IMMUNODIAGNOSTIC STUDIES IN A CHOSEN FRESH WATER FISH ADMINISTERED WITH A MEDICINAL AQUATIC FERN *MARSILEA QUADRIFOLIA*

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IN

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CERTIFICATE

This thesis entitled "**IMMUNODIAGNOSTIC STUDIES IN A CHOSEN FRESH WATER FISH ADMINISTERED WITH A MEDICINAL AQUATIC FERN *MARSILEA QUADRIFOLIA"*** submitted by **A. SIVAGURUNATHAN** for the award of Degree of Doctor of Philosophy in Zoology of Manonmaniam Sundaranar University is a record of bonafide research work done by him and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University or Institution.

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DECLARATION

I hereby declare that the thesis entitled "**IMMUNODIAGNOSTIC STUDIES IN A CHOSEN FRESH WATER FISH ADMINISTERED WITH A MEDICINAL AQUATIC FERN *MARSILEA QUADRIFOLIA"*** submitted by me for the Degree of Doctor of Philosophy in Zoology is the result of my original and independent research work carried out under the guidance of **Dr. B. XAVIER INNOCENT**, Associate Professor and Head, PG and Research Department of Zoology, St. Xavier's College (Autonomous), Palayamkottai – 627002 and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University or Institution.

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CONTENTS

ABBREVIATIONS

%	-	Percentage
α	-	Alpha
β	-	Beta
γ	-	Gamma
µg	-	Microgram
µl	-	Microliter
AFB_1	-	Aflotoxin B_1
$AgNO_3$	-	Silver Nitrate
ALP	-	Alkaline Phosphatase
ALT	-	Alanine Amino Transferase
AST	-	Aspartate AminoTransferase
CCl_4	-	Carbon tetra chloride
Cfu	-	Colony forming units
$CHCl_3$	-	Chloroform
$CuSO_4$	-	Copper Sulphate
dl	-	Deciliter
DNA	-	Deoxyribo nucleic acid
DPPH	-	2,2,diphenyl-1-picrylhydrazyl
g	-	Gram
GC-MS	-	Gas chromatography – Mass spectrometry
H_2O	-	Water
H_2SO_4	-	Sulphuric Acid
ha	-	Hectare
Hb	-	Haemoglobin
HCl	-	Hydrochloric acid
$HgCl_2$	-	Mercuric Chloride
HNO_3	-	Nitric Acid
Hrs	-	hours
Ht	-	Haematocrit
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
Kcals	-	Kilocalories

KDa	-	Kilo Dalton
Kg	-	Kilogram
LC_{50}	-	Lethal concentration for 50 percent mortality
M	-	Molar
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration
MCV	-	Mean corpuscular Volume
mg	-	Milligram
ml	-	Milliliter
N	-	Normality
Na_2So_4	-	Sodium sulphate
Nacl	-	Sodium Chloride
NaoH	-	Sodium hydroxide
OD	-	Optical Density
PBS	-	Phosphate buffered saline
PCV	-	Packed Cell Volume
ppt	-	Parts per thousand
PUFA	-	Poly unsaturated Fatty Acids
Rf	-	Relative front
RNA	-	Ribo nucleic acid
SD	-	Standard Deviation
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGOT	-	Serum glutamic oxaloacetic transaminase
SGPT	-	Serum glutamate pyruvate transaminase
TEC	-	Total Erythrocyte count
TLC	-	Total Leucocyte Count

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CHAPTER - 1

General introduction

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CHAPTER - 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Hunger and malnutrition are the most devastating problems facing the world's poor. With exploding human population 3,500 million people are undernourished as on 2012. An adult human being needs 1680 – 1990 Kcals energy and 58 grams of proteins per day (Currie 2014).

1.1.1 Live stock as Protein source

Live stock (Dairy, Poultry, Fish, Beef and Sheep & Goat) provides 12.9% of energy and 27.9% protein world wide. Fish provides 16.6% of global animal protein.

From just 10, 40, 10 million tonnes of poultry, fish and sheep & goat production in 1967 it has grown to 100, 130, 18 million tonnes respectively in 2011. Parellely, from the present consumption level of 110 and 80 million tonnes of poultry and fish respectively, it was estimated to raise upto 200 million tonnes at 2050. Thus significant growth potential in fish as animal protein is envisioned (Currie 2014).

Another reason for the better prospect for fish as important source as animal protein is, out of the total available fresh water, 70% is used in agriculture, 11% for municipal requirements and 19% for industrial purposes. Thus from the available limited amount of fresh water, production of 1kilogram of chicken requires 4000 litres and 1 kilogram of mutton production requires 9000 litres of water when compared with the little amount of water required for fish production.

Similarly, to gain 1kilogram of weight cattle needs 7kilogram of grain, poultry needs 2kilogram and fish requires only 1kilogram of grain only. Thus fish converts feed

into protein much efficiently than land animals. Thus fishes are cheap source of good Protein (Currie 2014).

1.1.2 Health benefits of Fish

Fish have a highly desirable nutrient profile providing an excellent source of high quality animal protein that is easily digestible and of high biological value. Fishes are also rich in vitamin A & D and minerals (especially calcium, phosphorus, iron, selenium and iodine). The fats and fatty acids in fish, particularly the long chain n-3 fatty acids (n-3 PUFA), are highly beneficial and difficult to obtain from other food sources. Of particular importance are eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) FAO-2003).

The oil extracted from fish are also extremely rich in essential fatty acids including Omega-3 poly unsaturated fatty acids (PUFAs) which improves health of brain, improves eye sight and reduces cardiovascular diseases by reducing blood clots, lowering blood fats and boosting good cholesterol. It also lowers risk of dementia including Alzheimer's disease, reduces the risk of cancer by 50%, lowers diabetes and relieves symptoms of rheumatoid arthritis, psoriasis and autoimmune diseases. People can easily digest 93.2% and 93.7% of fish protein and fat respectively (Olusola *et al* 2013).

1.2. Aquaculture – Present Status

Aquaculture is the fastest growing food-producing sector in the world. A great proportion of this production comes from the developing world (91.2% in 2000), currently all major aquaculture producing countries are in Asia. They are China, India, Japan, Indonesia, Thailand, Bangladesh and Vietnam.

The production from world capture fishery has not improved significantly for the past two decades, ie in 1990 it was 80 million tonnes and in 2010 it has increased to 92 million tonnes only. But the global aquaculture production has increased from 10 million tonnes in 1990 to 65 million tonnes in 2010. It was expected that the global capture fishery contribution may decline to 47% at 2050 from the present contribution of 60%, whereas the culture fishery is expected to grow to 53% at 2050 from the present contribution of 40%.

In the present global aquaculture production China tops with 62% followed by India with 7%. Carp production accounts for 40% of the aquaculture production. Thus there exists a great demand for the farmed fish (Currie 2014).

1.2.1 Aquaculture in India

India has 8,118 kilometers of marine coastline, 3,827 fishing villages, and 1,914 traditional fish landing centers. India's fresh water resources consist of 195,210 kilometers of rivers and canals, 2.9 million hectares of minor and major reservoirs, 2.4 million hectares of ponds and lakes, and about 0.8 million hectares of flood plain wetlands and water bodies. However, less than 10 percent of India's natural potential is used for aquaculture currently (Wikipedia – Fishing in India).

India is the second largest country in aquaculture production in the world. Fish production has increased from 41.57 lakh tonnes (24.47 lakh tonnes for marine and 17.10 lakh tonnes for inland fisheries) in 1991-92 to 82.90 lakh tonnes (32.20 lakh tonnes for marine and 50.70 lakh tonnes for inland fisheries) in 2010-11 (dahd.nic.in).

Top ten fish producing states in India as on 2008 is West Bengal, Andhra Pradesh, Gujarat, Karnataka, Tamil Nadu, Maharastra, Orissa, Punjab, Bihar and Karnataka totally producing 6,255,330 metric tonnes.

1.2.2 Aquaculture in Tamil Nadu

The state Tamil Nadu has 3.7 lakh hectare of water spreads suitable for fish culture. It comprises of major reservoirs (52,000 hectares), big/small irrigation tanks (98,000 hectares), small lakes and rural fishery demonstration tanks (1,58,000 hectares) and brackish water areas, swamps, estuaries (63,000 hectares) apart from 7400 kilometer length of rivers and canals offering good scope for fisheries development. These water bodies can be effectively utilized to improve the fish production (Fisheries.tn.gov.in).

Inland fish production in Tamil Nadu has increased from 459.43 thousand tonnes (2005) to 596.547 thousand tonnes (2011) (dahd.nic.in).

The Tirunelveli district has 1114 reservoirs, 875 tanks and 10,297 small and big seasonal tanks to the tune of 11,386 hectare area which produced 4352.369 tonnes of fishes in 2004-2005 (Fisheries.tn.gov.in) and 1343 tonnes during 2010-2011 (nellai.tn.nic.in).

1.3 Challenges faced by Aquaculturists

Besides Industrialization, urbanization, deforestation, mining etc, Environmental degradation and increasing land and water scarcity are the greatest threats to inland fish production.

In order to exploit the available land, aquaculturists practice Composite fish culture and Integerated fish farming such as Paddy cum Fish Culture, Pig Cum Fish Culture etc. In fish farming, production always depends on ecosystem management and disease management.

Ecosystem management mainly focuses on water quality, soil quality and stocking density, adverse changes in them induces 'Physiological stress' to the fish, which weakens the immune system of the fish making them more susceptible to infection

which may either bring out mortality or morbidity (decreased growth), both decreases the production. The loss of fish production from infectious diseases accounts for 60% of all diseased (Mishra 2010).

Many potential fish disease pathogens are opportunistic pathogens, which are continually present in the water, soil, air, or fish in nature. Fishes are often resistant to these pathogens when they are healthy. However, when the pathogen level increases due to external factors natural resistance of the fish cannot cope up and becomes vulnerable for infection. Similarly, when the fish is weakened due to physiological stress or injury also they are infected. Thus Fish diseases may cause severe losses on fish farming by

- Reduced fish growth and production
- Increased feeding cost caused by lack of appetite and waste of unfed diet
- Increased vulnerability to predation
- Increased susceptibility to low water quality
- death

Infectious disease in fish is mainly caused by the pathogens of virus, bacteria, fungi or unicellular algae. For instance, bacterial ulcerative disease, bacterial enteritis, bacterial gill rot/ fin rot and bacterial erythema etc. Most Gram negative bacteria belonging *to Aeromonas, Vibrio, Pseudomonas, Yersinia, Edwardsiella, Pasteurella, Cytophaga* causes disease in fish. Again bacterial pathogens of infectious diseases are not strictly parasitic microorganisms. If the condition for parasitism is unsuitable, it will lead a saprophytic life. *Aeromonas hydrophila* and other *Aeromonas species* are responsible for much of disease problems in fish, causing Aeromoniasis, haemorrhagic septicaemia and Epizootic ulcerative syndrome (EUS) in India. So far 20 species of pathogenic bacteria have been isolated from affected fishes of which *A.hydrophila* has been consistently found along with fungus *Saprolegnia.* The latest investigations point

out the prime causative agent to be a fungus called *Aphanomyces* sp. The important fungal pathogen in freshwater fishes is *Saprolegnia*. It is usually a secondary invader of damaged or stressed fish and also infects fish eggs.

Invasive diseases are caused mostly by fish parasites, like Trichodinasis, Ichthyphthiriasis, Lernaesis, Argulusis, etc. Fish carrying parasites or corpse of diseased fish are the direct sources of invasive disease. It is called the primary source, objects accompanied with direct source, such as contaminated feeds, gears, pond water and silt etc. are called indirect source, or secondary source for infection. *Trichodina sp* (Protozoan) infection causes gill rot, *Gyrodactylus sp* and *Dactylogyrus sp* (Nematode) attaches to gills, fins or body surface of the fish and Lernae sp (Crustacean) fixes to the musculature of the host causing inflammation leading to secondary infection (Mishra 2010).

1.4 *Aeromonas hydrophila* – Infection to fish

Aeromonas hydrophila is a heterotrophic, Gram-negative, rod-shaped bacterium mainly found in fresh or brackish water areas with a warm climate. This bacterium causes tail and skin rot and fatal hemorrhagic septicemias in fish and soft-tissue wound infection and diarrheic diseases in humans.

Mechanism of action

When the bacterium enters the body of the victim, it travels through the bloodstream to the first available organ. It produces Aerolysin Cytotoxic Enterotoxin (ACT), a toxin that can cause tissue damage, dermo necrotic factors, hemolysins, proteases and haemagglutinins. It was recently reported that it secretes a specialized protein secretion machinery known as Type III secretion system (TTSS) which exports virulence factors directly to host cells. These factors subvert normal host cell functions to

the benefit of invading bacteria which leads to interruption of NF-κB pathway, cytoskeletal damage and apoptosis.

When infected with *Aeromonas hydrophila*, fish develop ulcers, tail rot, fin rot, and hemorrhagic septicemia. Hemorrhagic septicemia causes lesions that lead to scale shedding, hemorrhages in the gills and anal area, ulcers, exophthalmia, and abdominal swelling. Other organs commonly affected with this disease include the gills, kidneys, liver, spleen, pancreas, and skeletal muscle (Wikipedia-Aeromonas hydrophila, Lu 1992). Thus infection brings about either immediate mortality or decreased growth due to prolonged illness. Hence maintaining the health of the fish is of prime importance in fish culture.

A.hydrophila has been chosen for the present work as it poses great threat to Aquaculture.

1.5 Disease Management

For sustainable farming, the health management programme should focus on two main approaches like Ecosystem management: environmental management with optimum water, soil quality maintenance, with proper stocking density, devoid of stress to animals and Disease management.

Disinfection of the pond with lime, formalin etc is widely practiced to control the disease spreading. Secondly, the infected fishes are treated with copper sulphate solution, ferrous sulphate solution, formalin solution, Malachite green solution, potassium permanganate solution etc by dipping, a labourious process. Thus the fish disease control should be based on “all-round porphylaxis” (Li shaoqi - FAO.org).

1.5.1 Chemotherapy

Drug bath

The first step in disease prevention is to disinfect the pond and gears. To control *Costia* (Protozoan parasite) and *Discocotyle* (Trematode) calcium cyanamide and formalin, to control Lernaea, Argulus (Crustaceans) and Gyrodactylus (Trematode) organophosphates, to control Saprolegnia (Fungus) Malachite green and to control bacteria copper sulphate can be used at appropriate concentration and dilution, can be applied to the pond or the fishes are dipped in them for appropriate time (Ram C. Sihag 2012). However toxicity of these chemicals increases when water temperature increases above 25^{o}C, decrease in pH and alkalinity and dissolved oxygen (below 5mg/l).

Application of Common salt kills several disease organisms but also have positive effect on the fish by stimulating appetite and increasing mucus secretion, improving resistance to handling, however excess levels are stress to fish, especially, cyprinids are more susceptible.

Antibiotics

Antibiotics like Erythromycin, Deoxycycline, Oxytetracycline (to control parasitic diseases), Sulphadiazine, Nitrofurans and Trimechoprim (to control bacterial / fungal diseases) etc can be used to treat/prevent bacterial infection (Pathak 2000). Antibiotics like these can either be injected to individual fish or dip treatment can be followed. Nowadays use of antibiotics in aquaculture is declining due to the problems like development of resistant pathogens and residual poisoning.

Medicated feeds

Parasitic diseases can be cured with medicines mixed into feed or with premixes. This type of therapy is especially effective against tapeworms (Bothriocephalosis in carp

farms), nematodes and coccidia. For the treatment, feed containing 0.1–0.2% Devermin can be fed for 1–3 days. In bacterial diseases, Furane derivatives (furazolidone, nitrofurazone, sulphonamides), sometimes in combination with trimethoprin, or antibiotics (oxytetracycline, chloramphenicol, neomycin), are applied with feed. Before starting the treatment, tests for drug resistance should be done. In applying these drugs, the recommended dosage and period of treatment should be respected, so as to avoid bacterial resistance. For myxobacterial infections, the drugs are routinely administered mixed with feed (Pathak 2000). However, they are non-specific and of low efficacy.

1.5.2 Vaccination

Vaccines are preparations of antigens derived from pathogenic organisms, rendered non-pathogenic by various means, which will stimulate immune system of the animal to increase the resistance to the disease when naturally encountered with pathogens. Once stimulated by a vaccine, the antibody-producing cells, called B lymphocytes, remain sensitized and ready to respond to the agent should it ever gain entry to the body. Based on the preparations of the antigens the vaccines are classified as Killed vaccines, Attenuated vaccines, Toxoid vaccines, Conjugate vaccines and recently recombinant vaccines and DNA vaccines (Yanong 2008).

Vaccines are administered to the fish through different routes. They are

Oral vaccination

It results in direct delivery of antigen via the digestive system of the fish. It is the easiest method as stress on the fish is minimal, and no major changes in production are required. Prior to feeding, vaccine is mixed, top-dressed, or bio encapsulated into the feed. But it conveys relatively short immunity (compared to the other methods) such that additional vaccination may be required (Yanong 2008).

Immersion vaccination

This permits immune cells located in the fish's skin and gills to become directly exposed to antigens. These immune cells may then mount a response (e.g., antibody production), thus protecting the fish from future infection. Other types of immune cells in the skin and gills carry antigens internally, where a more systemic response will also develop. Immersion vaccination occurs by dip or by bath. Dips are short, typically 30 seconds, in a high concentration of vaccine. Baths are of longer duration—an hour or more—and in a much lower concentration of vaccine. In practice, dips are logistically more practical for large numbers of small (1- to 5-g) fish. Unfortunately, protection using immersion methods may not last long and a second vaccination may be required (Yanong 2008).

