CHAPTER THREE

MATERIALS AND METHODS

The present study was carried out at the Fish Nutrition Laboratory, Department of animal and fish production, Faculty of Agriculture (Saba-Basha) and Reproductive Toxicity Laboratory, Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University. This study comprises of two experiments to investigate the following:

1- The effects of supplementation of some dietary natural phytochemicals on growth performance, anabolic steroid hormone levels, antioxidant enzymes and physiological response of Nile tilapia, *Oreochromis niloticus*, fingerlings.

2- The effects of supplementation of some dietary natural phytochemicals in comparison with 17 α-methyl testosterone (MT) on sex reversal, growth performance, feed and protein utilization and survival rate of Nile tilapia, *O. niloticus*, fry.

3.1. First experiment:-

Two levels of each phytochemicals supplementations (ginseng extract, Tribulus extract and date palm pollen) were compared with control on growth performance, feed utilization, whole body composition, anabolic steroid hormone levels, oxidative stress, antioxidant enzymes, hematological, plasma biochemical and histological changes of Nile tilapia fingerlings.

3.1.1. Experimental fish:

Apparent healthy 140 tilapia fingerlings, with an average initial body weight of 3.67±0.02 g/fish. Fish were obtained from a private commercial fresh water fish farm sited in Motobas, Kafr–El Sheikh Governorate, Egypt. Experimental fish were kept for two weeks in indoors circular fiberglass tanks (1 cubic meter) as an acclimation period and fed on diet contained 32% crude protein prior to the start of the experiment.

3.1.2. Experimental facilities:

Fourteen glass aquaria with dimensions of 100×30×40 cm and 100 L volume of water/aquarium used. Water temperature averaged 26±2°C. Continuous aeration maintained in each aquarium using an electric air blower. Manual method for removal of excreta conducted every day before the first feeding by siphoning half of the water volume and replaced by an equal volume of ground water.

3.1.3. Experimental design:

Seven treatments applied at fourteen aquaria (two aquaria/treatment), each stocked with ten fish. The following treatments were used: 1-control (basal diet, BD); 2- BD supplemented with ginseng extract (0.2 g/kg diet); 3- BD supplemented with ginseng extract (0.4 g/kg diet); 4- BD supplemented with ginseng extract (0.2 g/kg diet) Tribulus extract (0.625 g/kg diet); 5- BD supplemented with Tribulus extract (1.25 g/kg diet); 6- BD supplemented with date palm pollen (3g/kg diet) and 7- BD supplemented with date palm pollen (6 g/kg diet). The levels were selected according to the literature data (Goda, 2008; Gauthaman and Ganesan 2008; Hassan *et al.,* 2012). The experiment was lasted for 84 days.

Fish weights were recorded at the beginning of the experiment and every week. Meanwhile fish total length (cm), blood samples, dissection and organs sample (viscera, liver, testes and spleen) for homogenization and histology carried out at the end of the experiment. Fish samples were collected at the beginning and the end of the experiment and store frozen at -20°C until whole body chemicals analysis.

3.1.4. Experimental diets:

The experimental diets containing 32% crude protein were prepared in the laboratory. All feed ingredients were purchased from the local market (Table, 2) and finely ground, mixed well then incorporated in to the diet. Diets were supplemented with vitamins and minerals.

The formulated experimental diet were pelleted (0.5 mm diameter) using small mincer, dried for 48 hrs using 40-50ºC oven and stored at room temperature. The chemical composition (%) of the formulated diets was analyzed according to AOAC, (1995). All supplementations add to the diets by excluded the same portion of wheat flour; the used phytochemicals gained or prepared as flow:

Table (2): Ingredients and chemical composition (%) of the first experimental diets.

