1. MATERIALS AND METHODS

The materials and methods adopted in the present investigation titled "Feed formulation and its impact on growth and immune response of *Oreochromis mossambicus* (Peters, 1852) is presented under following headings:

Phase I

Phytochemical analysis and characterization of bioactive compounds in the leaf extracts of *Coleus aromaticus* and *Ocimum basilicum*

- Selection, collection and identification of plant sample
- Preparation of leaf extracts
- Phytochemical analysis in the leaf extracts
- Characterization of bioactive compounds in the leaf extracts

Phase II

Growth characteristics, feed utilization efficiencies and proximate composition of *O.mossambicus* fed with the leaf extracts of *C.aromaticus* and *O.basilicum* as supplemented feed

- Selection, collection and acclimatization of experimental animal
- Preparation of fish feed
- Determination of growth characteristics in *O.mossambicus*
- Evaluation of feed utilization efficiencies in *O.mossambicus*
- Estimation of proximate composition in *O.mossambicus*

Phase III

Haematological and biochemical parameters of *O.mossambicus* fed with the leaf extracts of *C.aromaticus* and *O.basilicum* as supplemented feed

- Selection of experimental animal
- Determination of haematological parameters in *O.mossambicus*
- Estimation of biochemical parameters in *O.mossambicus*

Phase IV

Physiological and immunological parameters of *O.mossambicus* fed with leaf extracts of *C.aromaticus* and *O.basilicum* as supplemented feed

- Maintenance of *A.hydrophila*
- Antibacterial activity of leaf extracts against *A.hydrophila*
- Determination of physiological and immunological parameters in *O.mossambicus* challenged with *A.hydrophila.*

PHASE I

Phyto-chemical analysis and characterization of bioactive compounds in the leaf extracts of *C.aromaticus* and *O.basilicum*

Selection, collection and identification of plant sample

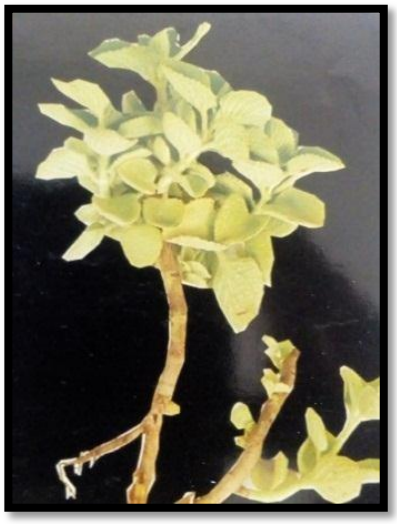
The plant samples chosen for the present study were *Coleus aromaticus* and *Ocimum basilicum* (Plate 1 & 2). The plant samples were collected from Sundarapuram (Latitude- 10.96420 N and Longitude- 76.97010 E), Coimbatore district, Tamilnadu, India (Plate-1). The plant samples were identified and validated by botanist in the Botanical Survey of India, Southern Regional centre, Coimbatore, Tamil Nadu, India. The herbarium registered wide variety was once BSI/SRC/5/23/2018/Tech/2465-2466.

Coleus aromaticus* / *Plectranthus ambonicus

Coleus aromaticus or *Plectranthus ambonicus* or Indian borage is a culinary herb belongs to the family lamiaceae. Most culinary with a pungent oregano like taste and odor. Also cultivated as ornamental plant in gardens and in indoor pots (Plate 1).

Plate 1

Plant sample selected for the present study - *Coleus aromaticus*



Systematic position

Kingdom : Plantae

Order : Lamiales

Family : Lamiaceae

Genus : *Coleus/ Plectranthus*

Species : *aromaticus/ ambonicus*

Binomial name: *C.aromaticus/P.ambonicus*

C.aromaticus is a aromatic perennial herb, grows up to 1 meter height, has lengthy fleshy stem with rigid hairs, leaves are simple, fleshy and ovate with glandular hairs giving frosted appearance, flowers are pale purplish, spike like raceme. It is distributed throughout India.

C.aromaticus is folkloric medicinal herb. It is used to treat various diseases such as malaria, disorders in alimentary canal, renal and vesicle calculi, respiratory disorders including asthma and bronchitis, convulsions in colon and neurological disorder (Chopra *et al.,* 1956; Kirtikar and Basu, 1975 and Nadkarni 1996). The leaf extract is used for skin treatment such as pores, thickening, itching and irritation.

Ocimum basilicum

Ocimum basilicum is a culinary herb. It is also cultivated as ornamental plant . Sweet basil is native to Indo-Malayan region. It is the king of herbs and has bioactive compounds with large dietary as well as antioxidant competencies (Jayasinghe *et al.,* 2003) (Plate 2).

Plate 2

Plant sample selected for the present study - *Ocimum basilicum*

Systematic position

Kingdom : Plantae

Order : Lamiales

Family : Lamiaceae

Genus : *Ocimum*

Species : *basilicum*

Binomial name: *O. basilicum*

It has shown unique health defending property due to the presence of flavonoids and volatile oils. The flavonoids found in basil offers protection at cellular level (Nyak and Uma, 2005). Essential oil found in *Ocimum basilicum* leaves have the potentiality to inhibit a number of resistant species of pathogenic bacteria (Opalachenoiva and Obreshkova, 2003).