Injection vaccination

It allows direct delivery of a small volume of antigen into the muscle (intramuscular (IM) injection) or into the body cavity (intracoelomic [Ice], intraperitoneal [IP] injection), allowing for more direct stimulation of a systemic immune response. Injection vaccines normally include an oil-based or water-based compound, known as an adjuvant that serves to further stimulate the immune system. Injection is effective for many pathogens that cause systemic disease; and the protection is much longer than by other methods. Another advantage is that multiple antigens (for different diseases) can be delivered at the same time. However, vaccination by injection is logistically the most demanding of all three methods. Fish must be anesthetized to minimize stress. Injection requires more time, labour, and skilled personnel. The correct needle size is important. The vaccine may incite a more severe reaction if it is injected into the wrong portion of the fish. And finally, smaller-sized fish (under 10 g) may not respond well to this method (Komar et al 2004). Examples of fish vaccines developed are

Vaccines	Species	Disease
Aeromonas salmonicida Bacterin	Atlantic salmon	Furunculosis
Vibrio anguillarum - *Yersinia ruckeri* Bacterin	Rainbow trout	Vibriosis, Yersiniosis (enteric red- mouth disease)
Yersinia ruckeri Bacterin	Salmonids	Yersiniosis (enteric red-mouth disease)
Vibrio salmonicida Bacterin	Salmonids	Vibriosis
Vibrio anguillarum-salmonicida Bacterin	Salmonids	Vibriosis
Aeromonas salmonicida Bacterin	Salmonids	Furunculosis
Edwardsiella ictaluri Bacterin	Catfish	Enteric septicaemia
Spring viraemia of carp virus	Common carp	Spring viraemia of carp
Koi herpes virus (KHV)	Koi carp	Koi herpes virus (KHV) disease
Biofilm and free-cell vaccines of *Aeromonas hydrophila*	Indian major carps	Dropsy
Streptococcus agalactiae vaccine	Tilapia	Streptococcosis

Currently, vaccines are available for some economically important bacterial and viral diseases. Vaccines for protection against parasitic and fungal diseases have not yet been developed. Vaccination should be considered as a part of comprehensive fish

health management scheme, and not the only solution for a disease problem (Arun Sudhagar-aquafind.com).

1.5.3 Probiotics

Live microorganisms administered in adequate amounts as feed supplements which have beneficial effects on the intestinal microbial balance of the host. The beneficial effects include disease treatment and prevention, improvement of digestion and absorption of the feed, they also stimulate the immune system of the fish to produce inhibitory substance against foreign antigen and the probiotic microbe colonizes in the gut of the fish there by resisting the colonization of the pathogenic bacteria. *Lactobacillus* sp, *Bifidobacterium* sp and *Streptococcus* sp are commonly used in probiotics. (Ram C. Sihag 2012). The probiotics are also non specific and their efficiency depends upon various factors including its colonization.

1.5.4 Immunostimulants

An Immunostimulant is a chemical, drug, synthetic or natural substance that elevates the non-specific defence mechanism or the specific immune response against those infectious agents (viruses, bacteria, fungi, and parasites), producing subclinical disease without risks of toxicity, carcinogenicity or tissue residues. They help to hasten the maturation of non-specific and specific immunity in young susceptible animals. They promote a greater and more effective sustained immune response.

The Immunostimulants can be administered either as injection or added with the feed in small doses. Plenty of reports are there that oral administration along with feed is also equally effective. Immunostimulants are effective against controlling

Bacteria:	*Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda, E. ictaluri, Vibrio anguillarum, V.vulnificus,V. salmonicida, Yersinia ruckeri, Streptococcus sp.*
Virus:	Infectious hematopoietic necrosis, yellow head virus, viral hemorrhagic septicemia.
Parasite:	*Ichthyopthirius multifiliis*. etc (Debtanu Barman *et al* – Aquafind.com)

The Immunostimulant substances may be

Bacterial derivatives	Muramyl dipeptide (MDP) (mycobacterium peptidoglycan derivative), lactoyl tetrapeptide (Streptomyces olivaceogriseus sp derivative) and Lipopolysaccarides (LPS) (preparation from gram negative bacteria)
Yeast derivatives	Glucans, long chain polysaccharide extracted from yeast
Algal derivatives	Laminaran is a B (1, 6)-branched B (1, 3)-D-glucan derived from *Laminaria hyperhorea.*
Synthetic compounds	Muramyl peptides, Levamisole, FK-565
Animal extracts	Chitin, (a polysaccharide forming the principle component of crustacean and insect exoskeleton and the cell walls of certain fungi), Chitosan (de-*N*-acetylated chitin)
Vitamins	Vitamin C & E
Hormones	Growth hormone (GH), Prolactin, Melanin stimulating hormone (MSH) and Melanin concentrating hormone (MCH)

Herbal Extracts The following are a few examples of medicinal herbs whose extracts have proven Immunostimulant property (Sagiv Kolkovski and Nutra-kol -2013).

Botanical Name	Biological Effect in Aquaculture
Eclipta electa	Hepato tonic, Immunostimulant, Antistress
Eclipta alba	Hepato tonic, Immunostimulant, Antistress,antiviral
Solanum trilobatum	Immunostimulant , Antibacterial
Aconus calamus	Immunostimulant , Antibacterial
Tinospora cordifolia	Immunostimulant , Antiviral
Daemia extensa	Immunostimulant , Antibacterial
Ipomea digitata	Immunostimulant , Growth promoter
Cymodon dactylon	Immunostimulant , Antibacterial
Emblica officinalis	Immunostimulant , Antibacterial
Picrorrhiza kurroa	Immunostimulant , Antistress
Withania somnifera	Immunostimulant , Growth promoter
Urtica dioica	Immunostimulant
Vernonia cinera	Immunostimulant
Viscum album	Immunostimulant
Zingiber officinale	Immunostimulant

1.6. Herbal Extracts as Immunostimulants – Advantage

The herbs are rich source of bio active compounds like volatile oils, saponins, phenolics, tannins, alkaloids, flavonoids, polysaccharide and polypeptides with various activities like anti-stress, appetizer, growth promoters, anti-microbial, anti-cancer and Imunostimulants. The antimicrobial potential of herbs is higher and they can act on broad spectrum of pathogens. The secondary metabolites also helps in breaking down the complex toxic substances to be eliminated into simple substances thus protects liver and kidney from stress. They also supplies pigments such as chlorophylls, carotenoids, xanthophylls and very complex vitamins in the most desirable form. Besides their medicinal property the other advantages of herbs are high availability, biodegradable, biocompatible and inexpensive. Above all they do not harm the physiological system and organelles of the organism and also do not leave residual poison.

The use of immunostimulants has opened a new chapter and a very promising area in aquaculture. Immunostimulants may be used to prevent losses from disease. Although vaccination is the most reliable method to control fish diseases, as yet, no effective vaccine against Bacterial Kidney disease or most viral infections and there is no single commercial vaccine available in India, and imported vaccine may not be effective because the strain which cause disease may be different in India. Immunostimulants are safer than chemotherapy and their range of efficacy is wider than vaccination. Therefore immunostimulants may be a powerful tool for controlling infectious diseases. (Mukesh K Bairwa *et al* 2012, Debtanu Barman *et a*l –Aquafind.com, Stephen Sampath Kumar and Anantharaja 2007).

1.6.1. Ferns as Immunostimulants

The pteridophytes are non-flowering, vascular and spore-bearing plants including ferns and fern-allies. The pteridophytes form a connecting link between the lower group of plants and the higher group of seed bearing plants. About 250 million years ago they formed the dominant part of earth's vegetation, but in present day flora, they have been largely replaced by the seed bearing plants. The world flora consists of approximately 12,000 species of pteridophytes of which around 1000 species are likely to occur in India (Dixit, 2000). The Western Ghats harbour about 320 species of ferns and fern allies with more species diversity in the southern part.

Humans have been dependent upon the plants as an important source of medicine since the time immemorial. Even today, many tribal communities and rural population is dependent heavily upon the natural resources obtained from the surrounding forest regions for treatment of various ailments and diseases. The Indian traditional medicine is based on different systems such as Ayurveda, Siddha and Unani used by various tribal communities (Gadgil 1996). Though, lot of studies focusing on the medicinal properties of plants, especially angiosperms, have been taken place, unfortunately limited amount of studies have been done to explore the medicinal potentialities of the pteridophytes. The medicinal qualities of ferns, are mentioned as early as 300 B.C. by the Greek philosopher Theophrastus and by his Indian contemporaries Sushrut and Charak. Medicinal properties of many ferns were listed out by many workers (Kamini Srivastava 2007, Benjamin and Manikam 2007).

Several investigators reported previously about phytochemical and biological activities of several fern and fern allies. Benerjee and Sen (1980) conducted an extensive survey of antibiotic activity among the ferns and reported about a hundred species having such property. A few are listed below (Mariadas and Raju 2010)

Scientific Name	Biological function
Huperzia hailtonii, H. phyllantha, H.squarrosa, H.phlegmaria, H.nilagirica and *H.hilliana*	Antibacterial
Selaginella delicatula, S.pulvinata, S.radicata, and *S.tenera*	Antibacterial
Equisetum ramosissimum	Antiviral, Antiseptic, Antidiabetic
Psilotum nudum	Antibacterial
Helminthostochys zeylanica	Antiviral, Antiinflammatory
Pteris vittata	Antiviral, Antibacterial
P.cretica	Antibacterial
Dryopteris cohleata	Antifungal
Actinopteris radiata	Antibacterial
Adiantum incisum and *A.lunulatum*	Antibacterial
Leucostegia immerse	Antibacterial
Nephrolepis auriculata	Antifungal
Sphaerostephanos unitus	Antibacterial
Athyrium pectinatum	Antibacterial
Tectaria zeilanica	Antioxidant
Stenochlaena palustris	Antibacterial
Drynaria quercifolia	Antibacterial
Salvenia molesta	Antifungal

Through review of available literature it was noticed that only a few ferns like *Azolla, Salvinia & Asplenium barteri* are incorporated in the feed of fishes that to for their protein value.

1.6.2. *Marsilea quadrifolia*

Marsilea quadrifolia is an aquatic fern present in shallow water or on land with rich medicinal value. Traditionally it is used to cure cough, bronchitis, diabetics, psychiatric diseases, eye diseases, diarrhea, skin disease and fever (Herbpathy.com). Juice made from the leaves of *M.quadrifolia* is diuretic, depurative, febrifuge and refrigerant and also used to treat snake bite and applied to abscesses etc (Duke and Ayensu 1985). *M.quadrifolia* also have the potentials like anti-inflammatory, Anodyne, Antidote, Antiphlogistic, Aphrodisciac, Astringent, Antibacterial, cytotoxic, antioxidant and anticancer activity (Schofield 1989, Herbpathy.com, Farhana *et al* 2009, Uma and Pravin 2013).

Being a medicinal plant it is rich in secondary metabolites like phenolic compounds, flavonoids, phytosterols, tannins, alkaloids and saponins which are responsible for its medicinal properties. Thirteen bioactive compounds with proven medicinal properties were identified among which Hexadecanoic acid ethyl ester, Phytol and 9,12-Octadecadienoic acid were predominant (Sivagurunathan and Xavier Innocent 2014).

It can be also used as feed for livestock as it contains 16.8%-36.2% crude protein, 4.6% Crude Fat, 23.4% Crude fibre, 0.55-1.4% Soduim, 1.74%-2.8% Potassium, 0.44%-0.6% Calcium, 0.1%-0.8% Phosphorus and 2.1-2.8% β-carotene (Precursor for Vitamin A) (Anjana and Matai 1990, Dewanji *et al* 1993).

Besides its proven medicinal properties, its aquatic habitat where both fish and *M.quadrifolia* were present made me to investigate its immunostimulant potential to fish.

1.7 Objectives of the present Investigation

- To identify and characterize the phytochemical constituents of the chosen plant "*Marsilea quadrifolia*" qualitatively, quantitatively and through GC-MS Analysis.
- To analyse the antibacterial and antifungal potential of *M.quadrifolia* extracts.
- To assess the growth promoting potential of *M.quadrifolia* through feeding trials by dietary incorporation and to validate its efficiency with a known immunostimulant β-Glucan incorporated diet.
- To estimate the sub lethal dosage of *Aeromonas hydrophila* to the experimental fish *Labeo rohita.*
- To evaluate the Immunostimulant potential of *M.quadrifolia* and β-Glucan incorporated diets in *Labeo rohita* through haematological and serological analysis challenged with *A.hydrophila*.
- To understand and infer the immune enhancement of non-specific humoral and cellular responses in *Labeo rohita* induced by *M.quadrifolia* and β-Glucan challenged with *A.hydrophila*.

CHAPTER - 2

Review of literature

CHAPTER - 2

REVIEW OF LITERATURE

2.1 INTRODUCTION

Inland fish culture has become a promising enterprise and efforts have been made to increase productivity per unit space by increasing the rearing density in different culture systems. Due to this practice a number of associated stressors like overcrowding, transport, handling, grading and poor water quality tends to adversely affect the health of cultured fish (Li *et al* 2004). These conditions produce poor physiological environment (including the suppression of the immune system) increasing the susceptibility of fish to infectious agents paving the way for the outbreak of a number of diseases due to an increasing range of pathogens, leading to substantial economic losses. The estimated annual economic loss due to diseases in aquaculture is more than US$ 400 million in China (1993), US$ 17.6 million in India (1994), and over US$ 500 million in Thailand (1996) (www.agriculture.de/acms1/conf6/ws9fish.htm) despite the partially successful preventive measures including sanitary prophylaxis, disinfection, antibiotics, vaccines and chemotherapy for the last 20 years. Hence immunoprotection of fish is of prime importance to overcome the stressors. Use of Immunostimulants in aquaculture is gaining importance as the other therapeutic measures like antibiotics, vaccines and chemotherapy have their own limitations.

An immunostimulant is a biological or synthetic compound administered either orally or through body fluids into the body of the fish or shrimp for enhancing the immune status of the host to over come the adverse environmental conditions, stress, pathogens and opportunistic microbes. Various natural and synthetic compounds known as immunomodulators or immunostimulants are being commonly used to potentiate the

immune system in every sphere of life. Application or use of immunostimulants is more common in animal husbandry and human health care. But in aquaculture the concept of immunostimulants is of recent origin and it is mainly to replace the use of antibiotics (Shodhganga-Inflibnet).

Immunostimulants may improve health and performance of farm animals, including fish and shrimp in aquaculture, if used prior to, situations known to result in stress and impaired general performance of animals, expected increased exposure to pathogenic microorganisms and parasites and developmental phases when animals are particularly susceptible to infectious agents. In fish non-specific defense mechanism plays an important role at all stages of fish infection. Fish particularly depend more heavily on these non-specific mechanisms than do mammals. Among the nonspecific defense mechanisms important in fish are the "Barriers in place" such as the skin and scales, and lytic enzymes of the mucus and sera, cellular aspects includes monocytes, macrophages, neutrophils and cytotoxic cells (Secombes 1992).

Immunostimulants may directly initiate activation of the innate defense mechanisms acting on receptors and triggering intracellular gene activation that may result in production of antimicrobial molecules (Bricknell and Dalmo 2005). Immunostimulants specifically bind to the receptor molecules present on the surface of phagocytes. Receptor for immunostimulants has been retained during evolution and is found in all animal groups. When the receptor is engaged by immunostimulants the cells become more active in engulfing, killing and digesting bacteria and at the same time they secrete signal molecules (cytokines) which stimulate the formation of new white blood cells which are producing antibodies (Shodhganga-Inflibnet).

The immunostimulants mainly facilitate the function of phagocytic cells, increase their bactericidal activities and stimulate the natural killer cells, complement,

lysozyme activity, and antibody responses in fish and shellfish which confer enhanced protection from infectious diseases. Leukocytes (white blood cells) are immune cells involved in defending the body against both infectious microbes and foreign materials. Leucocytes consist of neutrophil, eosinophil, basophil, monocyte, and lymphocyte. Neutrophils and monocytes are involved in the innate immune response as the first line of the defense system. Effector cells of the innate immune system circulate in the blood and migrate into tissues and kill microbes through phagocytosis. The innate immune cells also play a role in the inflammatory response by releasing cytokines. Lymphocytes including T cells and B cells are the only cells in the body capable of specifically recognizing and distinguishing different antigenic determinants (epitopes). When these adaptive immune effector cells recognize the microbes, the number of lymphocytes is significantly increased as a part of their effector functions. Although the number of the innate immune cells also expanded upon activation, the level of increase is much lower than that of the adaptive immune cells. The number of leukocytes in the blood is often an indicator of the activation of the immune system or of leukemia (Abbas *et al* 2010).

In order to research the humoral and cellular component activations it is necessary to study a number of biologically relevant assays such as complement activity, lysozyme production, phagocytosis, chemotaxis or the generation of microbicidal products like reactive oxygen species (ROS), due those are good choice for monitoring whether the innate immune system is activated.

2.1.1 Objectives of Immunostimulation

- To promote a greater and more effective sustained immune response to those infectious agents producing subclinical disease without risks of toxicity, carcinogenicity or tissue residues.

- To hasten the maturation of non-specific and specific immunity in young susceptible animals.
- To enhance the level and duration of specific immune response, both cell mediated and humoral, following vaccination.
- To overcome the immunosuppressive effects of stress and of those infectious agents that damage or interface with the functioning of cells of immune system.
- To selectively stimulate the relevant components of the immune system or non-specific immune mechanism that preferentially confers protection against microorganisms. For example via interferon release, especially for those infectious agents for which no vaccines currently exists and
- To maintain immune surveillance at heightened level to ensure early recognition and elimination of neoplastic changes in tissues.

2.1.2 Characteristics of an ideal Immunostimulant

- It should be non-toxic, even at a high dose rate.
- It should be non-carcinogenic or have no long term side effects.
- At therapeutic levels, it should have a short withdrawal period with low tissue residues.
- It should stimulate a wide range of non-specific immune responses against bacteria, fungi, virus, protozoa and helminthes.
- It should be capable of amplifying primary and secondary immune responses to infectious agents.
- Breakdown products of compound concerned should be either inactive or readily biodegradable in the environment.

- It should be having defined chemical composition of biological activity.
- It should be active by oral route and should be stable both in its native state and after incorporation into food and water.
- It should be compatible with array of drugs including antibiotics and antihelminthics and
- It should be inexpensive and either tasteless or palatable (Sajid Maqsood *et al* - Aquafind.com).