Ingredients	**%**
Fish meal (herring; 72%)	26
Corn glutin	15
Yellow corn	39
Rice bran	10
wheat flour	5
Corn oil	2.4
Vitamins and Minerals primex[1]	2
Calcium-mono phosphate	0.6
Total	100
Chemical composition (%) on dry matter basis	
Dry matter (DM)	96.2
Crude protein (CP)	32.38
Ether extract (EE)	12.64
Nitrogen free extract (NFE) [2]	43.54
Crude fiber (CF)	4.61
Ash	6.83
Gross energy (GE; kj/1 g DM)[3]	20.11
P/E ratio (mg CP: kj)[4]	16.10

[1]Composition of vitamin mineral mixture of 1 kg: Vitamin A - 50,00,000 IU; Vitamin D_3 - 10,00,000 IU; Vitamin B_2 - 2.0 g; Vitamin E - 750 units; Vitamin K - 1.0 g; Calcium pantothenate 2.5 g; Nicotinamide - 10.0 g; Vitamin B_{12} - 6.0 g; Choline Chloride - 150.0 g; Calcium - 750.0 g; Manganese - 27.5 g; Iodine - 1.0 g; Ion - 7.5 g; Zinc - 15.0 g; Copper - 2.0 g; Cobalt - 0.45 g.

[2]NFE: Nitrogen free extract calculated using the following equation: NFE = 100- (crude protein + ether extract + crude fiber + ash).

[3] GE: Gross energy calculated on the basis of 23.6, 39.4 and 17.2 k joule gross energy/g protein, ether extract and NFE, respectively (NRC, 1993).

[4]P/E ratio: protein energy ratio (mg crude protein/kj gross energy) = CP/GE x 1000.

Ginseng extract, *P. ginseng*:

Ginseng extract powder was kindly supplied via Pharco Pharmaceuticals Co., Alexandria, Egypt. The powder weight and mixed carefully into oil then added to basal diet with each respect levels.

Tribulus extract *(Tribulus terrestris*):

Tribulus extract (Trib Gold®) were purchased from Nerhadou International Co. for pharmaceuticals and nutraceuticals, Egypt. Powder of *Tribulus*extract in commercial capsule (250 mg) poured and mixed carefully into oil then added to basal diet with each respect levels.

Date palm, *P. dactylifera*, pollen:

Fresh pollen of date palm was collected in March 2013 from Edku city, Egypt. The pollen grains were separated from the kernels with a fine gauze sieve and dried at 40-50°C for 24 hrs, before weight and mixed carefully into oil then added to basal diet with each respect levels.

3.1.5. Feeding regime:

Fish in each aquarium were hand fed two times per day at 9.00 a.m. and 14.00 p.m. six days a week at a rate of 6% in the first four weeks, 5% in the second four weeks and 4% until the end of the experimental period (84 days). The feeding rates were adjusted according to fish live body weights weekly.

3.1.6. Measured parameters:

3.1.6.1. Growth performance parameters:

Final body weight (FW), weight gain (WG), average daily gain (ADG), specific growth rate (SGR), survival rate, length, length gain and condition factor were conducted according to the following equations:

Weight gain (g/fish): $WG = W_t - W_0$

Where:

W_0: initial mean weight of fish in grams.

W_t: final mean weight of fish in grams.

Average daily gain (g/fish/day): $ADG = W_t - W_0/n$

Where:

n: duration period.

Specific growth rate (%/day): $SGR = 100 \times (\ln W_t - \ln W_0)/ \text{days}$

Where:

ln: natural logarithm.

Survival rate (%) = 100 × (initial number of fish/ final number of fish)

Length gain (cm)= $L_t - L_0$

Where:

L_0: initial mean length of fish in cm.
L_t: final mean length of fish in cm.
Condition factor = 100 × (BW (g)/ L^3 (cm)).

3.1.6.7. Feed and nutrients utilization parameters:

Feed Intake (g/fish): This is the amount of feed given or supplied during the experimental period for each fish per gram.

Feed conversion ratio (FCR) = dry matter intake (g)/weight gain (g)

Protein efficiency ratio (PER) = weight gain (g)/protein intake (g)

Protein productive value (PPV %) = 100 × (P_t - P_0)/protein intake (g)

Where:
P_0: protein content in fish body at the start.
P_t: protein content in fish body at the end.

Energy gain (k joule) = E_t - E_0

Where:
E_0: energy content in fish body (Kcal) at the start.
E_t: energy content in fish body (Kcal) at the end.

