed *O. sanctum* diet to chicken. Alcoholic and aqueous extracts of *O. sanctum* increased haemagglutination (Mahajan *etal.,* 2013). These results suggest no side effects and positive impact of *O. sanctum* extracts on tilapia health

The liver is the primary organ for drug metabolism (Knights *et al.,*2016). Transaminases such as AST (aspartate aminotransferase) and ALT (alanine aminotransferase), which are well- known enzymes recognized as good indicators of liver function and biomarkers predictingprobable toxicity, are affected by drugs that cause toxicity in the liver (Ozer *et al.,*2008). High levels of the liver enzymes AST and ALT indicate cellular damage and hepatocellular membrane permeability (El-Moghazy *et al.,*2014). In our study, both AST and ALT did not show any treatment-related increase even at the 30g/kg dose followed by the control group. As a result, the lack of significant elevations in ALT and AST activities strongly suggests that treatment of O. sanctum had no effect on hepatocytes and, as a result, metabolism in the *O. mosambicus*. In the present study, *O. sanctum* decreased the serum level of LDH, AST, and ALT, but it was not significant. Ursolic acid is the active ingredient of *O. sanctum* which has hepatoprotective properties. Kumar *et al*. (2019) also found similar results in the rat when administrated different concentrations of *Ocimum sanctum*. Suanarunsawat *et al.* (2010) also reported that rat fed with a diet that contains *O.sanctum* has no significant effect on LDH, AST,

and ALT.

Several environmental stresses cause intracellular reactive oxygen species to be produced, creating oxidative stress in fish, as seen by the reduction of antioxidant enzyme activity (Abdel-Latif *et al.,*2021). Antioxidant systems can effectively remove reactive oxygen species, protect themselves from oxidative damage, and prevent a variety of pathogenic alterations (Dawood *et al.,*2019). One of the most important intracellular antioxidants systems is the glutathione redox cycle. Enzymes that combat free radicals SOD and CAT are considered the first line of defense against oxidative imbalance and tissue damage, with SOD removing excess hydrogen peroxide from the tissue and CAT scavenging superoxide molecules (Das *et al.,*2018).

In our study, when we increase *O. sanctum* level in the diet, antioxidant activity is also enhanced. It was the most significant increase in the T3 group, which fed 30g/kg *O. sanctum*. With an increase in *O. sanctum* in the diet, the LPO level also decreases significantly. The antioxidant properties of O. sanctum are clearly demonstrated by the decrease in TBARS and theincrease in GSH, SOD, POD, CAT, GPX, GSR, GSH, and GST. Similarly, Sethi *et al.* (2004) found that intake of *O.sanctum* leaves caused a significant increase in levels of GSH and SOD activity. According to Hussain *et al*., aqueous extract of *O. sanctum* mixed with rats' diet resultedin decreased LPO formation and increased antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione transferases (GT). It also raised antioxidant levels in the plasma, liver, lung, kidney, and brain of rats, such as reduced glutathione (GSH). *O. sanctum* has been discovered to have therapeutic potential as an anti-diabetic, anti-hyperlipidemic, and anti-oxidant *O. sanctum* contains eugenol, rosmarinic acid,and carvacrol all of these ingredients have antioxidant properties (Baliga *et al.,*2013). Other plantextracts are also known to enhance antioxidant activity in tilapia. Metwally (2009) reported that the use of *Allium sativum* in the diet enhances the antioxidant activities in tilapia. Gobi *et al.* (2016) reported that *O.mossambicus* fed with *P sidium guajava* leaf supplemented diet showedenhanced SOD, CAT, ROS, and GPX.

The Gonadostomic Index is a technique for determining an animal's sexual

maturity in relation to ovary and testes development. This index is used as an indication or evidence of potential endocrine disruption. Among the feeding groups, minimum GSI was obtained in the T3 group (male 0.915 and female 1.737) followed by T2 (male 1.034 and female 1.916) group whilemaximum GSI in T1 (male 1.197 and female 2.016) group along with control (male 1.418 and female 2.443) in both sexes. It clearly indicates that an increase in *O. sanctum* concentration resulted in decreased Gonadostomic Index. Ghanta et al. (2022) also reported similar results when they fed *O. sanctum* ingredients to Common carp. Other medicinal plant extracts have shown similar results in tilapia. Other medicinal herbs, such as neem (*Azadirachta indica*) and *Hibiscus rosa* sinensis, considerably lowered the GSI of O. niloticus females according to Jegede & Fagbenro (2008) and Ambreen and Javed (2018). Abdelhak *et al.* (2013) reported a decrease in GSI of females when fed paw paw (*Carica papaya*) seeds meal. This decrease in GSI with the *O. sanctum* diet shows its effectiveness as an effective antifertility agent to control prolific breeding associated with tilapia culture.