Some of the Immunostimulants evaluated against fish and shrimp (Debtanu Barman *et al* 2013, Galindo-Villegas and Hosokawa 2004) are

Types	**Immunostimulants Evaluated**
Synthetic Chemicals	Levamisole, FK-156, FK 565 (Lactoyl tetra peptide from *Streptomyces olvaceogriseus*), Quaternary ammonium compounds (QAC), Avridine, Bestatin, DW-2929, Fluoro-quindone
Bacterial derivatives	Muramyl dipeptide (from *Mycobacterium*), B-Glucan (MacroGard, Vitastim, SSG, Eco-Activa, Betafectin) Lipopolysaccharides, Fueund's complete Adjuvant (FCA), EF 203 (Fermented Egg Product), Peptidoglucan, *Clostridium butyrium* cells, *Achromobater stenohalis* cells, *Vibrio anguillarum* cells.
Yeast derivatives	Beta 1,3 Glucan, Beta 1,6 Glucan
Nutritional factors	Vitamin A,C & E, n-3 fatty acid, Nucleotides, Trace elements (Iron, Zinc, Copper & Selenium)
Hormones	Growth hormone, Prolactin, Tri-iodo thyronine
Cytokines	Interferon, Interleukin
Polysaccharides	Chitosan, Chitin, Lentinan, Schizophyllan, Oligosaccharides

Animal & Plant Extracts	Ete (Tunicate), Hde (Abalone), Glycyrrhizim, Firefly Squid, Quillaja, Saponin (Soap tree), Laminarian (Sea weed)
Others	Lactoferrin, Soyabean protein, Spirulina, Mucocircinelloides (Fungi)

Some commercial Immunostimulants available in the market of different countries are Immustim, Macroguard, Vitastim, Aqua-Mune, Penstim, Laminam, Calcium Spirulan, Sp604, Agrimos, Elorisan, DS1999, Levamisole, Lysozyme hydrochloride, Lactoferrin, Selenium yeast, Polypeptides fish hydrolysates, Blood Plasma, Fish Oils etc.

Some commonly used Immunostimulants and their potentials are discussed below

2.2.1 β-Glucan as Immunostimulant

Glucan

Glucans are high molecular-weight substances composed of glucose as building blocks, usually isolated from cell walls of bacteria, mushrooms, algae, cereal grains, yeast and fungi. Pharmacologically, they are classified as biological response modifiers (BRM). The common feature of immunomodulatory glucans is a chain of glucose residues linked by β-1.3-linkages, also called beta-glucans. Of the different β-glucans, the products known as β-1.3/1.6-glucans derived from baker's yeast, are suggested to be the most potent immune-system enhancers. β-1.3/1.6-glucans is characterized by side-chains attached to the backbone that radiate outward like branches on a tree. The primary structure of the β-1,3/1,6-glucan is determinant for its immune-enhancing ability (Zekovic and Kwiatowski 2005).

Glucan specific receptors are present on the surface of many cells especially on monocytes, macrophages, neutrophils and natural killer cells. When the glucans binds to glucan receptors all immune functions are improved, including diapedesis, phagocytosis, degranulation, initiating signal transduction pathways and induce gene expression synthesizing the immune related products, release of certain cytokines (Intercellular hormones), interferons to process the antigens. The cytokines also stimulates the formation of new white blood cells to enhance the immunity (Meena *et al* 2013).

EL-Boshy *et al* (2008) fed Tilapia (Injected with Aflotoxin B1) dietary Aflotoxin B1 (200μg/kg) and/or β1-3 glucan (0.1%) individually or combinedly for 3 weeks and analysed haematological, biochemical and immunological parameters. They have observed an increase in total erythrocytes, haemoglobin, total leucocytes, lymphocytes, packed cell volume, total serum protein, superoxide anion production, bactericidal activity, lysozyme concentration, neutrophil glass adhesion, lymphocyte transformation index and macrophage phagocytic index on β1-3 glucan diet fed fishes. Thus feeding β1-3 glucan can enhance the non-specific immunity not only in healthy but also in immunocompromised (with AFB1) Tilapia.

Jaya Kumari and Sahoo (2006) fed different groups of catfish (*Clarias batrachus*) with different immunostimulant incorporated feeds, *ie*, β 1-3-glucan (0.1%), Levamisole (50mg/kg), Lactoferrin (100mg/kg) and vitamin C (500 mg/kg) for 30 days. Each group is further divided into two groups, one is immune compromised by cyclophosphamide 3 doses (200mg/kg) and the other is non-immunocompromised. Fishes of all the groups were vaccinated on the first day with formalin killed *Aeromonas hydrophila*. After the experimental period the antibody titre and survival rate was found to be enhanced in both healthy and immunocompromised fishes. The immunostimulants were also graded based

on their immunostimulant potential as β Glucan > Levamisole > Lactoferrin > Vitamin C.

Sahoo and Mukherjee (2001) fed β-1,3 glucan (0.1%) incorporated diet for 7 days to healthy and aflatoxin B1-induced immunocompromised *Labeo rohita* in a 60 day trial and observed specific and non-specific immune responses. A marked rise in specific immune response parameters (bacterial agglutination titre, haemagglutination and haemolysin titre) and non specific immune parameters (phagocytic ratio, phagocytic index and serum bactericidal activity) were observed. Further increased survival against *Aeromonas hydrophila* infection was observed in glucan fed fishes. Thus feeding glucan has increased both specific and non-specific immunity against *A.hydrophila*.

2.2.2 Chitin and Chitosan as Immunostimulant

Chitosan is a deacetylation product of chitin (extracted from insect exoskeleton or crustacean shells and many cell walls of fungi).

Kiruba Aathi *et al* (2013) fed *Labeo rohita* with dietary Chitosan (0.5, 0.75, 1.0 and 1.25%) for 90 days and observed growth, haematological and serum parameters on 30^{th}, 60^{th} and 90^{th} day. Increase in feed conversion ratio, specific growth rate, total erythrocytes, haemoglobin, total leucocytes, total serum protein, carbohydrate, lipid and antibody titre were recorded. Thus chitosan enhanced growth and immunity.

Shimei Lin *et al* (2012) fed common carp (*Cyprinus carpio*) with dietary Chitosan Oligosaccharide (0.2%), *Bacillus coagulans* (0.1%) individually and combinedly for 8 weeks. Results indicated a highest increase in specific growth rate, total leucocyte counts, respiratory burst activity, phagocytic activity, lysozyme activity and super oxide dismutase activity and increased disease resistance to *Aeromonas veronii* in fishes fed with Chitosan and *B.coagulans* combined feed.

Saeed Meshkini *et al* (2012) fed rainbow trout (*Oncorhynchus mykiss*) with dietary chitosan (0, 0.25, 0.5 and 1%) for 8 weeks and haematological parameters were analysed on 2, 4, 6, 8th week and on 11th week the fishes were exposed to environmental (hypoxic & thermal) stress. They observed that serum glucose, total leucocytes, lymphocytes and neutrophils increased in fishes fed with 0.25% chitosan. Thus 0.25% level of dietary chitosan enhances the haematological parameters and resistance against environmental stress in Rainbow trout.

Sajid Maqsood *et al* (2010) fed *Cyprinus carpio* with dietary Chitosan (a linear polymer of β-(1,4)-2-amino-deoxy-D-glucose prepared by the alkaline deacetylation of chitin, a natural substance obtained from crab shell of any crustacean shell) at different doses (1%, 2% and 5%) for 70 days. They have injected *Aeromonas hydrophila* on 30th and 57th day and observed that the phagocytic index, phagocytic ratio, serum bactericidal activity, specific growth rate and relative percentage of survival have significantly increased at 2% concentration. Thus chitosan acts as a potential immunostimulant.

2.2.3 Levamisole as Immunostimulant (Levo-isomer of tetramisole - an antihelminthic drug)

Levamisole enhances cell mediated cytotoxicity, lymphokine production and suppressor cell function and stimulation of phagocytic activity of macrophages and neutrophils.

Nevien *et al* (2008) fed Tilapia (*Oreochromis niloticus*) with dietary Levamisole (225mg/kg diet) and Immunoton (commercial mix of Vitamin E and Vitamin C) for 2 weeks and analysed cellular and humoral innate immune responses. Fishes fed with levamisole enriched diet exhibited an increase in total leucocytes, lymphocytes, phagocytic index, lysozyme concentration and Y-globulin concentration when compared

with Immunoton and control feed. Thus levamisole have the potential in enhancing both cellular and humoral innate immune response and increased resistance to *Aeromonas hydrophila*.

Wijendra and Pathiratne (2007) fed *Labeo rohita* with dietary levamisole (0.05%) on every third day (6 doses) upto 16^{th} day, analysed haematological parameters 14 and 21 days post treatment and observed an increase in total leucocyte count, neutrophils, monocytes, phagocytic activity, phagocytic index and lysozyme activity, however total serum protein and immunoglobin levels exhibited no significant differences.

Jaya Kumari and Sahoo (2006) fed catfish (*Clarias batrachus*) with dietary levamisole (50, 150, 450mg/kg feed) for 10 days, analysed non-specific immune response after 1, 2 and 3^{rd} week and reported a significant rise in respiratory burst activity in neutrophils, myeloperoxidase activity and alternative complement activity at 50mg dose level. They also observed that there is no change in serum lysozyme, serum protein and natural haemagglutination titre. Thus reported that use of levamisole at 50mg/kg feed for 10 days as an immunostimulant to Asian catfish farming.

2.2.4 Omega-3 Fatty acid as Immunostimulant

Prathibha Bharathi and Sumanth Kumar Kunda (2011) assessed the immunomodulatory role of Omega-3 fatty acid to *Labeo rohita*. Dietary supplementation (1%, 3%, 5% and 7%) of omega-3 fatty acid to *L.rohita* for 60 days exhibited improved levels of total erythrocytes, haemoglobin, total leucocytes, serum total protein, serum albumin at 1% concentration. The infection studies with *Aeromonas hydrophila* (10^8 CFU/ml) exhibited an improved survival rate at 1% diet. Further, diet with 7% omega-3 fatty acid caused immunosuppression.

2.2.5 Vitamins as Immunostimulant

Syed Ali Fathima *et al* (2012) injected Vitamin A (0.025ml) to *Channa puntatus,* infected with *Aeromonas hydrophila* and analysed the blood parameters on 1, 2, 3, 4, 7, 14, 21 and 26th days and observed that the total erythrocytes, total leucocytes, lymphocytes, neutrophils and monocytes have increased significantly over control and also observed more antigen-antibody concentration on the 96 well microtitre plate. Thus indicating vitamin-A acts as an Immunostimulant.

German Bueno Galaz *et al* (2010) fed Parrot fish(*Oplegnathus fasciatus*) with dietary Vitamin E (0, 25, 50, 75, 100 and 500mg/kg diet) for 12 weeks and observed an increase in weight gain, feed conversion ratio, protein efficiency ratio, respiratory burst activity and serum myeloperoxidase activity at 25mg dose level itself. Further, when these fishes were bath challenged with *Vibrio anguillarum*, 500mg dose level produced higher survival rate. Thus, advocated that Vitamin E can enhance non specific immunity.

Rebeca Cerezuela *et al* (2009) fed gilt head Sea bream (*Sparus aurata*) with dietary Vitamin D_3 (3750, 18,750 and 37,500 units/kg) for 4 weeks and analysed immune parameters on 1st, 2nd and 4th week. They observed an increase in peroxidase content of head kidney leucocytes on 1st and 2nd week, phagocytic activity of head kidney leucocytes on 2nd week, natural cytotoxic activity of head kidney leucocytes from 1st week, but noted a minor decline on respiratory burst activity of leucocytes. Thus observed that supplementation of diet with Vitamin D3 has elevated cellular innate immune response with improved cytotoxic and phagocytic activity.

Ilmiah *et al* (2009) divided Groupers (*Epinephelus fuscoguttatus*) into 3 groups, performed bath treatment (60 minutes), one with vitamin C solution, another with *Aeromonas salmonicida* vaccine and the last group served as control. All the fishes were

challenged with *Aeromonas salmonicida* (3 x 10^7 cells). Phagocytic activity and antibody titre were analysed after 7, 14, 21, 28, 36 and 48 days of bath treatment. Results indicated that the phagocytic activity of head kidney leucocytes were higher in vitamin C treated fishes for all days and the antibody titer has increased after 42^{nd} day in both vitamin C and in Vaccine treatment. Thus vitamin C provided an increasing non-specific and specific immunity against *A.salmonicida* infection in grouper.

Janessa and Elisabeth (2006) fed fish Matrinxa (*Brycon amazomicus*) with dietary vitamin C (0, 100, 200, 400 and 800mg/kg feed) for 60 days and found no change in blood cortisol, chloride, total protein, haemoglobin, leucocytes, liver glycogen and hepatosomatic index. Further, these fishes were stress challenged by exposing to air for two minutes and blood was analysed after 5, 15, 30 and 60 minutes. After stress, an elevation in blood glucose after 60 minutes, decrease of serum sodium after 60 minutes and total erythrocytes after 5 minutes were observed.

2.2.6 Algae as Immunostimlant

Ahmadifar *et al* (2009) fed fish Beluga (*Huso huso*) with dietary Alginic acid (Ergosan) (Alginic acid is derived from several genera of brown algae including *Macrocystis*, *Laminaria*, *Lessonia*, *Ascophyllum*, *Alaria, Ecklonia, Eisenia, Neroecystis, Sargassum, Cystoseira* and *Fucus*) at different concentrations (0, 2, 4 and 6g/kg) for 60 days. The growth parameters exhibited an increase in weight gain and specific growth rate and in the haematological parameters lymphocytes and neutrophils has increased in the experimental diets and no significant change was observed in the parameters like haematocrit %, total erythrocytes, total leucocytes, haemoglobin, Mean corpuscular volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), monocytes and myelocytes. Thus dietary

supplementation of Alginic acid at 4g/kg can improve the growth and some immune components.

Hironobu *et al* (2006) orally administered (by intubation) 1, 10, 25 mg of spirulina (*Spirulina plantensis* – a blue-green algae) to *Cyprinus carpio* for 3 days and observed an increase in phagocytosis activity, superoxide anion production in phagocytic cells, expression of cytokine genes (IL-1β and TNF-α genes) in the head kidney leucocytes. Then the fishes were infected with *Aeromonas hydrophila* and their number was observed in liver and kidney after serial dilution. The number of *Aeromonas hydrophila* has decreased than control after 4, 8 and 12 hours of infection. Thus it was observed that spirulina has increased resistance against *A.hydrophila* infection.

2.2.7 Yeast as Immunostimulant

Arup Tewary and Patra (2011) fed *Labeo rohita* with dietary Baker's yeast (5%, 7.5% and 1.0%) for 60 days and different parameters were analysed at every 15 days interval. Increase in total erythrocytes, haemoglobin concentration, haematocrit, alkaline phosphatase, acid phosphatase, tissue protein, lipid, specific growth rate, phagocytic ratio, phagocytic index, leucocrit value and respiratory burst activity was recorded. Challenge studies against *Aeromonas hydrophila* have revealed a better survival rate. Thus yeast has stimulated the immune function and disease resistance in the fish rohu.

2.2.8 Levan as Immunostimulant

Levan is a natural polymer of fructose with β (2,6) linkages. Though plant fructans are available, many microorganisms like *Zymomonas mobilis, Bacillus subtilis, Bacillus polymyxa*, and *Acetobacter xylinum* also produce extracellular levan of high molecular weight when grown on sucrose which is serologically active, and capable of eliciting antibody formation.

Gupta *et al* (2008) fed *Labeo rohita* with dietary microbial derived levan (like glucan it is also a polymer of carbohydrate and a type of fructan consisting of β (2-6) linkages) in different concentrations (0, 0.25%, 0.5%, 0.75%, 1% and 1.25%) for 60 days and observed an increase in total erythrocytes, haemoglobin, total leucocytes, serum total protein, albumin globulin ratio, serum lysozyme and respiratory burst activity at 1% and 1.25% levels. Further, they also observed that when these fishes were infected with *A.hydrophila* no significant histo-architectural changes were observed. Thus levan significantly enhanced non-specific cell mediated immune parameters such as phagocytosis and oxidative extra and intra cellular radical production by leucocytes at 1.25% ameliorates the effects of infection by *Aeromonas hydrophila.*

Raivakhwada *et al* (2007) fed common carp (*Cyprinus carpio*) with dietary microbial levan (0, 0.1, 0.2, 0.5 and 1.0%) for 75 days and recorded an increase in total erythrocytes, haemoglobin, respiratory burst activity, lysozyme activity at 0.5% diet level, however no significant change was observed in total leucocyte counts, total protein and albumin/Globulin ratio. Further, survival was highest in 0.5% diet levan fed fishes when challenged with *Aeromonas hydrophila*.

2.2.9 Dimerized Lysozyme as Immunostimulant

Studnicka *et al* (2000) injected 10mg/kg body weight of Oxytetracycline, Oxolinic acid and Lindane (organochlorine pesticide) to suppress immune system in *Cyprinus carpio*, then immunized with *Yersinia ruckeri* vaccine and one group received dietary dimerized lysozyme (100µg/kg) before immunization & another group received it after immunization. Quantification of immunoglobulin secreting cells, antibody secreting cells, Immunoglobulin and specific antibody levels in the serum were studied after 5, 7, 14, 21 and 28 days. It was observed that dimerized lysozyme given both before and after vaccination has restored the cellular and humoral immune responsiveness and all

immunological parameters increased significantly. whereas, dimerized lysozyme introduced after vaccination also increased the cellular and humoral immune responsiveness but not significantly. Thus dimerized lysozyme, a natural immunostimulant, could be used for the restoration of cellular and humoral defence mechanisms and elimination of the immunodepressive effects of xenobiotics in aquaculture.

2.2.10 Nucleotides as Immunostimulants

Tahmasebi-Kohyani *et al* (2011) conducted a dose response trial on rainbow trout fingerlings in order to evaluate their effects on growth, humoral immune responses and resistance against *Streptococcus iniae.* A basal diet was supplemented with Optimun (Commercially available Nucleotides - Chemoforma, August, Switzerland) at 0.05%, 0.1%, 0.15% and 0.2% inclusion levels and fish were fed for eight weeks. Results showed that 0.05% dose was too low and thereafter increased growth, feed conversion ratio, alternative complement activity, lysozyme activity and IgM as nucleotide dose was augmented, reaching highest values for each of them at the highest nucleotide dose.

Lin *et al* (2009) reported the benefits of supplementing dietary nucleotides on growth and immune responses of grouper (*Epinephelus malabaricus*) juveniles. The first experiment assessed different inclusion levels of a nucleotide mixture containing equal amounts of IMP (Inosine Monophosphate), AMP (Adenosine Monophosphate), GMP (Guanosine Monophosphate), UMP (Uridine Monophosphate) and CMP (Cytidine Monophosphate). Weight gain and feed efficiency were highest in the group of grouper fed 0.15% of the nucleotide mixture; with head kidney leucocyte superoxide anion production ratio and plasma total immunoglobulin concentration also being higher than the fish fed with control diet. In the second experiment a diet containing 0.15% nucleotide mixture was compared against a control diet and also diets containing 0.15%

of IMP, AMP, GMP, UMP or CMP. From the second experiment it was concluded that juvenile grouper diets containing 0.15% AMP seemed to have better effects than other diets supplemented with different single-nucleotides. Overall conclusion showed that both growth and immune responses of juvenile grouper were enhanced in grouper diet with 0.15% nucleotide mixture.