Energy utilization (EU %) = 100 × (E_t - E_0)/ Energy intake (Kcal)

3.1.6.8. Fish whole body chemical composition (%):

Proximate chemical analyses (%; dry matter protein, ether extract and ash) of fish whole body (before and after the experiment) were performed according to the official methods of analysis of the AOAC (1995).

3.1.6.2. Organs somatic index:

The organs (viscera, liver, testes and spleen) somatic index (%) was calculated as g/100 g body weight as mentioned afterward:

Viscera index = 100 × (Viscera weight (g)/body weight (g)).

Liver index = 100 × (Liver weight (g)/body weight (g)).

Testes index =100 × (Testes weight (g)/body weight (g)).

Spleen index =100 × (Spleen weight (g)/body weight (g)).

3.1.6.3. Hematological parameters

Blood samples were taken from the caudal vein of anaesthetized (50 mg clove oil/l) fish using sterile syringe contain drop of heparin. Four pooling blood samples (four fish/sample) for each treatment were divided into two portions. The first

portion was subjected to determine the following hematological assays: red blood cells (RBCs), white blood cells (WBCs), hemoglobin, haematocrit (Hct), and calculate mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentrate (MCHC).

Manual method for counting RBCs and WBCs was performed using standard Neubauer cell counting chamber and Shaw's solution as dilution fluids, following the method of (Hesser, 1960). Haemoglobin determined colorimetically using commercial kits (Diamond, Egypt) according to (Van-Kampen and Zijlstra, 1961). The Hct determined by haematocrit tube, which filled with blood and the opposite end of the tube closed by special clay, centrifuged for 10 minutes at 10000 rpm (KREBS Micro-hematocrit centrifuge, BUNSEN, European Union). The hematocrit values were recorded by centrifuge's combo reader. Moreover, Blood indices were calculated according to the following formula:

$$\text{Mean corpuscular volume (MCV; } \mu m^3) = \frac{\text{Hct} * 10}{\text{RBCs count (million/mm}^3)}$$

$$\text{Mean corpuscular haemoglobin (MCH; pg)} = \frac{\text{Hb (g/100 ml)} * 10}{\text{RBCs count (million/mm}^3)}$$

$$\text{Mean corpuscular haemoglobin concentrate (MCHC; \%)} = \frac{\text{Hb (g/100 ml)} * 10}{\text{Hct (volume \%)}}$$

3.3.6.4. Blood biochemical parameters and enzyme activities

The second portion was centrifuged at 4000 rpm for 10 min. using cooled centrifuge (4ºC). The plasma sample were pipetted, kept in Eppendorf tube and stored frozen at -80ºC until the assays of hormones, plasma biochemical parameters and enzyme activities.

Stored plasma samples were analyzed for luteinizing hormone (LH) by Automated Enzyme Immunoassay system (AIA-360) called immulite/immulite 1000 system which based on the methods described by Beitens, (1976) and testosterone determine by Enzyme-linked immune sorbent assay (ELISA) according to (Abraham, 1977).

The activity of Glutathione peroxidase (GPx, EC. 1.1.1.9) was assayed by the method of Chiu *et al.,* (1976). Glutathione *S*-transferase (GST; EC 2.5.1.18) activity was determined according to Habig *et al.,* (1974). Catalase (CAT; EC 1.11.1.6) activity was determined using the Luck method involving the decomposition of hydrogen peroxide (Luck, 1974). Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to Misra and Fridovich (1972). Glutathione reduced (GSH) was determined according to the method of Beutler *et al.,* (1963). Plasma thiobarbituric acid-reactive substances (TBARS) were measured by the method of Tappel and Zalkin (1959).

Total proteins were measured by the Biuret method according to Gornal *et al.,* (1949). Albumin concentration was determined by the method of Doumas *et al.,* (1971). Globulin was calculated as the difference between total protein and albumin. Plasma concentrations of total lipids, cholesterol and triglycerides (TG) were determined according to the methods of Zollner and Kirsch (1962), Allain *et al.,* (1974) and Fossati and Principe (1982), respectively. High-density lipoprotein-cholesterol (HDL-c) was determined according to the methods of Grove, (1979). Low-density lipoprotein-cholesterol (LDL-c) was determined by the calculation (LDL-c= (cholesterol- (HDL+VLDL)) according to Warnick *et al.,* (1983). Very low-density lipoprotein-cholesterol (vLDL-c) was calculated by dividing the values of TG by factor of 5 according to Warnick *et al.,* (1983).

