CHAPTER III
RESEARCH METHODOLOGY

3.1 Experimental animal

Two thousand juvenile red tilapia (*Oreochromis niloticus* × *O. mossambicus*), which were obtained from Seang-Sawaang farm (Bangkok, Thailand), were acclimated to aquaria by feeding commercial pellets (commercial feed containing 32% of protein was purchased from Top Feed Mill Co., Ltd. Nakhon Pathom, Thailand) for three weeks. Prior to beginning of the experiment, 50 fish of approximately the same size were weighed for total initial body weight (initial body weight = 4.3 g/fish) and then randomly stocked into each of 20 aquaria (stock density = 1.8 g/L). Fish were fed with the experimental diets to near satiety, three times a day at 0900, 1300 and 1600 h on the workday, and once a day at 0900 h on the weekend for eight weeks. Mortality was recorded daily and feed intake was recorded weekly. Growth in weight of fish was measured at the 4-week and 8-week feeding interval.

3.2 Experimental conditions

This experiment was conducted at Applied Centre of Aquaculture Nutrition (ACAN), Biomin (Thailand) Co., Ltd. (Bangkok, Thailand). The culture system was the recirculated indoor system (the flow rate of water is proximately 1.5 m^3/ second/ tank (0.0015 L = 1.5 cm^2) that consisted of the 20 of 120-l aquaria, the water filter tanks (provided with a bio-balls, cloth filters, grit, and shells) and water storage tanks. Each aquarium was continuously aerated by blowers (air compressor, Puma Industrial Co., Ltd., Chang-Hwa, Taiwan) through an air-stone to maintain optimum dissolved oxygen (DO) level. Fecal pellets were removed from the sedimentation tank and cloth filters were washed every morning before feeding at the first time of the day. Aquaria and the sedimentation take were cleaned by scrubbing the tank surface and about 50% of the water volume in the system was replaced by the dechlorinated tap water weekly.

Temperature and DO were monitored daily by thermo scientific (Alpha DO 500 2-wire dissolved oxygen transmitter, Thermo Fisher Scientific Inc., MA, USA). Water quality parameters, including pH, ammonia (NH_3-H), nitrite (NO_3-N) and nitrate (NO_2-N), were weekly measured using test kits (the test solutions produced by Mars Fishcare North America, Inc.) The average values of each water quality parameter throughout the experiment were: DO = 4.44 ± 0.59 mg/L, temperature = 30.50 ± 0.6˚C, pH = 6.95 ± 0.16, NH_3-H = 0.42 ± 0.12 mg/L, NO_3-N = 20 mg/L, NO_2-N = 0.40 ± 0.13 mg/L.

3.3 Experimental diets

Mycotoxin source: wheat naturally contaminated with 41.0 ppm DON analyzed by Romers Labs, Biomin Singapore Pte. Ltd., Singapore (using High Performance Liquid Chromatography, HPLC) and clean wheat flour with 0.23 ppm DON later analyzed by IFA-Tulln, Austria (using Liquid Chromatography - *Tandem* Mass Spectrometry, LC-MS/MS). Both of the sources of wheat were referred to as "contaminated wheat" and "clean wheat", respectively. Five diets containing graded concentrations of DON at 0.07, 0.31, 0.50, 0.92 and 1.15 ppm DON were formulated by replacement of clean wheat with contaminated wheat.

Five isonitrogenous (24.6% dry matter basis) and isoenergetic (17.8 Kj/g) experimental diets (Table 6) were prepared at Aquaculture Centre for Applied Nutrition, Biomin (Thailand) Co, Ltd. (Bangkok, Thailand). The major milled-ingredients, such as cassava flour, soybean meal, clean wheat flour, rice bran, fish meal, were mixed for five minutes using a 20-litre Hobert type mixer (model B20, Guangzhou Youjia Machinery Co.,Ltd., China), and then contaminated wheat (for Diet 2-5), vitamin premix were mixed with major ingredients for 10 minutes, and soybean oil and fish oil were finally added and mixed for 10 minutes. Mixed diets were blended with approximately 50% of water per kg diet (v/w) for 10 minutes using a 20-litre Hobert type mixer in order to make spaghetti-like pellets using a hot meat grinder (TJ12A Meat Mincer, Guangdong Henglian Food Machinery Co., Ltd., Guangdong, China) in which temperature reached approximately 75-80 ˚C with 2 mm apertures and spaghetti-like pellets were broken by hands. The feed pellets were dried in an oven at 70˚C for two hours and left to cool at room temperature overnight before being packed with black plastic bags and kept in plastic containers to protect moisture, light and insects.