Jha *et al* (2007) tested yeast nucleotides, in the form of RNA (Sisco Research Laboratories, India), at 0.4% and 0.8% inclusion levels on *Catla catla* fingerlings to assess potential changes in haemato-immunological responses followed by a challenge trial against *A. hydrophila* after 60 days feeding. Feeding dietary nucleotides increased leucocyte counts, total protein, globulins, and albumin: globulin ratio, lysozyme activity and respiratory burst activity compared to controls. Fish fed 0.4% nucleotides also showed significantly higher survival than control fish.

2.2.11 Herbs (Plants) as Immunostimulant

Natural plant products have been reported as antistress, growth promotion, appetite stimulation, tonic, immunostimulation, and to have aphrodisiac and antimicrobial properties in finfish and shrimp larviculture due to the presence of active principles such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils. The other merits that herbs as immunostimulants are local availability, broad spectrum effect, cost effective, no side effects and biodegradable.

Iruthayam *et al* (2014) fed catfish (*Mystus montanus*) with dietary *Ocimum tenuiflorum*, *Zingiber officinale* and *Allium sepa* (0.5g/100g each) for 45 days and analysed growth, haematological and serum biochemical parameters. Specific growth rate, total erythrocytes, haemoglobin, total leucocytes, total serum protein, glucose, cholesterol, magnesium levels, serum amylase, alkaline phosphatase, SGOT, SGPT and

GGT have increased in all herbal supplemented fishes (especially in *Z.officinale*) than control. Thus concluded that all the three herbs have immunostimulant potential and among the three herbs *Zingiber officinale* is more potent.

Chinnasamy Arulvasu *et al* (2013) fed *Catla catla* with dietary *Zingiber officinale* (0, 0.1, 0.5 and 1.0g/kg diet) for 30 days, analysed haematological parameters on 10^{th}, 20^{th} and 30^{th} day and observed an enhanced levels of total erythrocytes, haemoglobin, total leucocytes, serum bactericidal activity, total serum protein besides enhanced specific growth rate. They also observed that the relative percentage survival was high at 1.0g/kg diet when infected with *Aeromonas hydrophila*. Thus ginger acts not only as immunostimulant but also growth promoter.

Annalakshmi *et al* (2013) fed *Labeo rohita* with dietary *Phyllanthus amarus* (10g/100ml aqueous extract) for 14 days and recorded an increase in total erythrocyte counts, haemoglobin concentration, total leucocytes, lymphocytes, monocytes and neutrophils. When these fishes were challenged with *Aeromonas hydrophila* significant increase in total erythrocytes, haemoglobin concentration, total leucocytes, lymphocytes and a steady population of monocytes and neutrophils were observed. Thus dietary incorporation of *P.amarus* helps to improve the general health and resistance of the fish.

Sivagurunathan *et al* (2012) fed *Cirrhinus mrigala* with dietary *Nelumbo nucifera* (1% and 2% ethanolic extract) for 40 days, infected with *Pseudomonas aeruginosa.* The haematological parameters were studied after 5 days of post infection, observed that there is an increase in specific growth rate and feed conversion ratio and also significant increase in total erythrocyte counts, haemoglobin, neutrophils, monocytes, lymphocytes, serum protein and albumin in *N.nucifera* fed fishes, a clear sign of improved general health.

Asadi *et al* (2012) fed Rainbow trout (*Oncorhynchus mykiss*) with dietary *Nasturtium nasturitium* (Water cress Plant) extract (0.1% and 1%) for 21 days and found elevated levels of alternative complement activity, plasma lysozyme, total plasma protein and globulin, indicating enhancement in non specific immunity.

Sivagurunathan *et a*l (2012) fed Tilapia with dietary Amla (*Phyllanthus emblica*) (1% and 2% ethanolic extract) for 40 days and found a significant increase in specific growth rate and feed conversion ratio. When the same fishes were infected with *Pseudomonas aeruginosa* (10^{-1} dilution) and blood parameters were analysed after 5 days, they recorded significant rise in total erythrocytes, haemoglobin concentration, total leucocytes, lymphocytes, neutrophils, monocytes, serum total protein, albumin and globulin levels. Thus Amla enhances general resistance in fishes also.

Fazlolahzadeh *et al* (2011) fed Rainbow trout (*Oncorhynchus mykiss*) with dietary garlic powder (0.3%, 0.45 and 0.6%) for 58 days and observed an increase (in 0.45% and 0.6%) in total erythrocytes, total leucocytes and lymphocytes. However no significant changes were observed in haemoglobin, haematocrit, Alanine aminotransferase (ALT) and Aspartate aminotransferase levels (AST). Thus they have concluded that use of garlic balanced and formulated in suitable doses can decrease mortality rate and increase immunity.

Mohammad H Ahmad *et al* (2011) fed Nile Tilapia (*Oreochromis niloticus*) with different concentrations (0%, 0.5%, 1% and 1.5%) of dietary Cinnamon (*Cinnamomum zeylonicum*) for 90 days and observed an increase in specific growth rate, total erythrocytes, haemoglobin concentration, packed cell volume (PCV) and serum total Protein and a decline in serum lipid, urea, creatinine, glucose, Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) levels. Further, cinnamon diet fed fishes recorded low mortality when challenged with *Aeromonas hydrophila*.

Thus cinnamon was found to have an antibacterial activity antagonistic to *Aeromonas hydrophila* infection in fish.

Prasad and Priyanka (2011) fed Catfish *Pangasianodon hypothalamus* with feed supplemented with different concentrations of aqueous extract of *Garcinia gummi-gutta* (Medicinal plant) (0, 500,1000, 2000 and 3000mg/kg) for 45 days and observed a decline in specific growth rate, plasma glucose, total cholesterol, triglycerides and low density lipids and increase in total erythrocytes, haemoglobin concentration, total leucocytes, thrombocytes, packed cell volume, Mean corpuscular haemoglobin concentration, serum protein and high density lipids. He has suggested that 2000mg/kg is the optimum level for dietary incorporation.

Xavier Innocent *et al* (2011) incorporated *Coriandrum sativum* powder (2%) with feed, fed *Catla catla* for 14 days and analysed the blood parameters on 1, 3, 7 and 14^{th} day. They observed an increase in total erythrocyte count, total leucocyte count and haemoglobin concentration than control feed fed fishes. Further, the *C.sativum* incorporated diet fed fishes after 14^{th} day were infected with *Aeromonas hydrophila* (10^{-3} and 10^{-5} dilution) and again haematological parameters were analysed on 1, 3, 7 and 14^{th} day, which observed an increase only in total leucocyte counts in fishes infected with 10^{-3} dilution and a significant increase in total erythrocyte counts, haemoglobin and total leucocyte counts in fishes infected with 10^{-5} dilution of *A.hydrophila*.

Behra *et al* (2011) injected intraperitoneally Curcumin (purified compound of *Curcuma longa*) at different doses (0, 1.5mg, 150, 15 and 1.5µg) to *Labeo rohita*, blood was collected after 7, 14, 21and 42 days. Myeloperoxidase, Respiratory burst, haemagglutination, haemolytic and bacterial agglutination has increased in 1.5µg level. In another experiment, formalin killed *A.hydrophila* injected fishes previously injected

with 15 µg of curcumin showed increased bacterial agglutination. Thus curcumin enhances the resistance against *A.hydrophila*.

Prasad and Mukthiraj (2011) fed *Oreochromis mossambicus* with dietary methanolic extract of *Andrographis paniculata* (0, 500, 1000, 2000 and 3000 mg/kg) for 45 days and found a significant increase in specific growth rate, total erythrocytes, haemoglobin, total leucocytes, thrombocytes, packed cell volume and Mean corpuscular volume (MCV) indicating an increase in non specific immune response.

Sudagar Mohamad and Hajibeglou Abasali (2010) mixed equal amount of 70% ethanolic extract of medicinal herbs *Inula helenium, Brassica nigra, Tussilago farfara, Chelidoniume majus* and *Echineacea purpurea* and incorporated in the diet at different concentrations (0, 100, 250, 500, 750 and 1000mg/kg), fed *Cyprinus carpio* for 60 days. The haematological, biochemical and immunological parameters were investigated at 20, 40 and 60 days of feeding. Results indicated that respiratory burst activity, serum bactericidal activity, lysozyme, serum protein, albumin, globulin, total leucocytes, total erythrocytes and haemoglobin content were enhanced in fish fed with herbal diets. 10^{th} day after post challenge with *Aeromonas hydrophila* exhibited high survival rate. Thus, these herbal extracts have the potential to improve the general resistance of the test organism.

Hajibeglou Abasali and Sudagar Mohamad (2010) incorporated equal portions of *Ocimum basilicum, Cinnamomum zeylanicum, Juglans regia* and *Mentha piperita* ethanolic extracts in the feed in different concentrations (0, 250, 500, 750, 1000 and 1250mg/kg), fed to *Cyprinus carpio* for 45 days, analysed the blood samples on 15^{th}, 30^{th} and 45^{th} day. They have observed an increase in total leucocytes, total erythrocytes, haemoglobin concentration, total serum protein, albumin, globulin, bactericidal activity, respiratory burst activity and serum lysozyme. However, the plasma glucose level has

decreased. In the challenge studies, the group fed with 1000mg of herbal extract exhibited a higher survival percentage when infected with *Aeromonas hydrophila*.

Soner Bilen and Musa Bulut (2010) fed Rainbow trout (*Oncorhynchus mykiss*) with dietary laurel (*Laurus nobilis*) leaf powder (0.5% and 1%) for 21 days and observed different parameters on 21st, 42nd and 63rd day and found that phagocytic activity of blood leucocytes has increased significantly, where as no significant changes were observed in respiratory burst activity (both intra cellular & extra cellular), plasma protein level and serum lysozyme levels.

Sunitha *et al* (2010) prepared a paste of the plant *Coleus aromaticus* with glycerin and distilled water, applied to *Channa marulius* with moderate lesions and found that the wound healed completely after 10 days of application. Thus they have inferred that *C.aromaticus* has immunostimulatory potential.

Prit Benny *et al* (2010) administered Banana (*Musa acuminate*) peel extracts orally to *Clarias batrachus* and studied blood parameters after 12, 24, 36 and 48 hours, found an increase in total leucocyte count and lymphocyte population and a decrease in neutrophil and monocyte population at 48 hours. They also observed that histopathologically there was no change in spleen, heart and lung architecture.

Ramasamy Harikrishnan *et al* (2010) fed *Cirrhina mrigala* with *Azadirachta indica* (0.2% ethanolic extract) incorporated feed and Probiotic feed (0.2% *Lactobacillus rhamnosus* incorporated) for 30 days, infected with *Aphanomyces invadens* on 7th day and studied blood parameters on 30th day. In control feed fed infected fishes they observed an increase in total leucocyte counts and decline in total erythrocyte counts, haemoglobin concentration, haematocrit, lymphocyte, monocyte, eosinophil, neutrophil, total serum protein, calcium, cholesterol and glucose levels. Whereas, no significant

changes were observed in fishes fed with experimental diet. Thus herbal supplementation in diets provided effective protection.

Alishahi *et al* (2010) fed *Aeromonas hydrophila* bacterin immunized *Cyprinus carpio* with dietary *Aloe vera* (0.5%) for 8 weeks (56 days) and blood was drawn for every 14 days. They observed that fishes fed with *Aloe vera* feed exhibited and enhanced lysozyme activity, bactericidal activity, specific antibody levels, total leucocyte counts, serum protein, globulin levels and improved survival. Thus they concluded that oral administration of *Aloe vera* is able to enhance some specific and non-specific immune response in the common carp.

Mohsen Abdel-Tawwab *et al* (2010) fed Tilapia (*Oreochromis niloticus*) with dietary green Tea (*Camellia sinensis* - 0, 0.125, 0.25, 0.5, 1.0 and 2g/Kg) for 12 weeks and observed an increase in specific growth rate, tissue protein, tissue lipid, total erythrocytes, total leucocytes, lymphocytes, serum glucose, protein, albumin, globulin, lipid and respiratory burst activity at 0.5 – 2.0g levels. A decrease was observed in monocytes and granulocytes. Further, when these fishes were infected with *Aeromonas hydrophila* (5×10^5 cells/ml) less mortality than control feed fed fishes was observed.

Kaleeswaran *et al* (2010) fed *Catla catla* with dietary *Cynodon dactylon* ethanolic extract (0.05%, 0.5% and 5%) for 45 days, observed a linear increase in specific growth rate, tissue protein and lipid levels. Further, elevated levels of amylase and protease activity were found in hepatopancreas on 30^{th} day feeding trial at 0.5% and 5% levels. Non-specific immune parameter of serum antiprotease activity was higher in 5% level. The result indicates that 5% inclusion of *C.dactylon* mixed diet improves the growth performance, feed efficiency, body composition, digestive enzyme and antiprotease activity in *C.catla.*

Aly and Mohamed (2010) fed Nile Tilapia (*Oreochromis niloticus*) with extracts of *Echinaecea purpurea* (1.0 ppt) and Garlic (3%) for 1, 2 and 3months as separate experimental groups and fed all the groups with control diet for another 4 months. They observed that the specific growth rate has increased in all the experimental groups, both *E. purpurea* and Garlic has elevated total leucocytes, lymphocytes, haematocrit values and neutrophil adherence with prolonged application. Further they also observed better protection to fishes fed with *E.purpurea* and Garlic for 3 months when challenged with *Aeromonas hydrophila*.

Immanuel *et al* (2009) studied the antimicrobial efficiency of medicinal herbs *Cynodon dactylon, Aegle marmelos, Withania somnifera* and *Zingiber officinale* against *Vibrio mimicus* and *Vibrio vulnificus* and found the efficiency in decreasing order as *W. somnifera* > *Z.officinale* > *C.dactylon* > *A.marmelos*. Then prepared different diets incorporating 1 gram acetone extract of *Cynodon dactylon, Aegle marmelos, Withania somnifera* and *Zingiber officinale* and fed to *Oreochromis mossambicus* for 45 days. They observed an increase in growth, serum total protein, albumin, globulin, packed cell volume, leucocrit and phagocytic activity in all the experimental feed fed fishes and found that *Zingiber officinale* incorporation in diet produced pronounced results than other three herbs. Herbal diet fed fishes also exhibited higher survival rate when they are infected with *V.vulnificus*.

Dorucu *et al* (2009) fed Rainbow trout (*Oncorhynchus mykiss*) with dietary black cumin seeds (*Nigella sativa*) (1%, 2.5% and 5%) for 21 days and recorded elevated levels of hematocrit, leucocrit, nitroblue tetrazolium cell activation, serum protein and immunoglobulin at 2.5% levels. Thus black cumin seeds can be incorporated in diet to boost the non specific immune response in fishes.

Aly *et al* (2008) fed Garlic incorporated diet (0%, 10% and 20%) to *Oreochromis niloticus* for 1month and 2 months and analysed the haematological parameters after 8th month. Fishes fed garlic incorporated diet for 2 months exhibited a significant increase in growth, heamatocrit, monocyte population and respiratory burst activity, however no change was observed in total leucocyte counts. Further the fishes fed with garlic for 2 months and infected with *Aeromonas hydrophila* (10^8cfu/ml) exhibited an increase in survival than control feed fed fishes, better shelf life was also noted in garlic feed fed fishes.

Yin *et al* (2008) fed one set of fishes with *Lonicera* extract (1%) incorporated feed, another set with *Ganoderma* extract (1%) incorporated feed, another set with both *Lonicera* and *Ganoderma* extracts (0.5%+0.5%) and another set with control diet for 3 weeks. Blood parameters were analysed for 1, 2 and 3rd week and found that fishes fed with *Lonicera* and *Ganoderma* combined feed produced high phagocytic activity, high respiratory burst activity and high levels of plasma lysozyme concentrations. Further, when these fishes were infected with *Aeromonas hydrophila* ($3x10^6$ cells/ml) low mortality was observed in the fish group fed with *Lonicera* and *Ganoderma* combined feed. Thus incorporation of *Lonicera* and *Ganoderma* improved non specific immune response and longevity.

Abo-Esa (2008) evaluated LC_{50} of (96 hour) *Zingiber officinale* against catfish *Clarias gariepinus* as 192mg/l. The bath treatment with ginger at 20mg/l concentration can effectively treat the protozoan ectoparasites like *Trichodina* and *Epistylis* species but not suitable for treating Monogean *Gyrodactylus* species. Further, he observed an increase in dissolved oxygen content and a decrease in total ammonia in the ginger added water. Thus ginger also improved the quality of water.

Jaya Kumari *et al* (2007) fed *Labeo rohita* larvae on plankton, ImmuPlus (ImmuPlus a poly herbal formulation containing the extracts of selected natural Indian medicinal plants viz., (*O.sanctum, W.somnifera, T.cordifolia* and *E.officinalis*)-mixed compound feed, and plankton plus Immuplus-mixed compound feed (ImmuPlus added at three dose levels of 0.25, 0.5 and 0.75g/kg feed) from 4th day to 14th day. ImmuPlus-mixed diets enhanced growth of larvae, survival and disease resistance against *Aeromonas hydrophila* challenge. In two other experiments, advanced rohu larvae and fingerlings were fed with ImmuPlus-mixed compound feed (at 0, 0.5, 1.0 and 2.0g/kg) for 15, 30 and 45 days. At the end of 45 days for advanced larvae and 30 days for fingerlings, the fish fed with ImmuPlus at 1g/kg level showed significantly higher growth and disease resistance against *A.hydrophila* challenge. In a separate experiment, juveniles of rohu were fed with 1g/kg of ImmuPlus incorporated feed for 15 days and 30 days. At the end of the trial, the Immuplus fed fish showed enhanced non-specific immunity (as measured through nitroblue tetrazolium reduction assay, serum lysozyme activity, serum haemolysin titre and resistance against *A.hydrophila* challenge in non-vaccinated fish as well as specific immunity levels (as measured through bacterial agglutination titre against *A.hydrophila* in vaccinated fish). Thus incorporation of ImmuPlus at 1g/kg level in the diet of rohu may be beneficial for enhancing disease resistance.

Samuel Sudhakaran *et al* (2006) injected petroleum ether extracts and ethanolic extracts of *Tinospora cordifolia* (0.8, 8 and 80mg/kg body weight) to Tilapia (*Oreochromis mossambicus*), followed by heat killed *Aeromonas hydrophila* injection. Prolonged peak of primary antibody titre in all groups upto 3 weeks, secondary antibody titre has increased in ethanolic extract at the dose of 8mg/kg and petroleum ether extract at the doses of 0.8 and 8mg/kg, further the neutrophil activity has enhanced in all the

ethanolic extracts. They also observed that injection with petroleum ether or ethanolic extracts of *T.cordifolia* at 8mg/kg protected against experimental infection with virulent *Aeromonas hydrophila*.