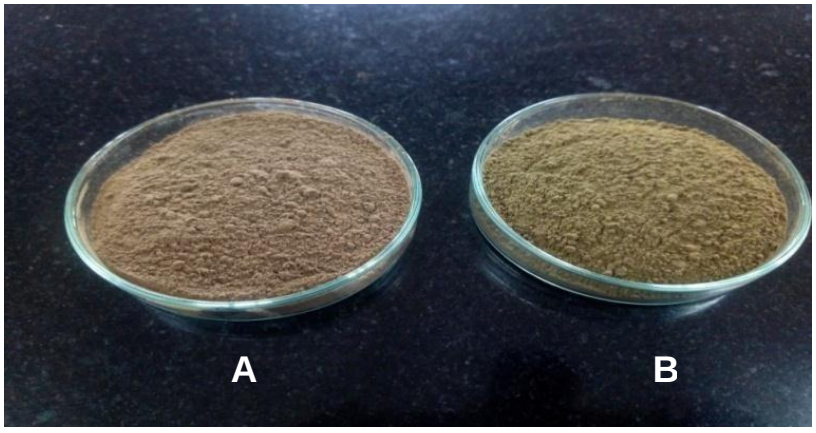
Ocimum basilicum possess stimulant, carminative, antispasmodic, diuretic, demulcent effects. The leaves and its oil have various biological properties such as insecticidal, nematicidal, anti-fungal, anti-bacterial and anti- inflammation (Takano, 1993).

Processing and preparation of leaf powder

Fresh leaves of *C.aromaticus* and *O.basilicum* were collected, washed and dried. The dried leaves were finely powdered and stored for further use (Plate 3).

Plate 3

Pulverized leaf powders



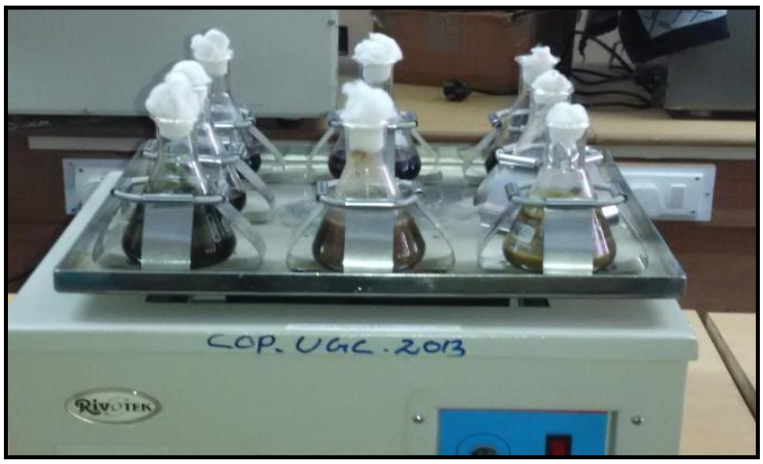
a) C.aromaticus* and b) *O.basilicum

Aqueous extraction

Ten grams of leaf powder (*C.aromaticus* and *O.basilicum*) were taken separately and soaked in 50ml water in a conical flask stoppered with sterilized cotton and left undisturbed for 48 hours in shaker‟s incubator. The extract was filtered, evaporated at 100°C and the extract was stored in refrigerator for further use (Farombi *et al.,* 2003) (Plate 4).

Plate 4

Process of aqueous extraction of selected leaf extracts of *C.aromaticus* and *O.basilicum*



Qualitative analysis of phytochemical constituents

Phytochemical analysis of the aqueous extracts of *C.aromaticus* and *O.basilicum* leaves were carried out to identify the presence of phytochemicals. Qualitative tests for flavonoids, phenols, tannins, terpenoids, steroids, saponins, alkaloids and glycosides were carried out by using standard reference techniques (Trease and Evans 1989; Tiwari *et al.,* 2011) (Appendix 1).

Characterization of bioactive compounds in the leaf extracts of *C.aromaticus* and *O.basilicum*

UV-visible absorption spectrum

UV visible spectroscopy was carried out for qualitative evaluation and identification of certain classes of compounds such as aromatic and aliphatic molecules. *C.aromaticus* and *O.basilicum* two leaf extracts were analyzed by double beam UV spectrophotometer (Shimadzu UV – 2102 PC). The instrument was once set to scan mode and the absorption spectrum was bought in the range of 200nm to 800nm.

FT-IR spectral analysis

FT-IR spectrum of *C.aromaticus* and *O.basilicum* leaf extracts was done by using KBr disk technique. The samples of *C.aromaticus* and *O.basilicum* were scanned by using infrared radiations with the range of 3500–500 cm^{-1} using FTIR spectrophotometer (Shimadzu, IR Affinity 1, Japan). The bands were obtained in contrast with the reference chart.

Phase II

Growth characteristics, feed utilization efficiencies and proximate composition of *O.mossambicus* fed with leaf extracts of *C.aromaticus* and *O.basilicum* as supplemented feed

The present phase of the study deals with the supplementation of *C.aromaticus* and *O.basilicum* leaf extracts at various concentrations viz., 2.5gram/ kilogram feed, 5gram/ kilogram feed and 10gram/kilogram feed for forty five days of experiment on growth, survival rate, feed utilization efficiencies and proximate composition of *O.mossambicus*.

Selection of Experimental fish: *Oreochromis mossambicus* (Peters, 1852)

The fish selected for the phase I of the study was fresh water edible fish, *Oreochromis mossambicus* (Peters, 1852) (Plate 5).

Plate 5

Experimental fish selected for the present study
Oreochromis mossambicus
(Peters, 1852)

Biology of *Oreochromis mossambicus* (Peters, 1852)

Oreochromis mossambicus is a freshwater teleost fish. In Greek, “oreos” means “of the mountains” chrom skill “color” (Boschung and Mayden, 2004).

It is positioned taxonomically as follows:

Phylum	: Chordata
Sub phylum	: Vertebrata
Division	: Gnathostomata
Super class	: Pisces
Class	: Osteichthyes
Order	: Perciformes
Family	: Cichilidae
Genus	: *Oreochromis*
Species	: *mossambicus*

Habitat

O. mossambicus has a vast salinity tolerance (Trewevas, 1983) and it can survive at excessive range of salinity up to 40 ppm (Knaggs, 1977; Dial and Wainright, 1983). It can additionally endure high water temperature, low dissolved oxygen and excessive ammonia concentration. It breeds in all sorts of waters such as saline, brackish, fresh or foul and also in waters at an altitude of 7000 feet above sea level (Sterba, 1962).