Five-hundred gram of fish dietary sampling of each experimental diet were ground to a fine and were sampled as representatives to be analyzed for chemical compositions (Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand) and the contamination of mycotoxins by LC-MS/MS (Austria) and HPLC (Singapore).

3.4 Mycotoxin analysis

3.4.1 A wheat sample as the mycotoxins source and the five experimental diets were analyzed for DON, ZON, FB_1, fumonisin (FB_2), OTA, aflatoxin B1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) using HPLC at Romers Labs (Biomin Singapore Pte. Ltd., Singapore). HPLC procedure for mycotoxin determination was carried out according to Rodrigues and Nährer (2012) and Binder et al. (2007).

3.4.1.1 Sample extraction: milled samples were well-mixed and then extracted with 100mL of acetonitrile:water (84:16) with 250mL blender jars. The extract was filtered through a folded filter (#595 1/2, Schleicher & Schull, Germany) and 4mL of the filtrate was slowly pressed through a Mycosep®#227 (trichothecenes) or #226 (zearalenone, aflatoxin B_1) column. MycoSep®#226 AflaZon clean-up cartridges, MycoSep®#227 trichothecene clean-up cartridges and MultiSep®#216 columns were used.

Zearalenone extracts were evaporated at 60 ◦C and reconstituted in 200 µL HPLC mobile phase. Aflatoxin B1 extracts (100 µL) were transferred into silanized amber autosampler vials and diluted with 440 µL of deionized water. For determination of DON, 4mL of the Mycosep®#227 purified extract was applied to a preconditioned Multisep®#216 column and eluated with 9mL of acetonitrile/water (90/10) mixture. After evaporation at 60◦C the residue was dissolved in 400 µL mobile phase and centrifuged (10 000 rpm, 5 min). For OTA, 25 g of ground sample was extracted with 100mL of acetonitrile:water (60:40) by blending and filtered as indicated earlier. The sample extract (4 mL) was diluted with 28mL of phosphate buffered saline (PBS, 8.0 g NaCl, 1.44 g Na2HPO4, 0.2 g

KH2PO4, 0.2 g KCl, adjusted to pH 7.4 with HCl or NaOH) and applied to an OchraStar® immuno-affinity column at a flow rate of 2-3 mL/min. After washing with 2×10mL 0.2Mof ammonium acetate solution, OTA was eluted with 3mL of methanol:acetic acid (98:2). The solvent was evaporated at 60 ◦C and the residue was redisolved in 400 µL mobile phase. Extraction of fumonisins was accomplished by adding 100mL of a mixture of acetonitrile:water (50:50) to each 25 g sample and then blending for 3 min. After filtration the pH was adjusted within a range of 6–9 with 0.5N sodium hydroxide, and 3mL of sample extractwas diluted with 8mL of methanol:water (3:1). The entire 11mLwas passed through a preconditioned Multisep®#211 column (Romer® Labs. Inc., MO, USA). After rinsing with 8mL of methanol:water (3:1) fumonisins were eluted with 10mL of methanol:acetic acid (99:1) and the solvent was evaporated at 60 ◦C.

3.4.1.2 Performance Liquid Chromatography (HPLC): HPLC analyses were performed using an HPLC series 1100 from Agilent® technologies (Germany), comprising a micro vacuum degasser, a binary capillary pump, a micro autosampler, column oven as well as an API-ES interface in case of T-2 toxin and fumonisin analysis, a variable wavelength detector for DON, and a fluorescence detector for ZO, derivatized fumonisins, AFB_1 and OTA determination.