Vasudeva Rao and Rina Chakrabarti (2004) fed *Achyranthes aspera* (0.5% Aqueous extract) incorporated diet to *Labeo rohita* for 4 weeks, immunized with 500µl of chickenRBC and analysed the non specific an specific immune parameters after 7, 14, 21 and 28th day. *A.aspera* fed fishes maintained high level of serum anti-trypsin activity (Non specific immune response), antigen specific antibody levels, serum globulin levels and RNA/DNA ratios (Specific immune response). Thus *A.aspera* has improved both specific and nonspecific immune response in *L.rohita*.

Suheyla Karatas *et al* (2003) fed Rainbow trout (*Oncorhynchus mykiss*) with aqueous extracts of dietary mistletoe (*Viscum album*), Nettle (*Urtica dioica*) and Ginger (*Zingiber officinale*) for 21 days and observed no change in specific growth rate but significant increase in respiratory burst activity and phagocytosis of blood leucocytes in ginger diet fed fishes. They also observed that the total plasma protein has increased in all the experimental diets except ginger.

2.3.1 Animal Studies in Ferns

Ferns and their allied plants being the major constituent of biodiversity their role in the system of nature cannot be overlooked or neglected in the global changing scenario (Sharpe 2011). There is not much information was available in the literature about the medicinally important ferns except a few studies (Karthik *et al* 2011). The specific knowledge of plants as a source of medicine dates back to the time of “Rig Veda”. Much of this knowledge remained secret and often passed on from one generation to another through words of mouth instead of records. This knowledge is

vanishing on account of rapid industrialization and modernization. Documenting the indigenous knowledge through ethanobotanical studies is important for the conservation and utilization of biological resources.

Kamini Srivastava (2007) has reported the ethanomedicinal values of 26 ferns and Benjamin and Manickam (2007) has reported the ethanomedicinal values of 61 ferns of Western ghats which are collected from the tribal communities and Medicine man. Animal studies has been conducted only in few ferns.

Reginald and Timothy (2014) fed *Clarias gariepinus* with different levels of fern (*Asplenium barteri*) (0%, 5%, 10%, 15%, 20% and 25%) for 56 days and analysed the growth parameters. The results indicated an increase in %weight gain, feed conversion, protein efficiency ratio and fish condition. Thus dietary supplementation up to 10% level can be used as protein supplement.

Gautam *et al* (2013) studied antiallergic activity and antistress (adaptogenic activity) of *Actionpteris radiata* in wistar rats. Rats were injected with ethanolic extracts of *Actionpteris radiata* (25, 50 and 100mg/kg) intra peritoneally to wistar rats one hour before milk induced eosinophilia. After 24 hours decrease in eosinophil counts (100mg dose) and significant inhibition of leukocytosis exhibited the potential antiallergic and antistress activity of *A.radiata*.

Naga Sravanthi *et al* (2012) administered different doses of ethanolic extracts of *Actinopteris radiata* (100, 200 and 400mg/kg) to CCL_4 induced hepatotoxicity wistar Albino rats once daily for 7 days and observed at doses 200 and 400mg/kg offered significant hepatoprotective activity by reducing the serum marker enzymes like SGPT, SGOT, ALP, bilirubin.

Naik and Jadge (2010) injected intraperitoneally 300mg/kg ethanolic extract of *Actinopteris radiata* to writhing induced (by injecting 1% acetic acid) albino mice, found a decrease in writhing. Thus *A.radiata* extracts exhibits potent analgesic activity.

Gayathri *et al* (2005) administered orally different extracts (Aqueous, Ethanolic and n-Hexane) of different species of the fern Selaginella (*S.involvens, S.delicatula & S.wightii*) to mice immunized with sheep RBC. After 7 days, they observed that the fern extracts have not influenced the humoral antibody titre and antibody secreting spleen cells, but remarkable increase in the weight of thymus of mice administered with aqueous extract of *S.involvens*. As thymus is known to be one of the leading regulator of ageing and age-dependent decline in thymus dependent immune functions, there is a scope to use this fern as an agent to prolong life span and reduce old age related health complications.

Ofelia and Armando (2003) fed Abalone (*Haliotis asinina*) gastropod with different leaf incorporated diet (*Moringa oliefera, Azolla pinnata, Carica papaya* and *Leucaena leucocephala*) for 120 days. *M.olifera* and *A.pinnata* diet fed Abalone produced more specific growth rate. Thus they have concluded that *M.olifera* and *A.pinnata* are promising alternative feed ingredients (Protein substitute) for practical diet for farmed Abalone. As *Azolla* can take up heavy metals from polluted water it can be also used in bioremediation. (Sanyahumbi *et al* 1998).

2.3.2 Animal Studies in *Marsilea quadrifolia*

Prafulla Soni and Lal Singh (2012) explored the traditional tribal knowledge about the fern *Marsilea quadrifolia*. Information collected from the "HO" tribes – Jaduguda-Jharkand revealed the different medicinal properties of *M.quadrifolia*. They have reported that *M.quadrifolia* is used to relieve hypertension, sleep disorders,

headache, cough, convulsive condition of leg and muscle, respiratory troubles, epilepsy, infantile diarrhea and migraine. This fern is also being used to improve lactation after childbirth and to treat lesions in mouth and tongue and rheumatism.

Uma and Pravin (2013) evaluated the *in vitro* cytotoxic activity of methanol, aqueous and ethyl acetate extracts of leaves of *M.quadrifolia* on MCF-7 cells (Human Breast Adenocarcinoma) and found that methanol and ethyl acetate extracts demonstrated strongest antioxidant and anti-proliferative activities. Thus *M.quadrifolia* has a vast potential as medicinal drug especially in breast cancer treatment. Similar anti-oxidant activity by DPPH (α α Diphenyl β-picryl hydrazyl) free radical scavenging was also reported by Mathangi and Prabhakaran (2013).

Aswini *et al* (2012) administered two doses of (250mg/kg, 500mg/kg) ethanolic extract of *Marsilea quadrifolia* for seven successive days in separate groups of mice, both the doses significantly improved the learning and memory in mice and significantly reversed the amnesia induced by scopolamine. This beneficial effect on learning and memory was due to facilitation of cholinergic transmission in mouse brain. Thus *M.quadrifolia* acts as a memory enhancing agent.

Reddy *et al* (2012) reported that the hydro alcoholic extract of whole plant of *Marsilea quadrifolia* significantly potentiated the phenobarbitone induced sleeping time suggesting probable tranquilizing action as well as central nervous system (CNS) depressant action in Wistar rats. The extract was also found to cause reduction in spontaneous activity, decrease in exploratory behavioural pattern by swimming and pole climbing test, reduction in the muscle relaxant by traction test. Thus *M.quadrifolia* possess minor tranquilizing effect.

Aquatic and semiaquatic macrophytes play an important role in maintaining the riverine ecosystem (Gopal and Zutshi 1998). *Marsilea quadrifolia* an aquatic fern which is commonly available in the habitat were the protein producers like fish are living. As no work has been reported in literature about the immunostimulant role of *M.quadrifolia* especially to fish, the present work has been undertaken. Further *M.quadrifolia* is found in aquatic habitats and decaying parts of the fern may be dispersed in water and act as Immunostimulant to fishes in native waters. Hence an indepth study has been proposed in this investigation to identify and characterize the immunostimulatory role of *Marsilea quadrifolia*.

CHAPTER - 3

Phytochemical analysis of marsilea quadrifolia and its antimicrobial potential

Sivagurunathan A, Immunodiagnostic studies in a chosen fresh water fish administered with a medicinal Aquatic fern *Marsilea quadrifolia,* Ph.D. Thesis, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India.

CHAPTER – 3

PHYTOCHEMICAL ANALYSIS OF *MARSILEA QUADRIFOLIA* AND ITS ANTIMICROBIAL POTENTIAL

3.1 INTRODUCTION

India is a mega biodiversity country not only with rich source of medicinal plants, but also with valuable information on traditional medical practices (Pauline *et al* 2012). The history of herbal medicine starts from the ancient human civilization. The wealth of India is stored in the enormous natural flora which has been gifted to Indians (Dixit 1974, Dixit and Vohra 1984).

Traditional healers and their plant medicines provide the only health care to majority of people in a curative rather than a preventive approach in the developing countries for common ailments (Gabriel *et al* 2007). From time immemorial herbal products were used for curing diverse type of bacterial, fungal and viral diseases. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for discovery of new drug because of the unmatched availability of chemical diversity. According to WHO, 80% of the world's population primarily those of developing countries rely on plant-derived medicines for their healthcare needs (Gurib 2006). Plant products and related drugs are used to treat 87% of all categorized diseases (Grover *et al* 2002). The ready availability and economy of plants as direct therapeutic agents make plants more attractive when compared to modern medicine (Agbo and Ngogang 2005). As a result, vast literature now exist on the use of traditional medicine with botanist reporting description of plants used for disease treatments, the phytochemist on the chemical constituents and the pharmacologist on the effectiveness of particular plant compound or extracts (Gabriel *et al* 2004). According to WHO (2000)

medicinal plants are plants, which when administered to man or animal exert a sort of pharmacological action on them. Herbs make up most of the plant sources for the production of useful drugs that are being utilized by people worldwide (Agbo *et al* 2000).

Phytochemicals are chemical compounds formed during the plants normal metabolic processes. These chemicals are often referred to as "Secondary metabolites" of which there are several classes including alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids (Okwu 2005). In addition to these substances, plants contain other chemical compounds. These can act as agents to prevent undesirable side effects of the main active substances or to assist in the assimilation of the main substances. Plants have an almost limitless ability to synthesize aromatic substances, mainly secondary metabolites of which 12,000 have been isolated, a number estimated to be less than 10% of the total (Mallikharjuna *et al* 2007). These active components serve as molecules of plant defence against attack by microorganisms, insects and herbivores and at the same time also exhibit medicinal properties for treating several ailments.

Pteridophytes (ferns and fern allies) are called as reptile group of plants and are one of the earliest groups of vascular plants. Most of the indigenous people are not well aware of the uses of pteridophytes since it is not easily available like flowering plants. Pteridophytes have an important role in the earth's biodiversity (Karthik *et al* 2011). It is the demand of present time to discover new alternative antimicrobial compounds with diverse chemical structure and novel mechanism of action for new and reemerging infectious diseases (Rojas *et al* 2003).

In the present chapter the aquatic fern *Marsilea quadrifolia* with proven medicinal properties was evaluated to identify the presence of different secondary

metabolites through phytochemical screening and GCMS analysis. Further its ability to resist the growth of microbes was evaluated through antimicrobial studies.

3.2 MATERIALS AND METHODS

The plant selected for the present study is an aquatic fern *Marsilea quadrifolia.*

3.2.1 Systematic Position

Kingdom	-	Plantae
Division	-	Pteridophyta
Class	-	Pteridopsida
Order	-	Salviniales
Family	-	Marsileaceae
Genus	-	Marsilea
Species	-	*M.quadrifolia*

3.2.1.1 Description

Marsilea quadrifolia Linn is an aquatic fern which belongs to the family (Marsileaceae) commonly named as Aaraikeerai in Tamil, Neeraral in Malayalam and Cauptiya, Sunsuniya in Hindi. It is an aquatic fern bearing 4 parted leaf resembling '4-leaf clover', and the leaves float in deep water or erect in shallow water or on land. It possesses long stalked petiole with 4 clover like lobes and are either held above the water or submerged (Plate – 1). The plant prefers light (sandy) and medium (loamy) soils. It can grow in semi-shade (light woodland) or no shade. It requires moist or wet soil and can grow in water. *Marsilea quadrifolia* can be grown as a potted plant, either just with soil kept wet, or semi-submerged, with fronds emergent from the water, or fully submerged, with the fronds floating on the surface of the water. In the aquarium, water clover is grown fully submerged, usually in the foreground where it spreads by means of

runners. It normally seems to be unfussy as to light and water conditions, and doesn't need a rich substrate.

Marsileas are very easy to germinate from their sporocarps. However, the sporcarps must be abraded, cracked, or have an edge sliced off before submerging them in water so that the water can penetrate to swell the tissues, and germination is infrared-light dependent. Full sunlight is fine for this purpose (Wikipedia – *Marsilea quadrifolia*).

The fresh plants of *M.quadrifolia* were collected from natural habitat of Papanasam at Western Ghats, Tirunelveli, Tamil Nadu, India. The collected plants was identified by Prof.A.Saravana Gandhi, PG Department of Botany, Rani Anna Government College for Women, Tirunelveli in association with Foreau herbarium, Centre for Biodiversity and Biotechnology, St.Xavier's College, Palayamkottai, TamilNadu, India (Voucher specimen Number KG001).

The collected plants were washed well with deionized water repeatedly to remove the adhered mud and other impurities, shade dried for about 10 days, powdered, packed air tightly and stored in refrigerator. Plant extracts were prepared by dissolving the plant powder in the solvents Ethanol, Benzene and distilled water (Aqueous) in the ratio of 1:10. The containers were intermittently agitated for 48 hours and filtered through whatman No-1 filter paper. The filtrate was condensed using rotary evaporator and stored air tightly in the refrigerator.

3.2.2 Qualitative Analyses of Phytochemical Constituents

The phytochemical analyses of Steroids, Reducing sugar, Alkaloids, Phenolic compounds, Flavonoids, Saponins, Tannins and Amino acids were carried out following the procedure described by Brindha *et al* (1981) and the presence of Proteins, Chloride, and vitamin C were tested following Sofowra 1993 and Harborne 1973.

Plate - 1 : *Marsilea quadrifolia*

S.No	Experiment	Expected Observation	Inference
1	Test solution+ a few drops of $CHCl_3$ + 3-4 drops of acetic anhydride and one drop of Con.H_2SO_4.	Purple colour changing to blue or green	Presence of Steroids
2	Test solution + 2ml of Fehling's reagent and 3ml of water and boil	Red or Orange colour	Presence of reducing sugars
3	Test solution taken with 2N HCl Aqueous layer forked decanted and to which are added one or two drops of Mayer's reagent.	White turbidity or precipitate	Presence of Alkaloids
4	Test solution in alcohol + one drop of neutral ferric chloride	Intense colour	Presence of Phenolic compounds
5	Test solution in alcohol + a bit of magnesium and one or two drops of con .HCl	Red or Orange colour	Presence of Flavonoids
6	Test solution + H_2O + and shaken well	Foamy lather	Presence of Saponins
7	Test solution + H_2O + Lead acetate solution	White precipitate	Presence of Tannins
8	Test Solution + 1% Ninhydrin in alcohol	Blue or violet colour	Presence of Amino acids
9	Test solution + few drops of 4% NaOH + few drops of 1% $CuSO_4$	Violet or Pink colour	Presence of Proteins
10	Diluted Test solution + 1 drop of 5% sodium nitroprusside + 2ml dil.Sodium hydroxide + 0.6ml HCl added dropwise	Yellow colour turns Blue	Presence of Vitamin C
11	Test Solution prepared in HNO_3+ few drops of 10% $AgNO_3$	White precipitate	Presence of Chloride
12	Test solution + 2ml Acetic anhydride + 1or 2 drops of Con.H_2SO_4	An array of colour change	Presence of Phytosterols

3.2.3 Quantitative Analysis of Phytochemical Constituents

The total Carbohydrates was estimated by Anthrone method (Yemm and Willis 1954), Total Proteins by Lowry's Method (Lowry *et al* 1951), Total Flavonoids by Aluminium Chloride method (Aiyegroro and Okoh, 2010), Total Amino acids by Ninhydrin Method (Moore & Stein 1948) and Saponins by the method described by Obadoni and Ochuko (2001).

3.2.3.1 Estimation of total carbohydrates

The carbohydrates concentration was measured by Anthrone method (Yemm and Willis 1954). 100 mg of plant sample was weighed and taken into a boiling tube, hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and the mixture was cooled at room temperature. Using sodium carbonate it was neutralized and the volume was made up to100 ml. Then it was centrifuged and supernatant was collected. 0.5 ml of aliquots was used for estimation. The working standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard glucose solutions and '0' serves as blank. 0.1 and 0.2 ml of the test sample solution was taken in two separate test tubes. In all the test tubes the volume was marked up into 1 ml with water and blank was set with 1 ml of water. 4 ml of anthrone reagent was added to each tube. Mixed well and kept it in water bath for 10 minutes. Cool the contents rapidly and the absorbance were read at 630 nm and the amount of total carbohydrate present in the sample was calculated.

3.2.3.2 Estimation of Protein

The plant sample was homogenized in 10% cold Tri Chloro Acetic acid TCA (10 mg: 5 ml) and was centrifuged at 5000 rpm for 10 minutes. Supernatant was discarded and pellets were saved. Pellets were again suspended in 5 ml of 10% cold TCA and recentrifuged for 10 minutes. Supernatant was again discarded and the precipitate

was dissolved in 10 ml of 0.1 N NaOH 0.1 ml of this solution was used for protein estimation. In 1 ml of plant sample, total protein content was estimated using the protocol of Lowry *et al* (1951). A stock solution (1mg/ml) of bovine serum albumin was prepared in 1 N NaOH; five concentrations (0.2, 0.4, 0.6, 0.8 and 1ml) from the working standard solution were taken in series of test tubes. In another set of test tubes 0.1 ml and 0.2 ml of the sample extracts were taken and the volume was raised up to 1 ml in all the test tubes. To each test sample, 5ml of freshly prepared alkaline solution was added at room temperature and left undisturbed for a period of 10 min. Subsequently, to each of these mixture tubes 0.5 ml of Folin-Ciocaltcau reagent was rapidly added and incubated at room temperature for 30 minutes until the blue colour develops. The absorbances were read at 750 nm and the amount of total protein present in the sample was calculated.

3.2.3.3 Estimation of total flavonoids

Aluminium chloride colorimetric method was used with some modifications to determine flavonoid content. 1ml of sample plant extract was mixed with 3ml of methanol, 0.2ml of 10% aluminium chloride, 0.2ml of 1M potassium acetate and 5.6ml of distilled water and remains at room temperature for 30 minutes. The absorbance was measured at 420nm. Quercetin was used as standard (1mg/ml). All the tests were performed in triplicates. Flavonoid contents were determined from the standard curve and were expressed as quercetin equivalent (mg/g of extracted compound) (Aiyegroro and Okoh, 2010).