Sexual Maturity and Dimorphism

Adult fish live around six to eight years, however some of them with sturdiness of eleven to twelve years have been pronounced (Boschung and Mayden, 2004; Fryer and Illes, 1972). Male is large and darkish in nature it has longer anal, dorsal fins and greater sturdy jaws (Oloveira and Almada ,1995). The genital opening of male is circular in shape at the quit of the papilla, whereas it is in the form of transverse slit in the female (Pandian and Muthukrishnan, 1988). During the breeding season, the male develops a reddish color along the fringes of the dorsal, caudal and anal fins. The form of the dorsal and anal fin in adult female is spherical and in adult males they are pointed (Oliviera and Almada, 1995).

Reproduction and Fertilization

Males forms thin nests in the bottom of vegetation where fertilization of the eggs occurs (Bruton and Bolt, 1975). Many females lay eggs in the nest. Single female lay

around 50-1780 eggs, primarily based on individual‟s dimension and environmental circumstance (Trewevas, 1983). After fertilization, the female take the fertilized eggs into her buccal cavity (hence they are called African mouth breeders) and brood them till hatching process. Hatching occurs in about three to five days. Once hatched, the female continue to mouth-brood the fry until they are approximately fourteen to twenty one days old.

Feeding habit

O. mossambicus is classified as opportunistic omnivore by Boschung and Mayden (2004). This species is herbivorous in nature, consuming algae, diatoms, aquatic flowers and small fish (Moyle 1976; Chacko and Krishnamoorthi, 1954; Boschung and Mayden 2004). Juveniles are carnivorous; adults have a tendency to be herbivorous (Panikkar and Thambi, 1954).

Collection and maintenance of experimental fish

O.mossambicus weighing 7± 1gm two (advanced fingerlings) for growth study) and fishes weighing 20± 5gm (for haematology and immunology studies) were procured from Tamil Nadu Fisheries Development Corporation Limited, Aliyar fish farm, Pollachi, Coimbatore district, Tamil Nadu, India. The fishes were carefully carried from farm to the laboratory of our college in covers with water and well aerated oxygen. They had been acclimatized to laboratory prerequisites in cement tanks for about two weeks in non-chlorinated water. The fishes have been fed with prepared control feed once in a day. During the acclimatization period the water used to be changed (1/3 of water) every day to avoid accumulation of fecal material and to prevent microbial contamination.

Preparation of feed

Balanced feed was prepared with approximately 36% protein content to serve as feed by using rice bran, coconut oil cake and soyabean meal (Table 1 & Plate 6). The feed was prepared by using following the Pearson square method (New, 1989). Experimental feeds have been prepared by using supplementing the balanced feed with *C.aromaticus /P.ambonicus and O.basilicum* leaf extract at various concentrations (2.5, 5 and 10 gm/ kg of feed) on dryweightbasis.

Table 1

Composition of balanced feed supplemented with leaf extracts (g/kg)

Feed ingredients	Control	T1	T2	T3	T4	T5	T6
Rice bran	300	300	300	300	300	300	300
Coconut oil cake	300	300	300	300	300	300	300
Soyabean meal	400	400	400	400	400	400	400
Coleus romaticus	-	2.5	5	10	-	-	-
Ocimum basilicum	-	-	-	-	2.5	5	10

The ingredients were dried individually and ground in an electric grinder, sieved through a 200 µ mesh and mixed thoroughly with desired quantity of water to make dough. The dough was cooked at 2.7 kg pressure cm^2 for 15minutes. To the steam cooked feed leaf extracts at different concentrations (2.5, 5 and 10 gm/kg of feed) were added separately before pelletization and extruded through a hand pelletizer with perforated disc (3 mm diameter). The wet pellets were sun dried for two days. The dried pellets were broken into small pieces and were subjected to oven drying at 70°C for about 10 hours. Finally all the feeds were separately packed in polythene bags and stored at -18ºC for further use.

C Control feed

T1 Control feed+ 2.5 gm of *C.aromaticus/P.ambonicus*

T2 Control feed+ 5 gm of *C.aromaticus/P.ambonicus*

T3 Control feed+10 gm of *C.aromaticus/P.ambonicus*

T4 Control feed+2.5 gm of *O.basilicum*

T5 Control feed+5 gm of *O.basilicum*

T6 Control feed+ 10 gm of *O.basilicum*

Plate 6

Formulated feed prepared from selected leaf extracts

Estimation of proximate composition of feed

The proximate composition such as moisture, protein, carbohydrate, fat and ash content in the control feed and in the different experimental feeds supplemented with different concentrations of *C.aromaticus* and *O.basilicum* leaf extracts were analyzed by following standard procedures (Appendices 3– 5).

Crude protein content was estimated by Lowry *et al.*, method (1951) (Appendix 3), carbohydrate by Hedge and Hofreiter (1962) (Appendix 4), total fat content was determined by Folch *et al* method (1957) (Appendix 5) .