Chromatography of ZON was done with a Hypersil ODS column (2.1mm×100 mm, 5 µm; Agilent® technologies, Germany) using an isocratic mobile phase of water and acetonitrile (64:36) at a column temperature of 30◦C. Detector parameters were 235 nm (excitation) and 460 nm (emission). Chromatographic separation of AFB_1 was done by use of an Agilent Zorbax SB-Aq column (4.6mm×150 mm, 5-µm). The mobile phase applied was water:acetonitrile: methanol mixture (5:1:1), including 100 µL nitric acid and 0.3 g potassium bromide per liter. The flow rate was 2 mL/min, column oven temperature 30◦C, injection volume 100 µL. A Kobra cell was used for post-column derivatisation, fluorescence detector settings were 360 nm (excitation), 440 nm (emission). For fluorescence detection of fumonisins 0.5mL of derivatised sample were transferred to an amber autosampler vial and subjected to chromatography using a Hypersil ODS column (2.1mm×200 mm, 5-µm; Agilent® Technologies, Germany) at 40◦C. The mobile phase consisted of acetonitrile:water:methanol:acetic acid (48:46:5:1), at a flow rate of 0.5 mL/min. Fluorescence settings were 420 nm excitation and 500 nm emission wavelength.

In the case of mass spectrometric determination of fumonisins an Agilent® Eclipse XDB-C8 column was used for chromatographic separation, at a temperature of 30 ◦C and a flow rate of 0.6 mL/min. The mobile phase consisted of phase A (385 mg ammonia formate dissolved in 1 L water, pH 3.0) and phase B (acetonitrile) with the following gradient scheme: 0-8 min: increase of 25% phase B to 45%, 8-15 min: further increase to 55% B, decrease within the next 2 min to 25% phase B and hold for 3 min. The detector was an electro spray interface with positive ionisation mode with the following settings: nebulizer pressure 35 psig, drying gas flow 9 mL/min, drying gas temperature 350℃, quadrupole temperature 100℃, capillary voltage 3000V. Ion signals quantified were 722.5 (FB_1) and 706.5 (FB_2).

Chromatographic separation of B-trichothecenes was performed with a Hypersil® ODS column (2mm×125 mm, 5-µm; Agilent® Technologies, Germany) at 45◦C. The gradient mobile phase system consisted of a water:acetonitrile:methanol mix (mobile phase A: 96:2:2) and acetonitrile (mobile phase B) at a flow rate of 0.5 mL/min. The gradient was programmed as follows: start 100% A for 2 min, 98% A + 2% B 2 min, increase of solvent B to 7% within 4 min, held at 7% B for 4 min, increase to 15% B within the next 3 min and hold for another 3 min, decrease B to 0% within 3 min, and wash at 100% A for 5 min, which gave a total run time of 26 min. Injection volume was 30 µL. Detection was accomplished using a variable wavelength detector set at a wavelength of 220 nm.

Analytical determination of OTA was done after chromatographic separation using a HP Hypersil ODS column (2.1mm×100 mm, 3-µm; Agilent® Technologies, Germany) at 40◦C. The method used an isocratic mobile phase of acetonitrile:water:acetic acid (450:525:25), at a flow rate of 0.4 mL/min, and had a run time of 7 min. The fluorescence detector was set at an excitation wavelength of 330 nm, and emission at 460.

3.4.2 The same samples of five experimental diets as 4.1 were analyzed for OTA, DON, DON-3-Glucoside, ZON, zearalenon-4-Sulfat, alpha zearalenol, beta zearalenol, sterigmatocystin, averufin, 3-nitropropionic acid, moniliformin, siccanol, equisetin, apicidin, enniatin B, enniatin B1, enniatin A1, enniatin A, beauvericin, monocerin, aurofusarin, rubrofusarin, culmorin, 15-hydroxyculmorin, tenuazonic acid, alternariol, alternariolmethylether, macrosporin, tentoxin, secalonic acid D, chanoclavin, curvularin, tryptophol, brevinamid F, emodin, chrysophanol, ergometrin Ergometrin, ergosinin, ergosin, ergocornin, ergocorninin, ergotamines, ergocryptin, ergocryptinin, ergocristin, ergocristinin, linamarin, and lotaustralin using Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) at Christian Doppler Laboratory for Mycotoxin Research, IFA-Tulln, University of Natural Resources and Applied Life Sciences (Tulln, Austria).