The activities of plasma aspartate transaminase (AST; EC 2.6.1.1) were assayed by the method of Gella *et al.,* (1985). Alanine transaminase (ALT; EC 2.6.1.2) were assayed by the method of the international federation of clinical chemistry (Gella *et al.,* 1985). Alkaline phosphatase (AlP; EC 3.1.3.1) activity was determined in plasma according to the method of the International Federation of Clinical Chemistry (Rosalki *et al.,* 1993). Acid phosphatase (AcP; EC 3.1.3.2) activity was determined according to the method of Escribano, (1984). Urea and creatinine concentrations were measured by the method of Chaney and Marbach (1962) and Bartles *et al.,* (1972), respectively. Plasma total bilirubin was measured using the method of Pearlman and Lee (1974).

3.3.6.5. Organs biochemical parameters and enzyme activities:

At the end of the experiment eight fish for each replicate were dissected and the organs (liver, testes) separated, weighted and immediately, the liver and testes washed using chilled saline solution, kept on ice box then stored at -80°C until the homogenization process, also part of fresh testes preserved in formalin for histological study.

Frozen liver and testes were minced and homogenized (10% w/v) in ice cold sucrose buffer (0.25 M) in a Wise Tis® HG-15D Homogenizer, DAIHAN-SCIENTIFIC, India. The homogenate centrifuged at 10,000 rpm for 20 min at 4°C. The resultant supernatant of the organs collected and stored at -80 °C.

The organs homogenates were used for the analysis of the different enzyme activities and free radicals determination according to the previous methods. GST, SOD, GPx, CAT, GSH and TBARS were determined in liver and testes. While, AST, ALT, AcP and AlP were determined in liver.

3.1.6.6. Histological studies:

Testes were removed from experimental fish and rapidly placed in adequate amount of 10% neutral buffered formalin for at least 24 hrs. The fixed specimens were processed through the conventional paraffin embedding technique (dehydration through ascending grades of ethanol, clearing in chloroform and finally embedding in melted paraffin wax at 60ºC). Paraffin blocks prepared from which 5 microns thick

sections stained with Hematoxylin and Eosin (H & E) according to the method described by Culling, (1983).

3.2. The 2nd experiment:-

The second experiment was conducted to investigate the effect of different sex-reversal agents (ginseng extract, Tribulus extract, date palm pollen) in comparison with 17 α-methyl testosterone (MT) on sex reversal, growth performance, feed and protein utilization, and survival rate of Nile tilapia, *O. niloticus*, fry.

3.2.1. Experimental fish and facilities:

Nile tilapia fry one day old obtained from private commercial hatchery sited in Motobas, Kafr–El Sheikh Governorate, Egypt. Initial body weight averaged 0.02±0.00068 g/fish and average initial total length was 1.13±0.01 cm/fish. The fry kept in glass aquaria for one day after transportation without feeding. Fish reared under the same condition of the first experiment, except the siphon tube closed by hapa net to prevent fry escaping.

3.2.2. Experimental design:

A total of 800 tilapia fry randomly distributed on 16 glass aquaria (100×30×40 cm and 100 L volume). Starting fish density was 50 larvae per tank with two replicates per treatment. The following 8 treatments were used: 1-control (basal diet containing, BD); 2- BD supplemented with 17 α-methyl testosterone; 3- BD supplemented with ginseng extract (0.2 g/kg diet); 4- BD supplemented with ginseng extract (0.4 g/kg diet); 5- Tribulus extract (0.6 g/kg diet); 6- BD supplemented with Tribulus extract (1.2 g/kg diet), 7- BD supplemented with date palm pollen (3g/kg diet) and 8- BD supplemented with date palm pollen (6 g/kg diet). The experimental fish received experimental diets for 28 days (end of experimental periods) and the fish of all groups fed control diet until reach 84 days of age (approximately 5-6 gm of weight) to be suitable for dissection.