Table 2

Proximate composition of control and leaf extracts supplemented feed

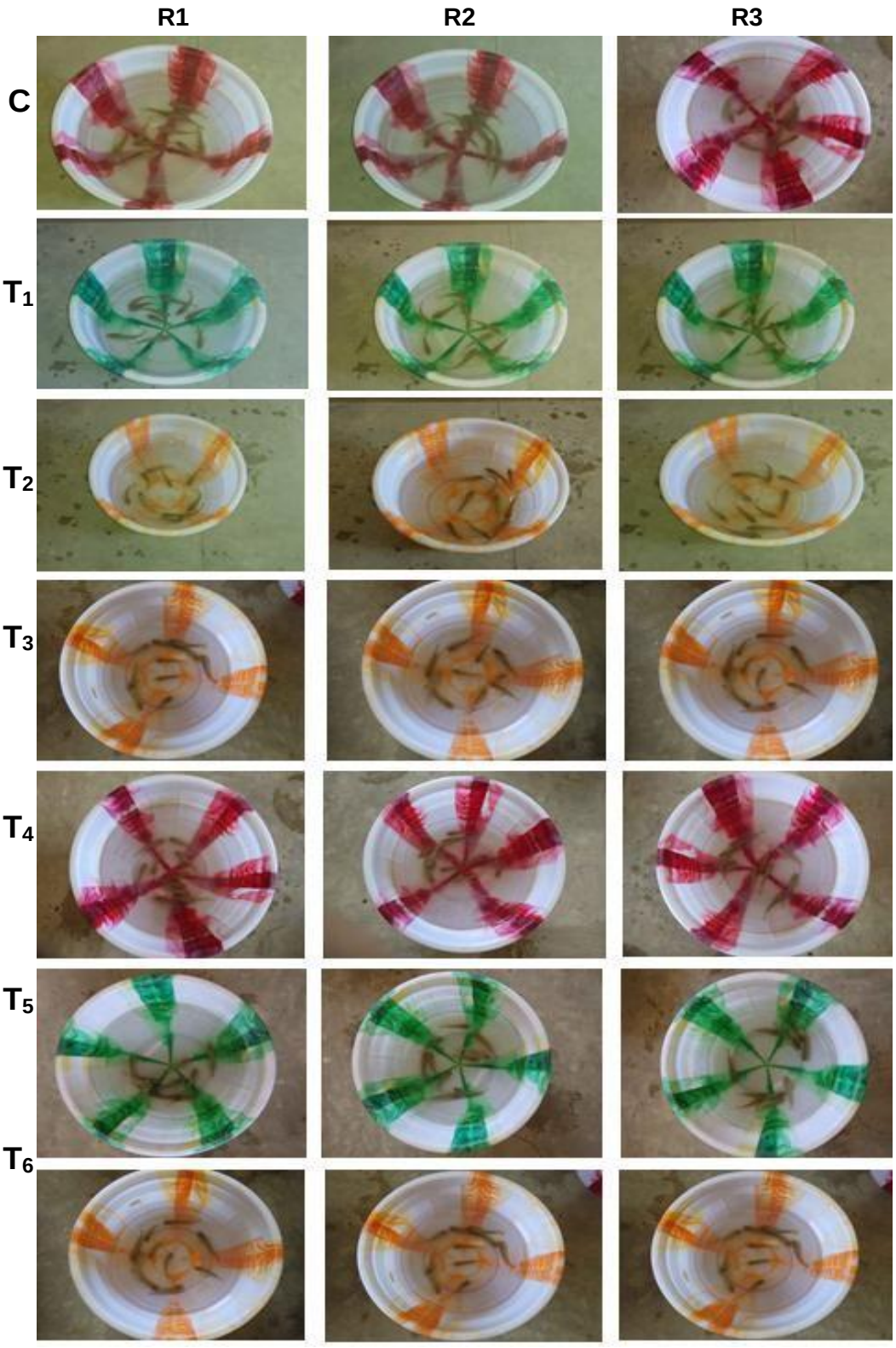
Feed	Treatments	Concentration (g/kg)	Protein	Carbohydrate	Fat
Control	C	….	36.12	12.79	14.07
C.aromaticus **supplemented feed**	T1	2.5	36.15	13.05	14.15
	T2	5.0	36.36	13.25	14.20
	T3	10.0	36.52	13.29	14.23
O.basilicum **supplemented feed**	T4	2.5	36.23	13.15	14.14
	T5	5.0	36.39	13.18	14.17
	T6	10.0	36.55	13.20	14.19

Experimental design

The fresh water edible fish, *O.mossambicus* (7 ± 1 g) were introduced in 50 L aerated plastic troughs. The fishes were divided into seven experimental groups (C – control and T1 to T6 – six different treatments) of 10 fishes in triplicate (7 x 10 x 3 = 210 fish). This experiment was carried out for a period of 45 days. During experimental period the fishes were fed with their respective feed at 5% of their body weight, once in a day. The unutilized feed and fecal matter were collected every day before feeding and kept for further analysis. The medium (water) was changed daily in order to prevent microbial contamination by replacing one-third of the water in the tanks daily throughout the experimental period after siphoning out the unutilized feed and fecal matter. The plastic troughs were washed and cleaned once in fifteen days when the fishes were removed from troughs for weighing. The survival rate was monitored during experimental period (Plate 7).

Plate 7

Experimental Setup for the determination of growth, feed utilization and proximate composition of *O. mossambicus* supplemented with selected leaf extracts



Determination of growth parameters

The weight of *O.mossambicus* in the control and six different treatments were measured individually and values were noted .

The fishes were weighed by using digital electronic balance with mg sensitivity. The fingerlings were released into water after body measurements (Hasan and Khan, 2013).The weight of individual fish were recorded initially and then at 15 days interval for 45 days. BW,FW,WG, GR, DWG,SGR and RGR were calculated in the control and treated fishes by using standard formulae (Petursewiez and Macfutyen, 1970).