All samples were analyzed for the presence and concentrations of fungal metabolites by LC-MS/MS according to Vishwanath *et al*. (2009) and Sulyok et al. (2007).

3.4.2.1 Sample extraction: extraction was carried out using a mixture of acetonitrile:water:acetic acid 79:20:1 (v/v/v), with ratios 3 mL solvent/g sample. After extraction, the samples were centrifuged, diluted with a 1:1 (v/v) mixture of extraction and dilution solvent. For injection, 5 µL of the diluted extract was injected into the LC-MS/MS system.

3.4.2.2 Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS): detection and quantification was performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini® C18 column, 150×4.6-mm i.d., 5-µm particle size, equipped with a C18 4×3-mm-i.d. security guard cartridge (Phenomenex, Torrance, CA, US). Both eluents contained 5 mM ammonium acetate and were composed of methanol:water:acetic acid 10:89:1 (v/v/v; eluent A) or 97:2:1 (eluent B), respectively. ESI-MS/MS was performed in the multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. Quantification was performed in the Selected Reaction Monitoring (SRM) mode.

Table 6 Ingredient composition of five isonitrogenous and isoenergetic diets and concentrations of detected *Fusarium* mycotoxins and other fungal metabolites in diets analyzed by LC-MS/MS

Ingredients	**Experimental diets**				
(g/ 100 g diet)	**1**	**2**	**3**	**4**	**5**
Cassava	15.0	15.0	15.0	15.0	15.0
Soybean meal	25.0	25.0	25.0	25.0	25.0
Clean wheat [a]	19.6	18.4	17.1	14.6	9.6
Contaminated wheat [b]	-	1.2	2.5	5.0	10.0
Rice bran	20.0	20.0	20.0	20.0	20.0
Fish meal [c]	15.0	15.0	15.0	15.0	15.0
Vitamin premix [d]	0.4	0.4	0.4	0.4	0.4
Fish oil	2.0	2.0	2.0	2.0	2.0
Soybean oil	3.0	3.0	3.0	3.0	3.0
Total	100	100	100	100	100
Proximate analysis (% dry matter basis)					
Dry matter (%)	94.4	95.6	94.9	95.5	94.6
Crude protein (%)	25.1	25.2	24.3	24.5	24.1
Lipid (%)	6.7	6.7	6.9	6.9	7.0
Ash (%)	8.5	8.5	8.5	8.5	8.6
Gross energy (Kj/g)	17.8	17.9	17.9	17.8	17.8
***Fusarium* mycotoxins ppm** [e]					
Deoxynivalenol (DON)	0.07	0.31	0.50	0.92	1.15
Zearalenol (ZON)	0.01	0.09	0.21	0.37	0.98
***Fusarium* metabolites**					
Aurofusarin	0.01	0.30	0.66	1.28	2.46
Rubrofusarin	0.02	0.05	0.12	0.23	0.49
Culmorin	0.02	0.18	0.26	0.50	1.39
15-Hydroxyculmorin	0.06	0.24	0.37	0.67	1.83
***Alternaria* metabolites**					
Alternariol	0.001	0.01	0.02	0.04	0.12
Alternariol methyl ether	0.01	0.02	0.04	0.06	0.14

[a] Clean wheat flour contains 0.23 ppm DON analyzed by LC-MS/ MS

[b] Wheat naturally contaminated with 41 ppm DON analyzed by HPLC

[c] Contains >60% of protein

[d] Vitamin premix (Premix BA 193C, Biomin) provides per kg diet: vitamin A, 2.000.000 IU; vitamin D3, 480.000 IU; vitamin E, 20 g; vitamin C, 10 g; vitamin K3, 3 g; vitamin B1, 2.5 g; vitamin B2, 3 g; vitamin B6, 3.5 mg; vitamin B12, 0.01 g; niacine, 5 g; panthotenic acid, 5 g; choline 60%; Zn, 70 g; Fe, 14 g; Cu 2.4 g; Mn, 12 g; Co, 0.025 g; I, 1 g; Se 0.12 g; folic acid, 1.7 g; Biotin, 0.05 g; inositol, 10 g.