3.2.3.4 Estimation of Amino acids

500mg of sample was weighed and ground in a pestle and mortar with a small quantity of acid-washed sand. To this homogenate, 5to 10ml of 80% ethanol was added. The supernatant was collected by centrifugation. Repeat the extraction twice with the

residue and pool all the supernatants. Reduce the volume if needed by evaporation and use the extract for the quantitative estimation of total free amino acids.

For the preparation of Standard 50mg leucine was dissolved in 50mL of distilled water in a volumetric flask. From that 10mL was diluted to 100mL in another flask for working standard solution. A series of volume from 0.1 to 1mL of this standard solution gives a concentration range 10µg to 100µg. Proceed as that of the sample and read the color. 0.1mL of plant extract was taken, to that 1mL of ninhydrin solution was added and made up the volume into 2mL with distilled water. The tube was heated in boiling water bath for 20min. 5mL of the diluents was added and mixed, after 15min the intensity of the purple color against a reagent blank in a colorimeter at 570nm was read. The color is stable for 1hour. The reagent blank was prepared as above by taking 0.1mL of 780% ethanol instead of the extract. A standard curve was drawn using absorbance versus concentration. The concentration of the total free amino acids in the sample was found and expressed as percentage equivalent of leucine (Moore and Stein 1948).

3.2.3.5 Estimation of Saponin

The method used was that of Obadoni and Ochuko (2001). 20g of sample powder was put into a conical flask and 100 ml of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation

the samples were dried in the oven to a constant weight and the saponin content was calculated as percentage.

3.2.4 GC-MS Analysis

GC-MS Analysis of the extract was performed using Perkin-Elmer GC Clarus 500 system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with a Elite-5Ms, fused silica capillary column (30mmx0.25mm X o.25µM df, composed of 5% Diphenyl/95% Dimethyl poly siloxane). For GC-MS detection, an electron ionization system with ionizing energy of 70eV was used. Helium gas (99.99%) was used as the carrier gas at constant flow rate 1ml/min and an injection volume of 1µl was employed (split ratio of 10:1); Injector temperature 250^{o}C; The oven temperature was programmed from 110^{o}C (isothermal for 2 min.), with an increase of 10^{o}C /min upto 200^{o}C, then 5^{o}C /min to 280^{o}C, ending with a 9 minute isothermal at 280 ^{o}C . Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 minutes. The relative % amount of each component was calculated by comparing its average peak area to the total area. The software adopted to handle mass spectra and chromatogram was Turbomass 5.2. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST)-version Year 2005. The spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test material were ascertained.

3.2.5 Anti-Microbial Activity Assay

Antimicrobial activities of plant extract were determined by well diffusion method (Anushia *et al* 2009). Five bacterial strains (*Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa and Aeromonas hydrophila*) and three fungal strains (*Aspergillus niger, Candida albicans* and *Pencillium*

notatum) were used in this investigation. The media used for antibacterial test were Nutrient Broth. The test bacterial strains were inoculated into nutrient broth and incubated at 37^0C for 24hrs. After the incubation period, the culture tubes were compared with the turbidity standard. Fungal inoculums were prepared by suspending the spores of fungus (as previously cultured) in saline water mixed thoroughly, made turbidity standard and used. Fresh bacterial culture of 0.1ml having 10^8 CFU was spread on nutrient agar (NA) plate using swab. The fungal strains are also spread similarly but the medium was Potato dextrose agar (PDA). Wells of 6 mm diameter were punched off into the medium with sterile cork borer and filled with 50µl of plant extracts by using micro pipette in each well in aseptic condition. Plates were then kept in a refrigerator to allow pre-diffusion of extract for 30minutes. Further the plates were incubated in an incubator at 37^0C for 24hours and 28-30^0 C for 3-4 days for bacterial and fungal cultures respectively. The antimicrobial activity was evaluated by measuring the zone of inhibition.

3.3 RESULTS

3.3.1 Qualitative Analysis of Phytochemical Constituents

Qualitative phytochemical analysis on benzene, Ethanol and Aqueous extracts of *Marsilea quadrifolia* was performed. In the benzene extract of *Marsilea quadrifolia* the results were positive for five compounds (Reducing sugar, amino acid, Phenolic compounds, Flavonoids and phytosterols). Whereas 8 compounds showed positive results in ethanolic extract (Reducing sugar, Protein, Tannin, Phenolic compounds, Flavonoids, Alkaloids, Phytosterols and saponins) and 7 compounds in aqueous extract (Reducing sugar, Amino acids, Tannins, Phenolic compounds, Flavonoids, Alkaloids and Phytosterols). The common compounds present in all the extracts were Reducing sugars, Phenolic compounds, Flavonoids and Phyto sterols, similarly the compounds present in

both ethanolic and aqueous extracts were Reducing sugars, Tannins, Phenolics compounds, Flavonoids, Alkaloids and Phytosterols. Proteins and Saponins were identified only in ethanolic extract (Table-1).

Table: 1. Qualitative Phytochemical analysis of *Marsilea quadrifolia*

Name of the tests	**Benzene extract**	**Ethanol extract**	**Aqueous extract**
Reducing sugar	+	+	+
Amino acid	+	-	+
Vitamin- C	-	-	-
Protein	-	+	-
Iron	-	-	-
Tannin	-	+	+
Phenolic Compound	+	+	+
Flavonoid	+	+	+
Alkaloid	-	+	+
Phytosterols	+	+	+
Steroids	-	-	-
Saponins	-	+	-
Total Compounds	**5**	**8**	**7**

'+' indicates presence of compounds; '-'indicates absence of compounds

3.3.2 Quantitative Analysis of Phytochemical Constituents

The quantitative analysis of *Marsilea quadrifolia* has revealed the presence of 200mg of carbohydrates, 51mg of proteins, 28mg of amino acids, 3mg of flavonoids and 2.8mg of saponins per gram of plant powder (Table-2).

Table:2. Quantitative Analysis of Phytochemicals of *Marsilea quadrifolia* (Mean±SD)

Contents	mg/g
Total carbohydrate	200±10.2
Total protein	51±3.4
Total amino acid	28±3.2
Total flavonoids	3±0.4
Total Saponin	2.8±0.5

3.3.3 GC-MS Analysis

GC-MS analysis of *Marsilea quadrifolia* whole plant extract revealed the presence of 13 compounds. The name of the compound with retention time, molecular formula, molecular weight, and concentration was listed in Table-3.

The thirteen compounds were Hexadecanoic acid ethyl ester (26.88%), Phytol (16.97%), 9,12-Octadecadienoic acid (Z,Z) (12.46%), 1,2-Benzenedicarboxylic acid, diisooctyl ester (8.62%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (6.71%), 2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl (ñ) (6.56%), Octadecanoic acid, ethyl ester (5.69%), 2-Piperidinone, N-[4-bromo-n-butyl (4.53%), Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-hydroxy-, (3á,17á) (4.16%), Z,Z,Z-1,4,6,9-Nonadecatetraene (3.86%), 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E) (1.82%), 10-Undecen-1-al, 2-methyl (1.20%), 5à-Androstan-16-one, cyclic ethylene mercaptole (0.53%) (Plate-2 & 3).

3.3.4Antimicrobial Activity

Antimicrobial activity for all the three extracts was studied against five bacterial strains (*Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, P.aeruginosa*

Plate-2 : GC-MS Chromatogram of *Marsilea quadrifolia*

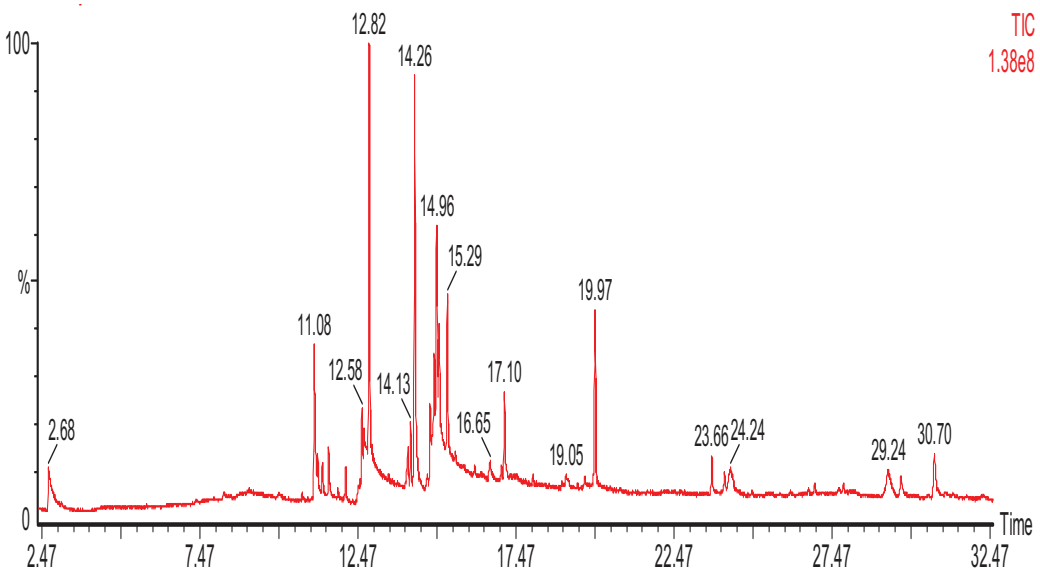
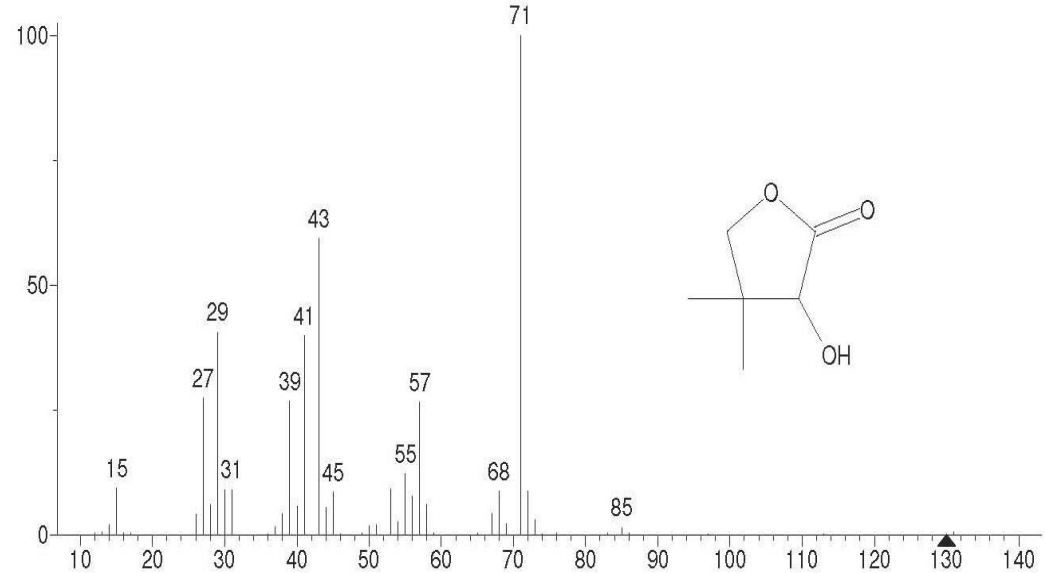
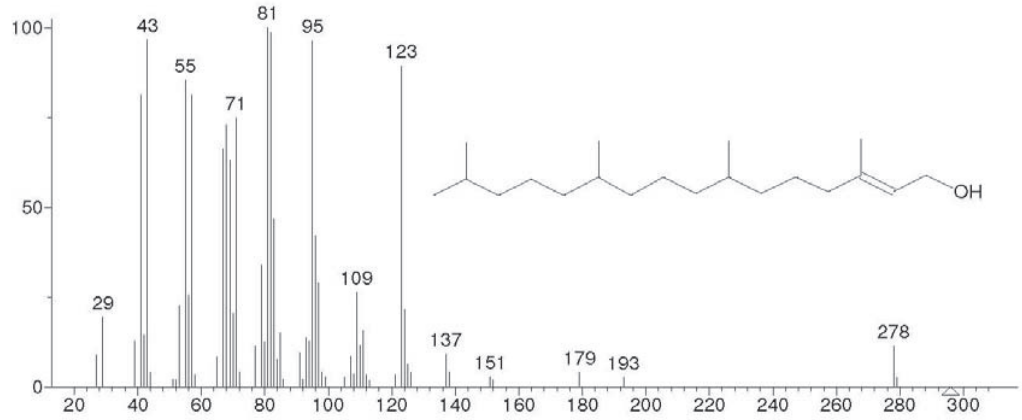


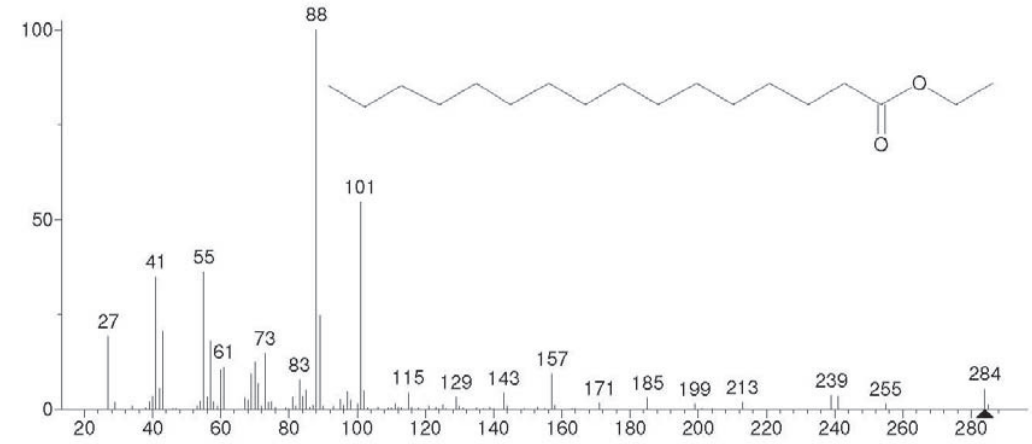
Plate – 3 : Structure of various chemical compounds identified in *M.quadrifolia*



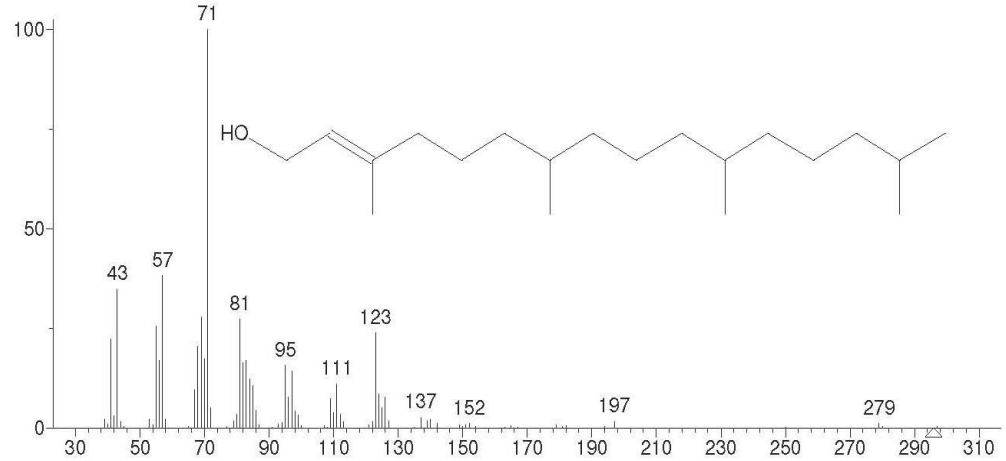
(mainlib) 2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/-.)-



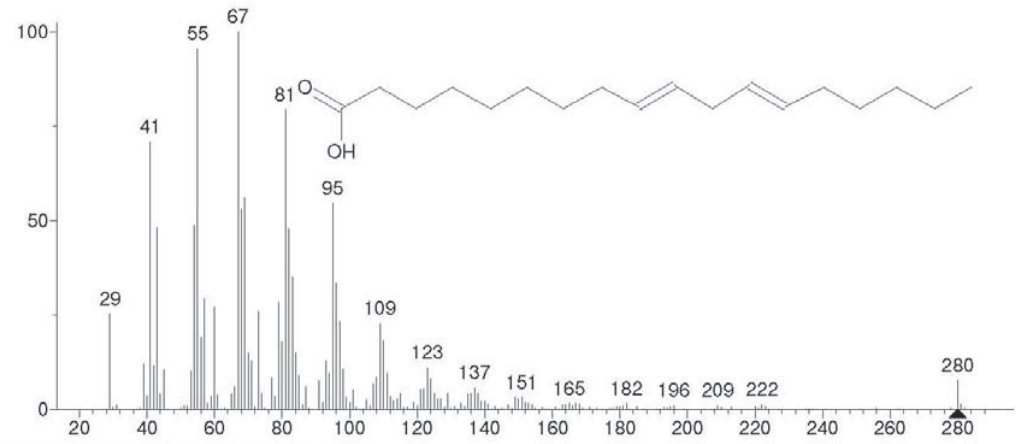
(mainlib) 3,7,11,15-Tetramethyl-2-hexadecen-1-ol