- Final weight (FW)(gm)
- Weight gain (WG) (gm) = FW(gm) – IW (gm)
- Growth rate (GR)(gm.day^{-1})= FW(gm) – IW (gm)/No. of days* IW
- Average daily growth rate (gm.day^{-1}) = FW(gm) – IW (gm)/ No. of days
- Relative growth rate (RGR) (%)= 100 x [FW (gm) – IW (gm)] / IW (gm)
- Specific growth rate (SGR)(% day^{-1}) = 100 x [In FW (gm) – In IW (gm)] /No. of days
- Survival rate (%) = 100 x Initial number of fishes – final number of fishes

Where,
IW – Initial weight of fishes
FW – Final weight of fishes

Determination of feed utilization efficiencies

The fishes were fed with their control and experimental feeds throughout for 45 days. Fecal matter and unutilized feed in the experimental troughs were removed daily with least disturbance.

The unutilized feed and fecal matter were collected and dried in hot air oven at 60°C and dry weight was recorded.

Feed intake (FI) (gm) = feed given (gm) – unutilized feed(gm)

Termination of the experiment

The feed utilization experiments were carried out for 45 days. The feed utilization efficiencies were calculated (Petursewiez and Macfutyen, 1970) every 15 days once based on the data recorded every day for feed given, unutilized feed and fecal matter collected.

The feed utilization parameters such as FER, FCR, PI,PER, LI and LER were calculated in the control and treated fishes by using standard formulae (Petursewiez and Macfutyen, 1970).

Feed conversion ratio (FCR) (gm) = FI (gm) / TWG (gm)

Feed efficiency ratio (FEC)(gm) = WG (gm) / FI (gm)

Protein intake (PI) (gm %) = FI (gm) x % of crude protein concentration in feed /100

Protein efficiency ratio (PER) (gm) = WG (gm) / PI (gm)

Lipid intake (LI) (gm%) = FI (gm) x % of lipid concentration in feed / 100

Lipid efficiency ratio (LER)(gm) = WG (gm) / LI (gm)

Estimation of proximate composition

The proximate composition of *O.mossambicus* was estimated initially before the start of experiment and after 45 days of experimental period. The muscle samples from control and six different treatments were used to analyze the proximate composition.

Five fishes from each group were taken and were anesthetized using chloroform. Fishes were starved for 24 hours prior to sampling. The sample fishes were sacrificed and the muscle tissues were taken out separately from the body of fish carefully. The separated muscle tissues were used for sample preparation. The samples were immediately processed for analyzing the proximate composition.

Moisture content was estimated by drying samples in hot air oven (AOAC,1999)(Appendix 2),crude protein content was estimated by Lowry *et al*., method (1951) (Appendix 3),carbohydrate by Hedge and Hofreiter (1962) (Appendix 4),total lipid content was determined by Folch *et al* method (1957) (Appendix 5) and ash content by AOAC (1999) (Appendix 6).

Phase III

Hematological and biochemical parameters of *O.mossambicus* fed with *C.aromaticus* and *O.basilicum* leaf extracts as supplemented feed

The third phase of study was planned to determine the hematological and biochemical parameters of *O.mossambicus* supplemented with *C.aromaticus* and *O.basilicum* leaf extracts supplemented feed at different concentrations viz., 2.5gram/kilogramg feed, 5gram/kilogram feed and 10gram/kilogram feed for a period of 45 days.

Selection and acclimatization of experimental animal

The fish selected for third phase of the study was fresh water edible fish, *Oreochromis mossambicus* weighing 20±5 gm . After two weeks of acclimatization in the laboratory condition the fishes were used for the experiment. Feeding was with held for 24 hours before the commencement of the experiment.