[e] Detected concentrations of mycotoxins or fungal metabolites analyzed by LC-MS/ MS

3.5 Sample collection

At the beginning of the experiment, 1,000 fish were weighed for the initially total body weight. Eaten diets were weighed to record for feed intake weekly. At the end of 4th and 8th week interval, all fish of each aquarium were weighed. Weight gain was calculated while three fish of each aquarium were anaesthetized by eugenol (Sigma-Aldrich Ptd., Ltd., Singapore) for individual body length measurement and blood was then withdrawn for hematological and biochemical analysis, namely Hct, ALT and AST. Afterward, organs of three fish of each aquarium were removed, such as liver, spleen, kidney and intestine, only livers were weighed for hepatosomatic index calculation (HSI = (liver weight / body weight) x 100), according to Dias et al., 1998), and intestines were measured for total gut length (TGL) to collect three portions of intestines at 20%, 50% and 80% of TGL before being fixed in 10% formalin with other organs for histopathological study. Muscles were collected to be stored at -20˚C until used for proximate analysis.

3.6 Laboratory analysis

3.6.1 Haematocrit test

Three fish of each aquarium were collected blood sample were collected from three fish of each aquarium by 1-ml-heparinized syringe. The needles were inserted under the lateral line behind the anal fin, pushed into muscles until reached the spinal column (back bone) and slowly withdrawn for 1-ml blood. The syringes were rotated softly to remove the needles and then the blood in the syringe was drained into 1 ml-eppendorf tube held on ice. The content was gently mixed by upside down inversion.

The heparinized capillary tubes were touched to the blood samples, allowed to fill three-quarters full and sealed with the plasticine. The capillary tubes were centrifuged for five minutes at 10,000 rpm in the micro HT centrifuge (DSC-100MH-2 Digisystem laboratory instrument Inc., Taiwan) and volume of each capillary tubes was measured by packed cells using the rotoreader (ACAN, Biomin (Thailand) Co., Ltd., Thailand).

3.6.2 Plasma aspartate aminotransferase (AST) and plasma alanine aminotransferase (ALT) activities

The same blood samples, which were used for haematocrit test, were kept in eppendorf tubes to clot under 4 ˚C for three hrs. The blood samples were monitored for the activities of AST and ALT using automated instrumentation (Bangkok R.I.A. LabCo., LTD, Bangkok, Thailand) based on the modified methods of Bergmeyer (1980) and Bergmeyer et al. (1976). The reaction of enzymatic activities are present in Figure 12, L-Aspartate and ∝-Ketoglutarate were catalyzed by AST in transamination to be L-Glutamate and Oxalacetat, which was then reduced to be Malate by Malate dehydrogenase (MDH), meanwhile NADH was reduced to be NAD. The catalytic activity of AST was investigated by measurement of the rate of NADH oxidation in reaction (Bergmeyer et al., 1976). For ALT activity (Figure 13), L-Alanine and ∝-Ketoglutarate were catalyzed by ALT to be L-Glutamate and Pyruvate, which was reduced to be Lactate by Lactate dehydrogenase (LDH); simultaneously, NADH was reduced to be NAD. The catalytic activity of ALT was determined by measurement of the rate of NADH oxidation in reaction (Bergmeyer, 1980).

$$\text{L-Aspartate} + \propto\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{L-Glutamate} + \text{Oxalacetate}$$

$$\text{Oxalacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}$$

Figure 12 The catalytic activity of AST (Adapted from Bergmeyer et al., 1976)

L-Alanine + ∝-Ketoglutarate ——— ALT ———> L-Glutamate + Pyruvate
Pyruvate + NADH ——— LDH ———> Lactate +NAD