In the control group (T0), the histological structure of the ovaries and testis tissues was normal. The effect of oleanolic acid, the active ingredient in *O. sanctum* responsible for sterility, may be attributed to structural changes in the ovary of females treated with a low dose (T1), necrosis in females treated with a medium-dose (T2), and atresia, necrosis, much vacuolated, andfusion of ova in females treated with a high dose of *O. sanctum* (T3). A high dose of *O. sanctum* has been shown to reduce tilapia fertility by altering the gonad structure. Abdelhak *et al.* (2013) also reported similar results when they fed pawpaw (*Carica papaya*) seeds meal to tilapia. Other therapeutic plants, such as *Hibiscus rosa sinensis* leaf meal (Temitope, 2010) and Aloe Vera Latex, (Tope-Jegede *et al*., 2019) had a comparable histological effect on ovaries. The ovaries of redbelly tilapia (*Coptodon zillii*) fed on neem (*Azadirachta indica*) leaf meal showed similar histological results by Geetha *et al*. (2019) and Jegede & Fagbenro (*O. niloticus* 2008).

In testis tissues treated with *O. sanctum*, deformation in seminiferous lobules of males treated with a low dose, degeneration in interlobular tissue in males treated with a medium dose, and deformation in both seminiferous lobules and interlobular tissue in males treated with a high dose were all observed. Spermatogenesis was probably

interrupted at the secondary spermatocyte stage, most likely as a result of hypogonadotropic levels. With prolonged treatment, Leydig cells were also damaged, and they became unable to manufacture testosterone. The testicles had lost weight due to a decrease in the amount of sperm and spermatogenic components. This result was confirmed by the work of Mankapure *et al.* (2013) when they administered *O. sanctum* to male albino rats. The treatment of monosex fish with 17^0 -methyltestosterone resulted in deformed interlobular tissues. Seminiferous tubules were seen in the testis of O. niloticus fed C. papaya, according to Geetha *et al*. (2019). Temitope (2010) & Tope-Jegede *et al*. (2019) found that *Hibiscus rosa* sinensis leaf meal and *Aloe Vera Latex* respectively had similar histological effectsin the testis of *O. niloticus*.

In the present study, testosterone level increased with increasing *O. sanctum* concentration with T3 having the maximum testosterone level. *O. sanctum* is well-known for its testosterone-boosting properties. It can, however, be used as an antifertility agent. While *O. sanctum* may increase testosterone levels, it also has the potential to inhibit the HPTA (Hypothalamic-Pituitary-Testicular Axis). Mondal *et al*., (2009) observed comparable findings when feeding *O. sanctum* to male albino rabbits at a rate of 2g/day, with testosterone levels rising from 303 ng/dL to 1500 ng/dL. However, luteinizing hormone (LH) synthesis ceased totally as a result, which appears to be similar to steroid-induced HPTA shutdown. One of the principal ingredients of *O. sanctum* leaves is ursolic acid, which is thought to be responsible for these hormonal effects. Ursolic acid is thought to have antifertility properties (Prakash & Gupta, 2005). Many other active components in *O. sanctum* have been reported to produce sterility in female rats, including oleanolic acid, rosmarinic acid, eugenol, carvacrol, linalool, and - caryophyllene. The injection of eugenol and *O. sanctum* leaf extract dramatically increased serum estradiol and progesterone levels, resulting in decreased ovulation frequency and reproductive impairment (Polli & challa, 2019). Mankapure *et al.* (2012) also reported similar results when albino rats were fed *O. sanctum*, it reduced fertility in male rats via damaging the gonads and epididymis

The hypothalamus-pituitary–testis axis, as well as accessory sex organs, are involved in the regulation of the male reproductive process. Both FSH and testosterone

are thought to be required for the initiation and maintenance of spermatogenesis. Testosterone has a significant impact on the growth and differentiation of germ cells. It also has a negative feedback effect on LH secretion and, to a lesser extent, FSH secretion, operating on the hypothalamic-pituitary axis.(Sethi & Chaturvedi, 2016). Even though *O. sanctum* boosted testosterone production, it was most likely owing to an androgenic/anabolic compound in the plant that increased testosterone levels in the gonads directly, rather than through gonadotropin (LH and FSH) signals from the brain. *O. sanctum* may reduce luteinizing hormone synthesis and effectively cease natural testosterone production, resulting in reduced follicle-stimulating hormone (FSH), which lowers sperm formation. The majority of plant components inhibit steroidogenesis by targeting the process of hormonal regulation of spermatogenesis via the hypothalamo-pituitary-gonadal axis. (Udoh *et al.,*2005).

Conclusion

The findings of this study indicated that the use of *O. sanctum* in fish diets is a growth promoter. It increases antioxidant activities and doesn't induce any stress on tilapia. A reduction in GSI andhistological results showing high doses of *O. sanctum* affecting the gonads in tilapia is an indication of impaired fertility is attributed to the direct effect of treatment with *O. sanctum*, which appears to suppress androgen activating hormones. There is a strong potential using *O. sanctum* alternative to synthetic steroids in controlling unwanted reproduction in tilapia culture. Further studies are needed to elucidate the hormonal level impacts and to determine the optimumtreatment regime for the induction of 100% infertility.

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