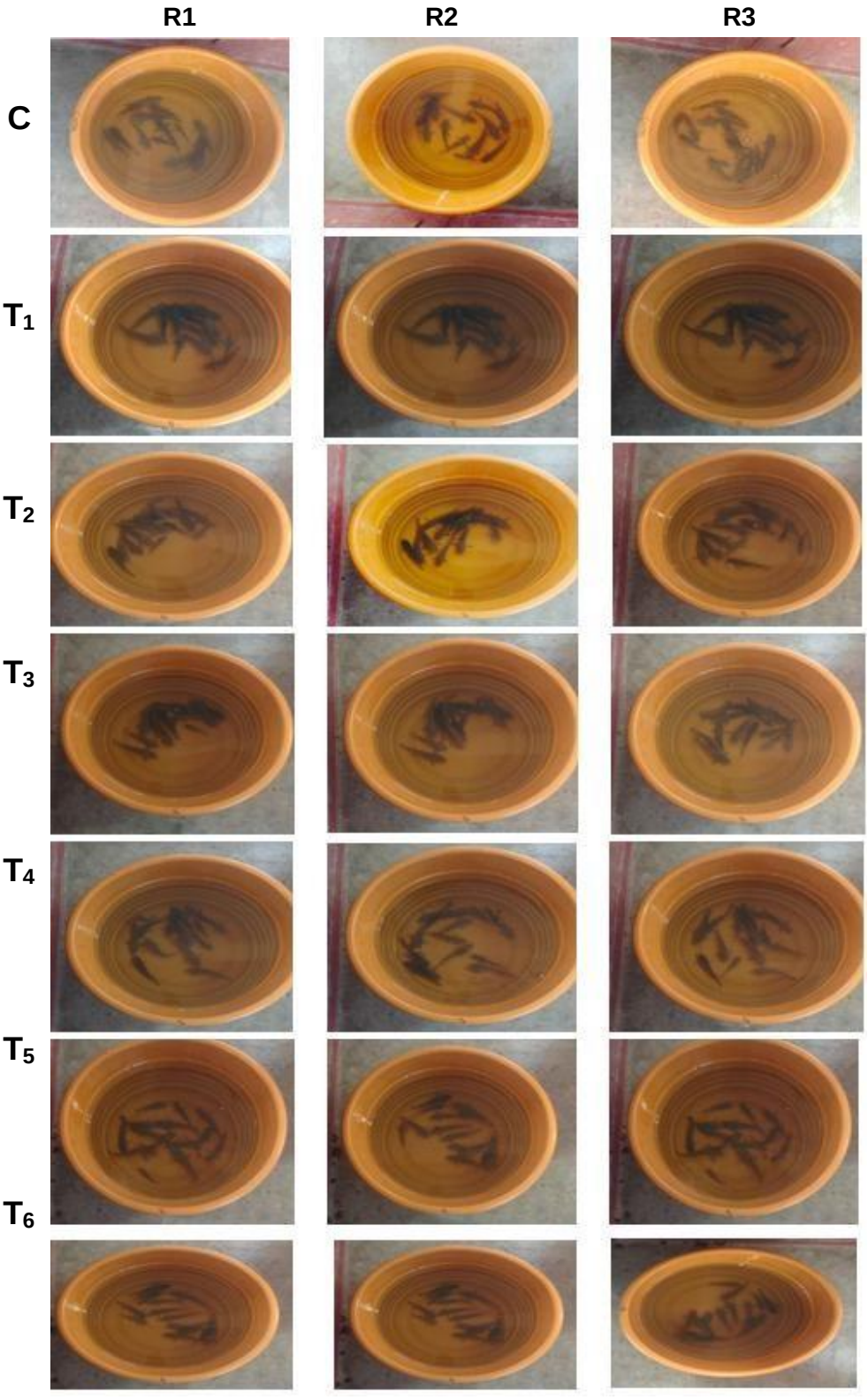
Experimental design

The fresh water edible fish, *O.mossambicus* (20 ± 5 g) were introduced in 50 L aerated plastic troughs. The fishes were grouped into seven experimental groups - control (C) and six different treatments (T1 to T6) of 15 each in triplicate (7 x 15 x 3 = 315 fish) (Plate 8). This experiment was carried out for 45 days. During experiment, the fishes were fed daily once with their respective feed at 5% of their BW. The unutilized feed and fecal matter were collected every day before feeding and kept for further analysis. The medium (water) was changed daily in order to prevent microbial contamination by replacing one-third of the water in the tanks daily throughout the experimental period after siphoning out the unutilized feed and fecal matter. The plastic troughs were washed and cleaned once in fifteen days.

This experimental set up was kept in two sets. First set was kept for hematological studies in phase III and second set for physiological and immunological studies in phase IV.

Plate 8

Experimental Setup for the determination of haemotological and biochemical parameters of *O. mossambicus* supplemented with selected leaf extracts



Collection of blood sample

The samples of blood from control and treatments were collected by a cardiac puncture method using disposable syringes and transferred into plastic vials. The blood was collected in EDTA rinsed small serological tubes and used for determination of hematological parameters. The blood (without anticoagulant) was collected and transferred to a centrifuge tube to form clot. After loosening the clot, the blood was centrifuged at 10,000 rpm for 10 minutes. The serum was collected and used for the estimation of protein, glucose and cholesterol.

Determination of hematological parameters

The hematological parameters such as erythrocyte count, leucocyte count, hemoglobin content, PCV, MCH and MCHC were analyzed in the blood of *O.mossambicus* initially and after 45 days of treatment with *C.aromaticus* and *O.basilicum* supplemented feed at different concentrations viz., 2.5gram/kilogram feed, 5gram/kilogram feed and 10gram/kilogram feed by following methods (Appendices 7 - 10). Erythrocyte and leucocyte count were determined by using hemocytometer (Davidson and Henry ,1969). Hemoglobin content (Hb) was determined by Acid – haematin method (Sahli, 1962). PCV was estimated by micro hematocrit method (Nelson and Morris, 1989). MCH and MCHC were calculated using to standard formula.

MCH (pg) = Haemoglobin concentration (gm /dL)/ RBC count in million*100

MCHC (g/dL) = Haemoglobin concentration (gm /dL)/Haematocrit (%)*100

Estimation of biochemical parameters

The biochemical parameters such as serum protein, glucose and cholesterol were analyzed in the blood of *O.mossambicus* initially and after 45 days of treatment with *C.aromaticus* and *O.basilicum* supplemented feed at different concentrations viz., 2.5gram/kilogram feed, 5gram/kilogram feed and 10gram/kilogram feed. The protein content of serum was determined by Lowry *et al* method (1951) (Appendix 11), serum glucose was determined by O- Toluidine method developed by Cooper and McDaniel *et al* (1970) (Appendix 12) and serum cholesterol was estimated by Zak *et al* (1954) (Appendix 13).

Phase IV

Physiological and immunological parameters of *O.mossambicus* fed with *C.aromaticus* and *O.basilicum* leaf extracts as supplemented feed

In phase IV, the experiment was conducted to study the antibacterial activity of leaf extracts against *A.hydrophila* and to determine the physiological and immunological parameters of O.mossambicus *fed* with *C.aromaticus* and *O.basilicum* feed at different concentrations viz., 2.5gram/kilogram feed, 5gram/kilogram feed and 10gram/kilogram feed for 30 days and after challenge with *A.hydrophila* on 31st and 38th day.

Fish pathogen - *Aeromonas hydrophila*

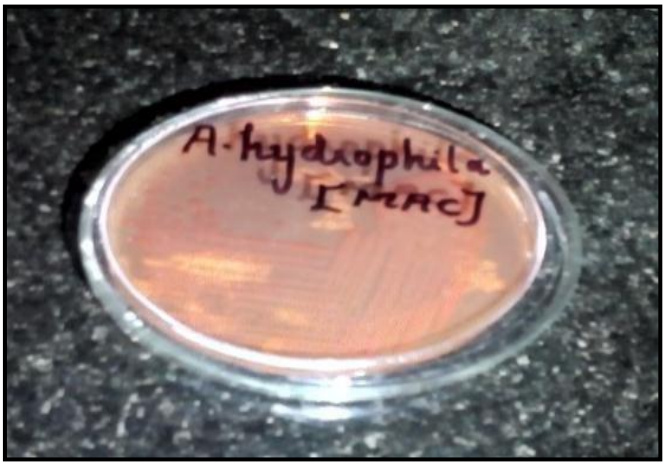
A.hydrophila is a rod shaped bacteria without spores, motile in nature bearing single polar flagella , gram negative bacteria comes under the family *Vibrionaceae.* This bacterium causes hemorrhagic septicemia in warm water fishes like channel cat fish, tilapia.Symptoms include hemorrhagic septicemia, abdominal dropsy, exophthalmia and fin rot (Austin and Austin, 1989).