Figure 13 The catalytic activity of ALT (adapted from Bergmeyer, 1980)

3.6.3 Proximate analysis of experimental diets and flesh of red tilapia

Experimental diets and flesh samples were analyzed for crude protein (CP), ether extract (EE, lipid), ash, moisture, dry matter (DM) and gross energy (GE) according to (AOAC, 1995) in Department of Animal Science, Faculty of Agriculture (Khon Kaen University, Thailand). Tecator Kjeltec digestion and distillation units were used for CP analysis and the percentage of total nitrogen was determined based on a dry matter basis (%N × 6.25). Lipid was extracted from samples using petroleum ether by Soxhlet apparatus and GE was analyzed using automated bomb calorimeter (AC500 Isoperibol bomb calorimeter, LECO Corporation, MI, USA).

3.6.4 Histopathological examination

At the end of 8-week experiment, three fish per aquarium were sectioned and organs include liver, kidney, spleen and intestines were removed and fixed for further histopathological analysis by modifying the method of Clark et al. (1973).

3.6.4.1 Fixation and Processing

The liver specimens were fixed in 10% buffer formalin for 24-48 hrs and then transferred to 10% ethanol. Prior to the processing, the tissue samples were observed and noted for lesion on tissues in the step of gross examination before cutting and trimming tissues (2×2 mm). Tissue processing was conducted by Shandon citadel 2000 tissue processor (International medical equipment Inc., CA, USA) (Department of Pathology, Faculty of Veterinary, Khon Kaen University) for 24 hrs. The 12 steps of processing are described in Table 7. In brief, tissues were dehydrated through 70%, 80%, 95% and 100% alcohol and acetone, respectively, and cleared in xylene; finally, placed with paraffin box at 60°C. Processed tissues were embedded in 55-65°C paraffin using Shandon embedding center (International medical equipment Inc., CA, USA).

Table 7 Twelve steps of the procedure of tissue processing in Shandon citadel 2000 tissue processor

Steps	Solutions	Duration (hour)
1	70% Ethyl alcohol	1
2	80% Ethyl alcohol	1
3	95% Ethyl alcohol	1.5
4	95% Ethyl alcohol	1.5
5	100% Ethyl alcohol	1.5
6	100% Ethyl alcohol	1.5
7	Acetone	2
8	Acetone	2
9	Xylene	2
10	Xylene	2
11	Paraffin (55-65 ˚C)	3
12	Paraffin (55-65 ˚C)	3

(Clark and Conn, 1973)

3.6.4.2 Histopathological examination

Paraffin sections were cut into a 4 µm thick with Leitz 1512 Rotary microtome and placed into the warm gelatin pot (1 teaspoon of gelatin powder/1 L of distill water) prior to placing on slide for haematoxylin and eosin staining (H&E staining method) .

The paraffinized tissues were stained with haematoxylin and eosin (H&E staining). H&E staining was conducted according to a diagram in Figure 14. Prior to H&E staining, the paraffinized tissues were incubated at 60°C for 30 minutes and deparaffinized in xylene for three minutes (two times) to remove the paraffin wax from the tissue and surrounding area on the slides. After deparaffinization, the tissues were hydrated to prepare tissue to be stained with a dye. Hydration was processed by passing through 100% (absolute) alcohol for two minutes, 95% alcohol for two minutes (two times), rinsed the tissues in the tap water. After dehydration, the tissues were stained with Harris's haematoxylin for 10 minutes and washed in running tap water for two minutes. The tissues were rapidly dipped in acid alcohol (1% hydrochloric acid in 70% ethyl alcohol) for three times to destained over haematoxylin in the tissues, washed in the tap water and then dipped in saturated lithium carbonate (1 g lithumcarbonate in 1 ml of distill water) for three to four seconds and rinsed in the running tap water. The tissues were stained with eosin solution for two to three minutes. The tissues were dehydrated through 95% alcohol for three minutes and absolute alcohol for three minutes (three times), and then cleared in xylene solution for three minutes (two times) to clear up the stained tissues, and mounted with mounting media. The stained tissues were monitored under the microscope and captured pictures by camera for histopathological study.