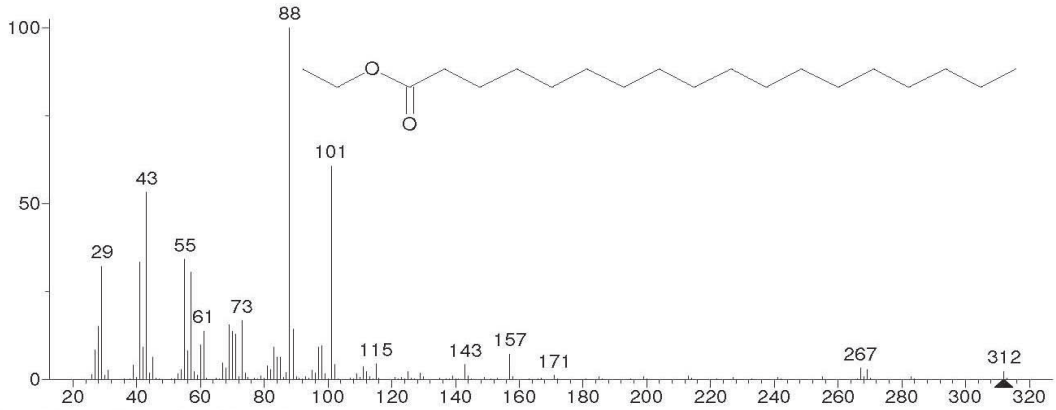
(replib) Hexadecanoic acid, ethyl ester



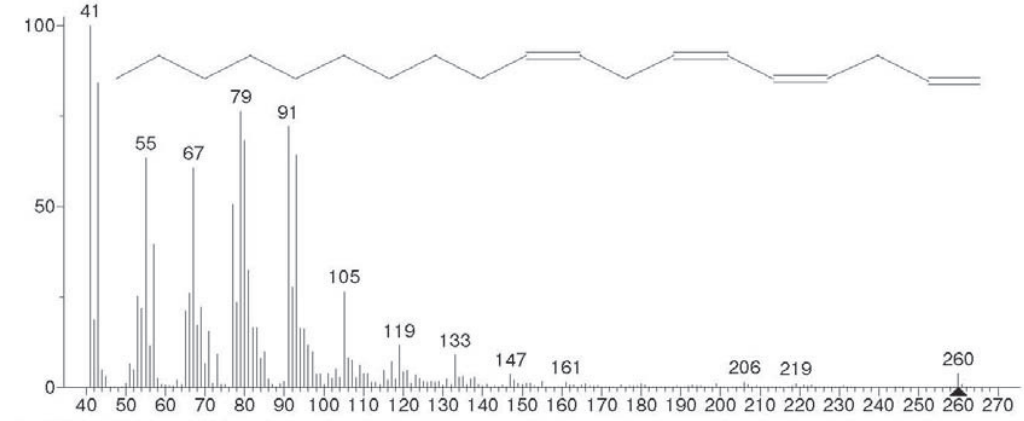
(replib) Phytol



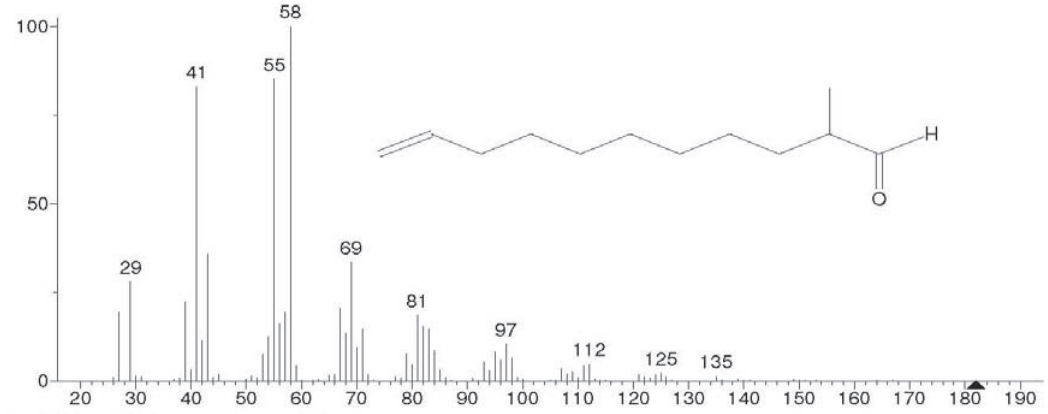
(replib) 9,12-Octadecadienoic acid (Z,Z)-



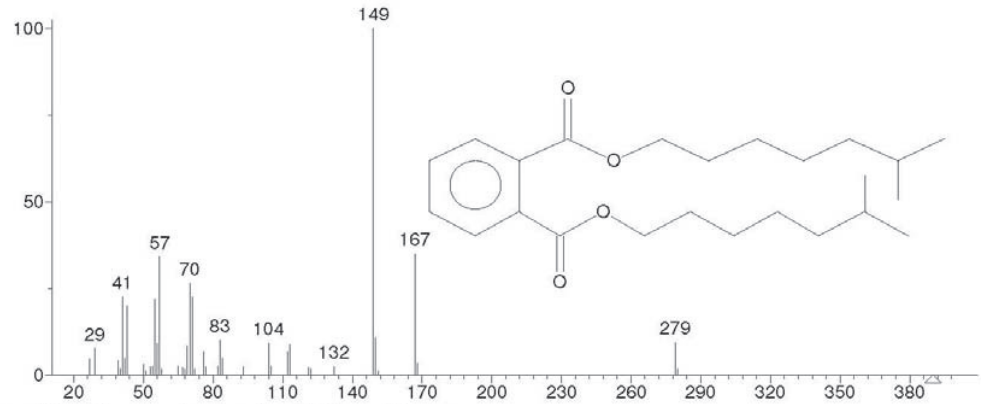
(replib) Octadecanoic acid, ethyl ester



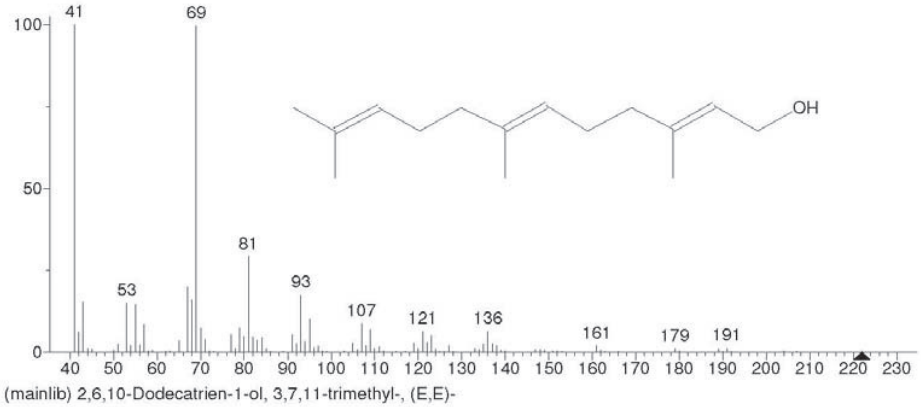
(mainlib) Z,Z,Z-1,4,6,9-Nonadecatetraene



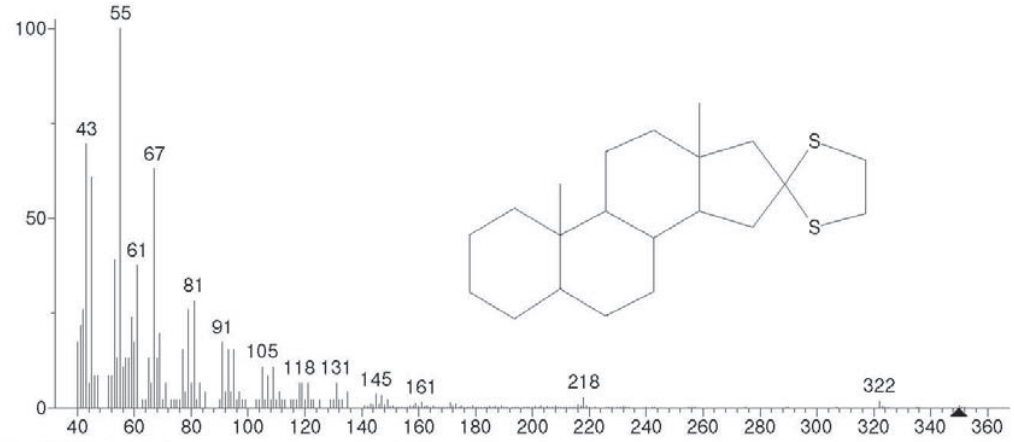
(mainlib) 10-Undecen-1-al, 2-methyl-



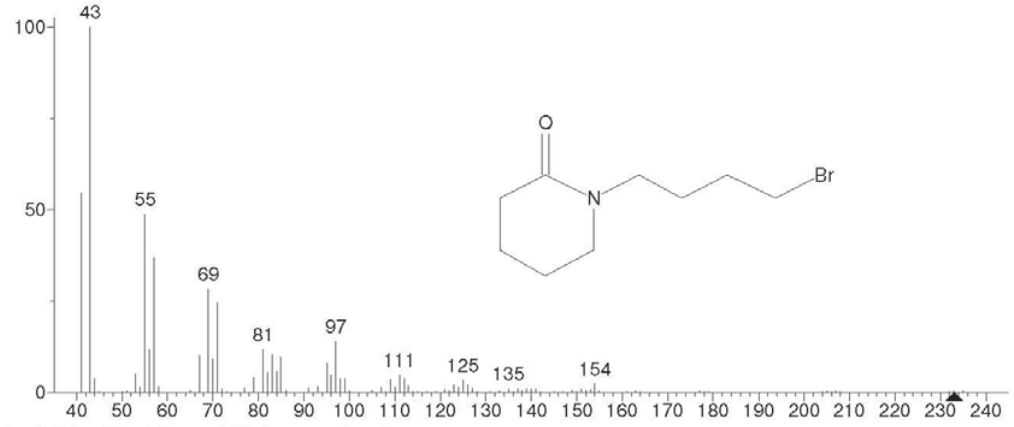
(replib) 1,2-Benzenedicarboxylic acid, diisooctyl ester



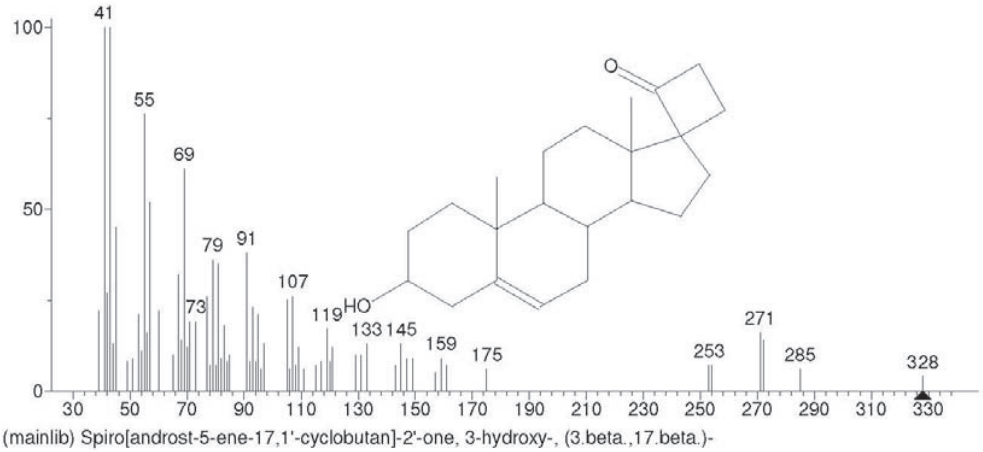
(mainlib) 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E)-



(mainlib) 5.alpha.-Androstan-16-one, cyclic ethylene mercaptole



(mainlib) 2-Piperidinone, N-[4-bromo-n-butyl]-



(mainlib) Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-hydroxy-, (3.beta.,17.beta.)-

Table-3. GC MS analysis of *Marsilea quadrifolia*. The components identified and their activity.

No	RT	Name of the compound	Molecular formula	MW	Peak Area %	Compound nature	**Activity
1	2.68	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (ñ)-	$C_6H_{10}O_3$	130	6.56	Ketone compound	No activity reported
2	11.08	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	6.71	Terpene alcohol	Antimicrobial Anti-inflammatory
3	12.82	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	26.88	Palmitic acid ester	Antioxidant Hypocholesterolemic Nematicide Pesticide Anti androgenic Flavor Hemolytic 5-Alpha reductase inhibitor
4	14.26	Phytol	$C_{20}H_{40}O$	296	16.97	Diterpene	Antimicrobial Anticancer Antioxidant Diuretic
5	14.96	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	12.46	Linoleic acid ester	Hypocholesterolemic Nematicide Antiarthritic

							Hepatoprotective Anti androgenic Nematicide 5-Alpha reductase inhibitor Antihistaminic Anticoronary Insectifuge Antieczemic Antiacne Anticancer
6	15.29	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312	5.69	Stearic acid ester	No activity reported
7	17.10	Z,Z,Z-1,4,6,9-Nonadecatetraene	$C_{19}H_{32}$	260	3.86	Alkene compound	No activity reported
8	19.05	10-Undecen-1-al, 2-methyl-	$C_{12}H_{22}O$	182	1.20	Aldehyde compound	Antimicrobial Anti-inflammatory
9	19.97	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	8.62	Plasticizer compound	Antimicrobial Antifouling
10	23.66	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E)-	$C_{15}H_{26}O$	222	1.82	Alcoholic compound	Antimicrobial
11	26.92	5à-Androstan-16-one, cyclic ethylene mercaptole	$C_{21}H_{34}S_2$	350	0.53	Steroid	Antimicrobial Anticancer Anti-inflammatory Antiarthritic

							Antiasthma Hepatoprotective
12	29.24	2-Piperidinone, N-[4-bromo-n-butyl]-	$C_9H_{16}BrNO$	233	4.53	Alkaloid	Antimicrobial Anti-inflammatory
13	30.70	Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-hydroxy-, (3á,17á)-	$C_{22}H_{32}O_2$	328	4.16	Steroid	Antimicrobial Anticancer Anti-inflammatory Antiarthritic Antiasthma Hepatoprotective

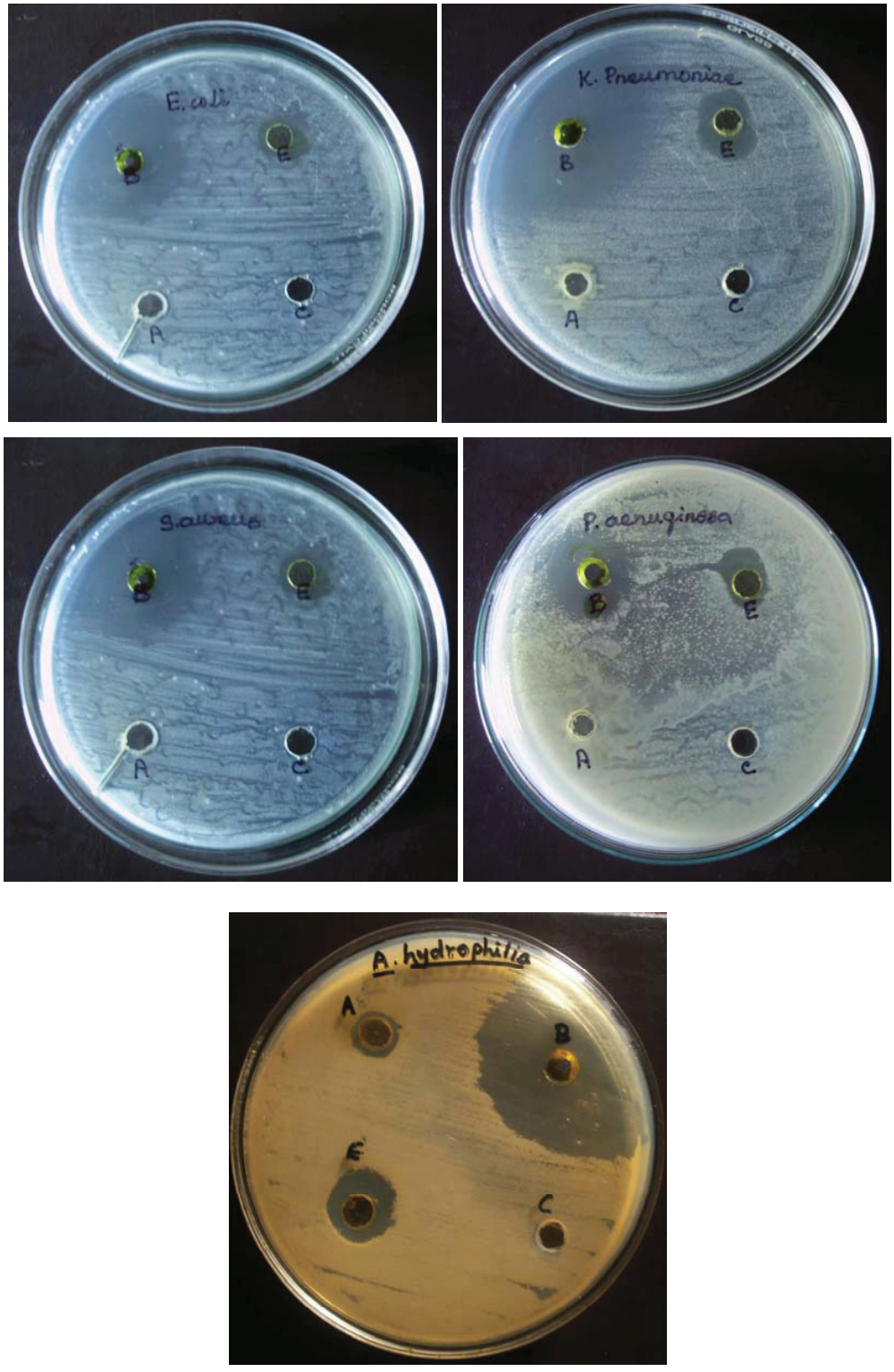
Activity source: **Dr. Duke's Phytochemical and Ethanobotanical Database

and Aeromonas hydrophila) and three fungal strains (*Aspergillus niger, Candida albicans* and *Pencillium notatum*). Pronounced anti-bacterial potential was observed in Benzene extract followed by ethanolic extract. It was also observed that both benzene and ethanolic extracts were more potent in resisting the growth of *Aeromonas hydrophila* followed by *Klebsiella pneumonia*, however better zone of inhibition in resisting the growth of fungus was observed with ethanolic extracts only, especially it was effective in resisting *Candida albicans,* and no visible antimicrobial activity was observed in aqueous extract and in control (Table-4 and Plate -4 and 5).