Collection and maintenance of *A.hydrophila* culture

Pure culture of *A.hydrophila* was procured from the Department of Microbiology, PSG Institute of Medical Sciences and Research, Coimbatore district, Tamilnadu, India. The culture was maintained on Muller –Hinton agar media (Hi –media) at 4°C for further experiments (Appendix -14) (Plate 9).

Plate 9

Maintenance *Aeromonas hydrophila* culture in Muller Hinton agar medium



Culture media

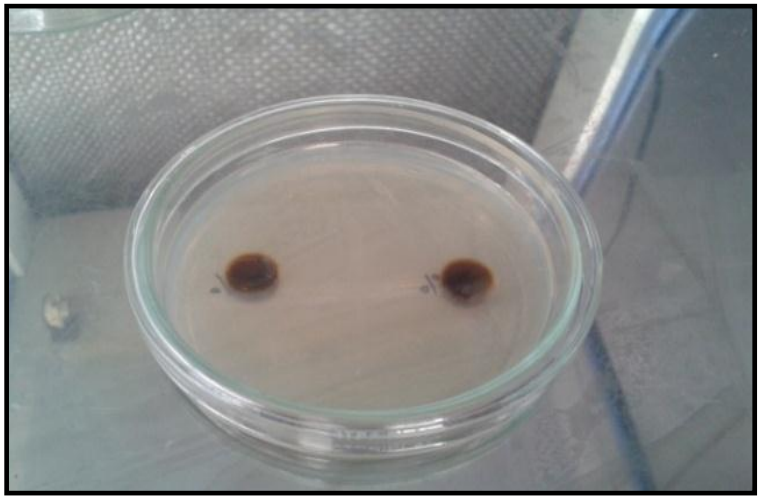
The culture media used for this present study was nutrient broth and nutrient agar media (Hi- media) (Appendices 15 - 16).

***Invitro* antibacterial activity - Well diffusion assay**

The antibacterial activity was assessed by well diffusion method (Plate 10). The petriplates with nutrient agar medium was inoculated using sterile cotton swabs with bacterial suspension. Wells were punctured in nutrient agar plates by using sterile cork borer. 20µl of each extract was added and incubated at 37°C in an incubator for 24 hrs and the diameter of inhibition was measured in mm. Positive control (chloramphenicol) and negative control (distilled water) wells were maintained simultaneously.

Plate 10

Antibacterial activity of selected leaf extracts against *A.hydrophila* by Well diffusion assay



Characterization of molecular structure by scanning electron microscopy (SEM)

Characterization of molecular structure and interaction of leaf extracts with *A.hydrophila* were determined by using SEM. Untreated (*A.hydrophila* culture) and treated samples (*A.hydrophila* culture treated with leaf extracts of *C.aromaticus* and *O.basilicum*) were mounted on to stubs, coated with gold palladium of 100-150 A° thickness and the samples were transferred to sample chamber of SEM and operated at 10kV.

Selection and acclimatization of experimental animal

The fish selected for fourth phase of the study was fresh water edible fish, *Oreochromis mossambicus* weighing 20±5 gm . After two weeks of acclimatization in the laboratory condition the fishes were used for the experiment. Feeding was with held for 24 hours before the commencement of the experiment.

Preparation of heat killed whole cell vaccine

The agar plate with single colony of *A. hydrophila* was inoculated in the nutrient broth . Overnight culture of *A.hydrophila* was subjected to 80˚ C for 30 minutes in a water bath. The cultures were centrifuged at 3000rpm for 15 minutes, supernatant was discarded, the packed cells in the bottom as sediments were washed carefully and required dose of bacterial cells were prepared using phosphate buffered saline (Sudhakaran *et al* ., 2006).