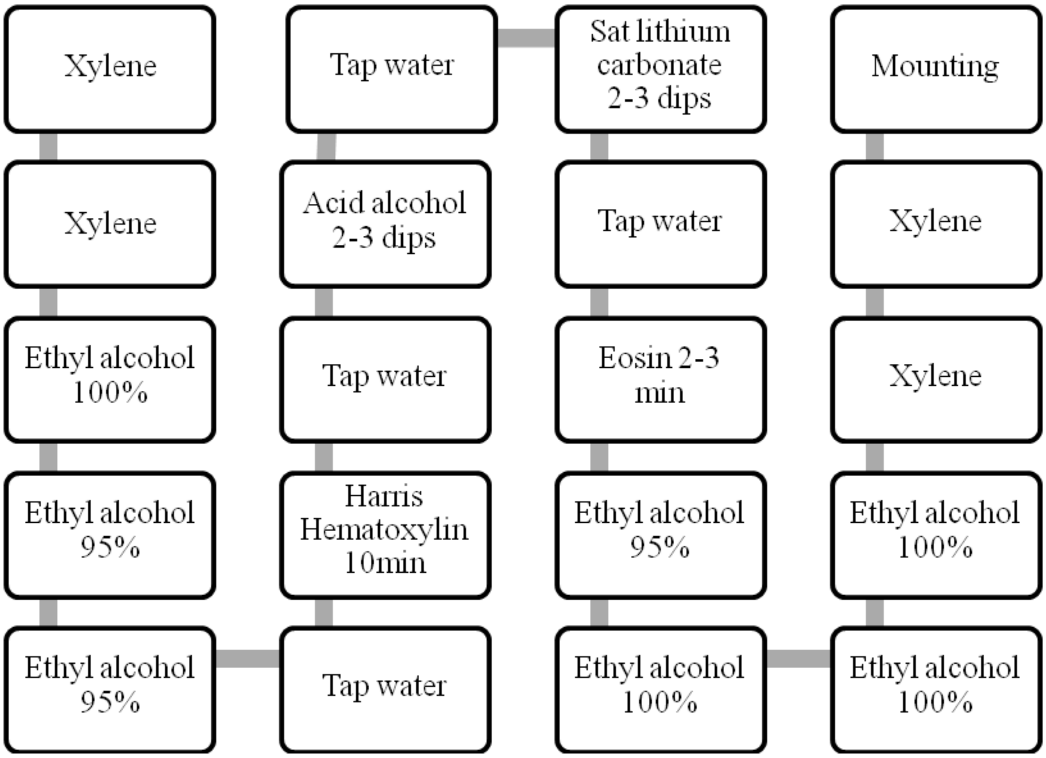

Figure 14 Chemicals and procedure used for staining the tissue specimens until Mounting (Modified from Clark and Conn, 1973)

3.7 Calculations and statistical analysis

Thermal-unit growth coefficient (TGC) was expressed as growth rate and calculated for each aquarium as: [100 × (FBW$_{1/3}$ – IBW$_{1/3}$) / Σ(Temp (°C) × number of days)], where: FBW = final body weight (g/fish); IBW = initial body weight (g/fish). Feed conversion efficiency (FCE) was calculated as live body weight gain/dry feed intake (FI), where: FI = total dry feed/ number of fish; live body weight gain = (FBW/final number of fish) – (IBW/initial number of fish). Liver weight of individual fish was referred as HSI and calculated as HSI = (liver weight (g)/FBW × 100). Mortality rate (%) was calculated as percentage of mortality (%) = (initial number of fish - final number of fish × 100) / initial fish number).

All parameters, such as WG, TGC, FI, FCE, mortality rate, HSI, Hct, AST and ALT are presented as mean of each treatment and standard error of mean (SEM). The GLM procedure of SAS for window (SAS version 9.2, SAS Institute Inc., Cary, NC, USA) was used to test for linear and quadratic orthogonal contrasts. The statement of statistical significance was declared at $P < 0.05$.