3.2.3. Experimental diet:

The experimental diet (45% crude protein) was formulated in powder form and its chemical composition is shown in (Table 3). The experimental diets prepared as follow: the solid ingredients grounded using house mixer, and sieved to get a fine powder, weighed and mixed manually in mixing bowl until the mixture became homogenous, then the oil was added slowly with continuous mixing and dried in an electric oven at a temperature of 40-50^{o}C for 24 hours. Fish were fed three times a day in a decreasing rate from 20% to 10% of live body weight for four weeks, and then the fish were fed at 8% to the end of the experiment, feed ratio adjusted every week. The chemical composition of the formulated diet analyzed according to AOAC, (1995).

The sex-reversal treatments were prepared according to the alcohol

evaporation method (Guerrero, 1975; Navarro-Martín *et al.*, 2009). The control diet treated with ethanol only and then the diet left to dry at room temperature in front of an electric fan for 12 hours.

The sex-reversal agents were MT (Argent Laboratories Inc., Philippines), ginseng extract (Pharco Pharmaceuticals Co., Alexandria, Egypt), Tribulus extract (Nerhadou International Co. for pharmaceuticals and nutraceuticals, Egypt) and date palm pollen: Fresh pollen of date palm was collected in March 2013 from Edku city, Egypt. The pollens were separated from the kernels with a fine gauze sieve and dried at 50°C for 24 hrs.

3.2.4. Measured parameters

Growth performance, feed and protein utilization parameters and survival rates were measured after 28 days as mentioned in the first experiments.

Sex ratio analysis was determined after 84 days of fry age via wet-squash method according to Guerrero and Shelton (1974). Briefly, the gonads were removed using fine forceps, placed on a slide, covered with few drops of (Nigrosin and Eosin) and squashed with a cover slip. The slide was then examined under light microscope using magnifications of 10*40 X.

For coupled squash and histological examinations, the gonads of 10 randomly chosen fish of each group were dissected, one fresh portion prepared and observed as above, while the other gonad fixed in formalin and treated using standard histological procedures. Gonads longitudinally sectioned to a thickness of 5µm and stained with H & E.

3.3. Statistical analysis:

In first and second experiments data were statistically analyzed with one-way ANOVA and Duncan's (1955) multiple range tests and expressed as mean values ± SE. Effects with a probability of $P<0.05$ were considered significant. Statistical analyses were performed using SPSS for Windows (Standard Version 17 SPSS Inc. Chicago, Illinois).

Table (3): Ingredients and chemical composition (%) of the second experiment diet.

Ingredients	**%**
Fish meal (herring; 72%)	56.40
wheat Flour	40.00
Corn oil	2.00
Vitamins and Minerals primex[1]	0.50
Vitamin C	0.50
Garlic Extract	0.10
Oxytetracyclin	0.50
Total	100
Chemical composition (%) on dry matter basis	
Dry matter (DM)	93.70
Crude protein (CP)	44.16
Ether extract (EE)	7.23
Nitrogen free extract (NFE)[2]	39.41
Crude fiber (CF)	3.17
Ash	6.03
Gross energy (GE; kj/1 g DM)[3]	20.05
P/E ratio (mg CP: kj)[4]	22.03

[1]Composition of vitamin mineral mixture of 1 kg: Vitamin A - 50,00,000 IU; Vitamin D_3 - 10,00,000 IU; Vitamin B_2 - 2.0 g; Vitamin E - 750 units; Vitamin K - 1.0 g; Calcium pantothenate 2.5 g; Nicotinamide - 10.0 g; Vitamin B_{12} - 6.0 g; Choline Chloride - 150.0 g; Calcium - 750.0 g; Manganese - 27.5 g; Iodine - 1.0 g; Ion - 7.5 g; Zinc - 15.0 g; Copper - 2.0 g; Cobalt - 0.45 g.

[2]NFE: calculated using the following equation: NFE = 100- (crude protein + ether extract + crude fiber + ash).

[3] GE: calculated on the basis of 23.6, 39.4 and 17.2 k joule gross energy/g protein, ether extract and NFE, respectively (NRC, 1993).

[4]P/E ratio: protein energy ratio (mg crude protein/kj gross energy) = CP/GE x 1000.