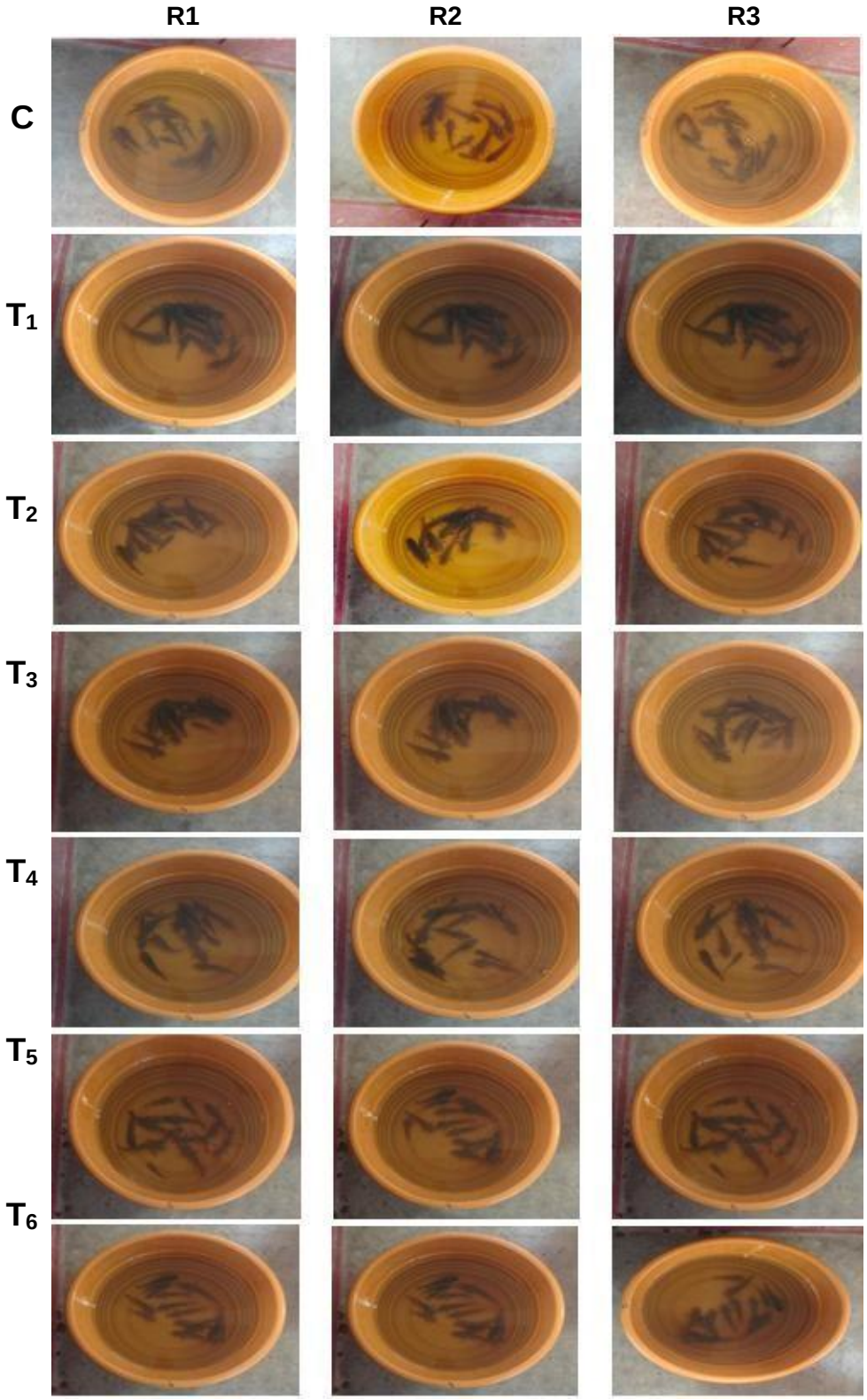
Experimental design

The freshwater edible fish, *O.mosambicus* (20 ± 5 g) were introduced in 50 L aerated plastic troughs. The fishes were divided into seven experimental groups (C – control and T1 to T6 – six different treatments) of 15 each in triplicate (7 x 15 x 3 = 315 fish). This experiment was carried out for 30 days .During experimental period the fishes were fed daily once with their respective feed at 5% of their body weight (Plate11).

After 30 days of treatment, all the fishes in control and treatment groups were fed with control food only. On 31^{th} day and 38^{th} day the fishes in the control and treatments were injected intramuscularly with heat killed 0.1ml of 10^{5} cfu / ml *A.hydrophila* suspension. Mortality rate was observed and recorded until 15 days after post challenge and dead fishes were removed.

Plate 11

Experimental Setup for the determination of physiological and immunological parameters of *O. mossambicus* supplemented with selected leaf extracts



Physiological studies

a) Cumulative percentage mortality

Cumulative percentage mortality was noted by observing the number of fish died throughout the experiment, expressed in percentage.

Mortality (%) = No. of death in a specific period/ Total population during that period x100

b) Survival rate

The survival rate of *O.mossambicus* was obtained by dividing the number of the fish survived to the total number of fishes expressed in percentage.

Survival (%) = No. of fishes survived / Total number of fishes x100

c) Relative level of protection

The potency of the leaf extract was determined by calculation of the relative level of protection (RLP) by the following formula:

RLP= 1 - Total number of mortality of fish in treatment / Total number of mortality of control x 100

Determination of serum antibody titer

Serial bleeding

The common cardinal veins situated just below the gills were used to bleed the fishes using one ml tuberculin syringe after 7^{nth} and 15^{nth} day after immunization .26-gauge needle is used for this purpose. Small micro centrifuge tubes (Torson) are used to collect the blood.

Collection of antisera

Blood was collected and kept at room temperature for 15 minutes and allowed to clot. After loosening the clot, the blood was centrifuged at 10,000 rpm for 10 minutes. The serum was collected and used for agglutination assay.

Antigen antibody agglutination assay

50 µl of physiological saline was added using a 50 µl dropper into all the wells of a clean microtitre plate. taken in the wells of microtitre plate 50 µl of antiserum was pipette out and was serially diluted in the wells of the microtitre plate of the first row up

to 11th well of the micro titer plate, leaving the 12th well as negative control. Similarly other serum samples were also diluted serially in each row of the microtitre plate. 50 µl of heat killed *A. hydrophila* (antigen) was added to all the wells. Gently shake the microtitre plate for efficient mixing of the reagent. Incubate the plates at room temperature for an hour. The highest dilution, which showed visible (macroscopic) agglutinations was noted and the values are expressed as log2

(Venkatalakshmi and Michael, 2001) (Plate 12).

Plate 12

Agglutination reaction in antigen antibody titer plate

Statistical analysis

The results of the entire study on growth characteristics, feed utilization efficiencies and proximate composition, hematological, biochemical, physiological and immunological parameters of *O.mossambicus* fed with *C.aromaticus* and *O.basilicum* leaf extracts as supplemented feed were analyzed statistically using one - way Analysis of Variance (ANOVA) and level of significance was defined at ($P \leq 0.05$) and the mean difference by Duncan‟s Multiple Range Test (DMRT).