Table :4. Invitro Antimicrobial activity of *M.quadrifolia* (zone of inhibition in 'mm')

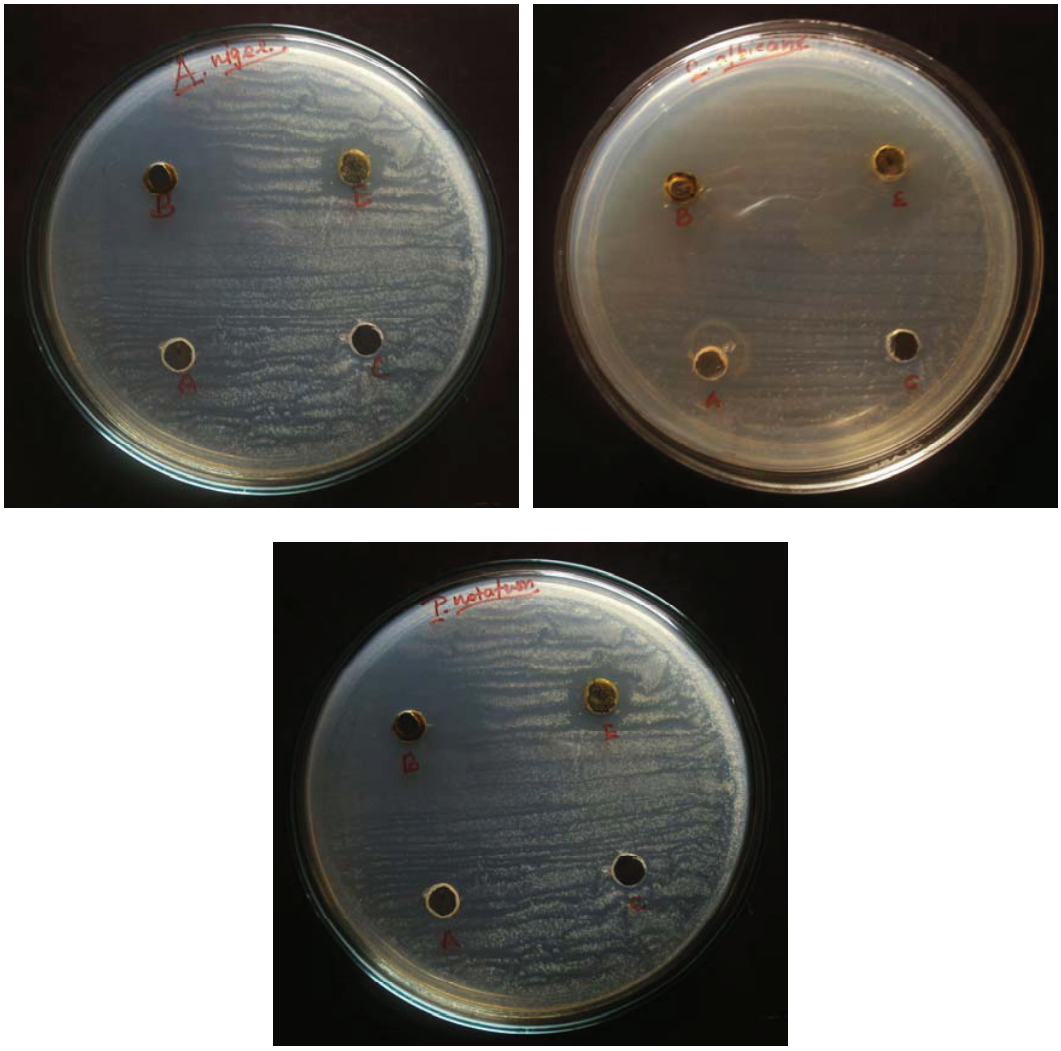
Test organisms	Benzene extract	Ethanol extract	Aqueous extract
Bacteria			
Escherichia coli	27±2.3	10±1.8	-
Klebsiella pneumoniae	32±4.1	15±2.1	-
Staphylococcus aureus	17±2.4	8±1.9	-
Pseudomonas aeruginosa	19±3.2	15±3.4	-
Aeromonas hydrophila	38±4.3	16±3.1	-
Fungi			
Aspergillus niger	12±2.5	13±3.1	-
Candida albicans	14±2.6	21±3.4	-
Penicillium notatum	11±2.4	13±3.8	-

Plate-4 : Zone of inhibition formed by Benzene, Ethanol and Aqueous Extracts of *M.quadrifolia* against Bacterial Pathogens.



A=Aqueous Extract, B=Benzene Extract, E=Ethanol Extract and C=Deionized water

Plate-5 : Zone of inhibition formed by Benzene, Ethanol and Aqueous Extracts of *M.quadrifolia* against Fungal Pathogens.



A=Aqueous Extract, B=Benzene Extract, E=Ethanol Extract and C=Deionized water

3.4 DISCUSSION

According to the WHO, 80% of the world's population primarily those of developing countries rely on plant-derived medicines for their healthcare needs (Gurib – Fakim 2006).

Plants are the storehouses and sources of safer and cheaper chemicals which are pharmacologically active, as they have limitless ability to synthesize aromatic substances, mainly secondary metabolites such as alkaloids, tannins, saponins, flavonoids and phenolics which play defensive role in plants and therefore they protect the plants from their invaders like fungi, bacteria, viruses, nematodes etc (Muraleedharan nair *et al* 2012). The herbal preparations are also known to have an important role in disease control due to their antioxidant, antimicrobial activities, and also they exhibit antistress, growth promotion, appetite stimulation, tonic, immune stimulation and aphrodisiac properties due to the presence of active principles such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils. The secondary metabolites from natural products show more drug likeness and biologically friendliness than total synthetic molecules (Bhambie and George 1972). Thus screening of plants for the presence of Phytochemicals is the first step in the discovery of a new drug.

Ethnopharmacological information can be used to provide three levels of resolution in the search for new drugs: (1) as a general indicator of non-specific bioactivity suitable for a panel of broad screens; (2) as an indicator of specific bioactivity suitable for particular high – resolution bioassays; (3) as an indicator of pharmacological activity for which mechanism-based bioassays have yet to be developed (Cox 1994).

Uma and Pravin (2013) has reported the presence of carbohydrates, proteins, amino acids, alkaloids, steroids, flavonoids, tannins, terpenes and resins in the

ethylacetate extract of *Marsilea quadrifolia* collected from Tanjore district, Tamil Nadu, however they have reported negative result for saponins. In the present study also positive results were obtained for all the above said phytochemicals including saponins. Aswini *et al* (2012) has reported the presence of only Carbohydrates, Steroids and Glycosides, and reported the absence of Proteins, Alkaloids, Steroids, Phenols, Flavonoids, Saponins and Terpences in the ethanolic extracts of *Marsilea quadrifolia* collected from Yellapur, Karnataka.

The quantitative analysis of *M.quadrifolia* has revealed the presence of carbohydrates, proteins and amino acids in sufficient quantity indicates that *Marsilea quadrifolia*'s role as nutritional supplement and the presence of significant quantities of flavonoids and saponins supports its role in health improving effect.

Alkaloids are antimicrobial as they intercalates into cell wall and DNA of parasites, antidiarrhoeal as they inhibits the release of autocoids and prostaglandins and antihelminthic as they possess anti-oxidating effects: thus reduces nitrate generation which is useful for protein synthesis, suppresses transfer of sucrose from stomach to small intestine, diminishing the support of glucose to the helminthes, acts on central nervous system causing paralysis. Phenols and Tannins are also antimicrobial as they binds to adhesions, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption, metal ion complexation, they are antihelminthic as they increases supply of digestible proteins by animals by forming protein complexes in rumen, interferes with energy generation by uncoupling oxidative phosphorylation, causes a decrease in G.I metabolism and antidiarrhoeal as they makes intestinal mucosa more resistant and reduces secretion, stimulates normalization of deranged water transport across the mucosal cell and reduction of the intestinal transit, blocks the binding of B subunit of heat-labile enterotoxin to GM_1, resulting in the suppression of heat-labile

enterotoxin-induced diarrhoea, astringent action. Similarly, Saponins are antidiarrhoeal as they inhibits histamine release in vitro, anticancer as it possesses membrane permeabilizing properties and antihelminthic as leads to vacuolization and disintegeration of teguments. The Flavonoids are antimicrobial as they complexes with the cell wall and binds with adhesions, and antidiarrhoeal as inhibits the release of autocoids and prostaglandins, inhibits contractions caused by spasmogens (Prashant *et al* 2011). Thus it is clear that though the activity of different bioactive compounds are same the mechanism of action is different. As *Marsilea quadrifolia* has many bioactive compounds with diversified mechanism of action will be a potent medicinal herb.

The bioactive compounds identified in the present study through GC-MS analysis with more percentage were Hexadecanoic acid ethyl ester (Palmitic acid ester) (26.88%), Phytol (Diterpene) (16.97%), 9,12-Octadecadienoic acid (Z,Z) (Linoleic acid ester) (12.46%) have wide range of bioactive potential. Among the thirteen identified compounds 8 showed Anti-microbial activity, 6 showed Anti-inflammatory, 4 have Anti-cancer and 2 other showed anti-oxidant and hypocholesterolemic activity. It was also observed that activity for 3 compounds have not been reported, a thrust area which has to be worked out.

Fatty acids always occur in plants. Fatty acids in plants (Hexadeconoic acid, Octo decadienoic acid etc) react with alcohols in an esterification reaction to form esters (William 2000). Unsaturated fatty acids are important to every cell in the body for normal growth, especially of the blood vessels and nerves and to keep the skin and other tissues youthful and supple through their lubricating quality (Okwu and Morah 2006). Fatty acids are nutrients which are invaluable for the production and movement of energy throughout the body, regulation of transportation of oxygen and are vital in maintaining the integrity of cell structure as well as the unique ability to lower

cholesterol levels of the blood (Okenwa Uchenna Igwa and Donatus Ebene Okwu 2013). Thus presence of Hexadeconoic acid, Octo decadienoic acid etc in *Marsilea quadrifolia* will be useful to maintain the general health and physiology of the fish.

Steroids are abundant in nature; many derivatives of steroids have physiological activity (Vollhardt 1994). Steroid hormones control sexual development and fertility in the human body (Okwu and Ighodaro 2010). Many steroids are used in medicine in the treatment of cancer, arthritis or allergies and in birth control (Vollhardt 1994, Okwu and Ighodaro 2010). They are also antidiarrhoeal as they enhance intestinal absorption of sodium and water (Prashant *et al* 2011). Besides antimicrobial, anticancer, anti-inflammatory and hepatoprotective activities detection of steroids in *Marsilea quadrifolia* may also improve the fertility of fish.

Phytols are the precursor for the manufacture of synthetic forms of Vitamin E and Vitamin K. Plants use phytol and its metabolites as chemical deterrents against predation (Wikipedia-Phytol). Phytol acts as effective adjuvant and also increases the titers of all major ImmunoglobulinG (IgG) subclass and are also capable of inducing specific cytotoxic effector Tcell responses (So-yon Lim 2006). Thus presence of sufficient quantity of phytol in *M.quadrifolia* may play a vital role in improving the immune response in the target fish.

Alkaloids have marked physiological activity. Pure isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agent for its analgesic, anti-spasmodic and anti-bacterial properties. For example quinine (alkaloid) extracted from Cinchona is used to treat malaria (Esimone 2008). They are also antidiarrhoeal and antihelminthic in function (Prashant *et al* 2011). Presence of alkaloid in *Marsilea* may fortify the antimicrobial activity.

Thus from the present GC MS study it is evident that *Marsilea quadrifolia* is a potent medicinal fern of pharmaceutical importance.

Bhuvaneswari and Balasundaram (2006) experimented the potential of different plants in inhibiting the growth of the bacterial pathogen *Aeromonas hydrophila*. The zone of inhibition formed by the ethanolic extracts of *Acorus calamus* was 13.3mm, *Indigofera aspalathoides* was 10mm, *Coleus aromaticus* was 8.3mm, *Acalypha indica* was 6.66mm and of *Heliotropium indicum* was 4.3mm. Veronica and Julian (2013) evaluated the antibacterial potential of crude extracts of different plants against different gram negative fish bacterial pathogens and reported that the extracts of the plant *Piper betle* formed 11mm, *Phyllanthus niruri* 10 mm and *Syzygium aromaticum* 9 mm diameter of clearing zone against *Aeromonas hydrophila.* Similarly, Hakan *et al* (2009) observed that the ethanolic extracts of Nuphar *lutea* 10mm, *Nymphaea alba* 10.5mm, *Stachys annua* 9.25mm, *Genista Lydia* 10.5mm, *Vinca minor* 9.25mm, *Filipendula ulmaria* 11.63mm and *Helichrysum plicatum* 8.25mm diameter zone of inhibition was formed against *Aeromonas hydrophila*, Whereas in this present experiment it was observed that the ethanolic extract of *Marsilea quadrifolia* was able to inhibit the growth of *A.hydrophila* to the extent of 16mm diameter proving its potential as potent growth inhibitor against *A.hydrophila.*

Through phytochemical analysis it was observed that ethanolic extract of *M.quadrifolia* is rich in phytochemicals like Reducing sugar, Protein, Tannin, Phenolic compounds, Flavonoids, Alkaloids, Phytosterols and saponins which are potent bioactive substances with pharmacological action. GC-MS analysis has revealed the presence of about 13 compounds most of which with a wide range of pharmacological activity and screening of antimicrobial potential of various extracts supports *Marsilea quadrifolia* as a potential medicinal herb whose Immunostimulant potential can be evaluated with fish species.

CHAPTER - 4

Marsilea quadrifolia as growth and immune enhancer in *labeo rohita*

Sivagurunathan A, Immunodiagnostic studies in a chosen fresh water fish administered with a medicinal Aquatic fern *Marsilea quadrifolia,* Ph.D. Thesis, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India.

CHAPTER – 4

MARSILEA QUADRIFOLIA AS GROWTH AND IMMUNE ENHANCER IN *LABEO ROHITA*

4.1. INTRODUCTION

The high susceptibility of fish to stress and the rapid spread of diseases in water have forced aqua-culturists to concentrate their efforts on maintaining their fish in good health in order to achieve sustainable economic performances. Among different practices, administration of immunostimulants is one, which along with good management practices will ensure high survival rates, improve the health status and enhance growth in intensive farming systems.

*Labeo rohita,*the Indian Major Carp, is one of the most preferred species in the Indian subcontinent, which contributes about 35% of the total carp production (FAO 2001). Development of an economical alternate to accelerate the growth and to maintain the health status of this species is of prime importance for sustainable carp culture. The present trend of intensification of aquaculture is a major concern for the out break of disease as fishes are more prone to stress and subsequent infection by pathogen.

Ethnomedicinal study deals with the study of traditional medicines. Since ancient times mankind has been using herbal plants, organic materials as well as materials from the sea, rivers etc. for its betterment. These substances have been used as food, medicine etc. Amongst them, the substances having medicinal value have been extensively used for treating various disease conditions. Herbs being easily available to human beings have been explored to the maximum for their medicinal properties (Mehta kavit *et al* 2013).

The world market for herbal medicine, including herbal products and raw materials, has been estimated to have an annual growth rate between 5 and 15%. The total global herbal drug market is estimated as US$62 billion and is expected to grow to US$5 trillion by the year 2050. In India, the value of botanical-related trade is about US$1.1 billion (Singh *et al* 2003); while China's annual herbal drug production is worth US$48 billion with an export of US$3.6 billion (Handa 2004). Presently, the United States is the largest market for Indian botanical products accounting for about 50% of the total exports. Japan, HongKong, Korea and Singapore are the major importers of traditional Chinese medicine, taking a 66% share of China's botanical drug export (Anon 2003).

Traditional herbal medicines seem to be the potential immunostimulator. Thus, the use of medicinal plants is an alternative to antibiotics in fish health management. The herbs are not only safe for consumers, but they also have a significant role in aquaculture. Many studies have proved that herbal additives enhanced the growth of fishes and protected them from diseases. The non-specific immune system of fish is considered to be the first line of defense against invading pathogens (Ahilan *et al* 2010). Many plant compounds have been found to have non-specific immune stimulating effects in humans and animals (Pandey and Madhuri 2010; Kolkovski and Kolkovski 2011) of which more than a dozen have been evaluated in fish and shrimp. Several plant products seemed to be potent antiviral agents against fish and shrimp viruses (Kolkovski and Kolkovski 2011). Therefore, the herbal plants may be used as potential and promising source of pharmaceutical agents against fish pathogens in organic aquaculture (Abdul Kader Mydeen and Haniffa 2011; Ravikumar *et al* 2010).

The pteridophyte plants play significant role on the ethnomedicinal point of view. In ancient Indian medicine several pteridophytes have been used as medicine by unani

physicians and local tribal people in India and Western Asia as they have great medicinal value. The pteridophytes show various economic values towards food and fodder indicators, biofertilizers, insect repellents, medicine and folk medicines (Ghosh *et al* 2004). Inspite of its great medicinal values less work has been carried out to elucidate their potential.

Marsilea quadrifolia an aquatic fern, has many medicinal properties. *M. quadrifolia* pacifies vitiated pitta, cough, bronchitis, diabetes, psychiatric diseases, eyediseases, diarrhea, skin diseases, antidote,antiphloristic, depurative, diuretic and febrifuge (Aswini *et al* 2012). However its immunostimulant potential is still unexplored, hence the present work has been undertaken.

Kolawole *et al* (2011) stated that one way to distinguish the appropriate or inappropriate prescription of medical plants is the assessment of their effects on haematological and biochemical parameters in experimental animals.

The objective of the present chapter is to evaluate the growth and health promoting potentials of the aquatic fern *Marsilea quadrifolia* incorporated diet in the experimental fish *Labeo rohita* through haematological, serological and immunological parameters. β-glucan, a known Immunostimulant has been chosen to compare and to validate the immunostimulatory qualities of *M.quadrifolia*.

4.2. MATERIALS AND METHODS

4.2.1. Experimental Fish

Rohu (*Labeo rohita*) is the most important among the three Indian major carp species used in carp polyculture systems. This graceful Indo-Gangetic riverine species is the natural inhabitant of the riverine system of northern and central India, and the rivers of Pakistan, Bangladesh and Myanmar. In India, it has been transplanted into almost all

riverine systems including the freshwaters of Andaman, where its population has successfully established. The species has also been introduced in many other countries, including Sri Lanka, the former USSR, Japan, China, Philippines, Malaysia, Nepal and some countries of Africa. The traditional culture of this carp goes back hundreds of years in the small ponds of the eastern Indian states (Talwar and Jhingran 1991).

4.2.1.1. Classification

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Actinopterygii
Order	-	Cypriformes
Family	-	Cyprinidae
Genus	-	Labeo
Species	-	*L.rohita* (Wikipedia-Rohu)

Rohu is a eurythermal species and does not thrive at temperatures below 14°C. It is a fast growing species and attains about 35-45 cm total length and 700-800 g in one year under normal culture conditions. Generally, in polyculture, its growth rate is higher than that of mrigal but lower than catla.The minimum age at first maturity for both sexes is two years, while complete maturity is reached after four years in males and five years in females. In nature, spawning occurs in the shallow and marginal areas of flooded rivers. The spawning season of rohu generally coincides with the south-west monsoon, extending from April to September. In captivity with proper feeding the species attains maturity towards the end of second year. However, breeding does not take place in such lentic pond environments; thus induced breeding becomes necessary.

4.2.1.2. Biological Features

Body bilaterally symmetrical, moderately elongate, its dorsal profile more arched than the ventral profile; body is covered with cycloid scales, head is without scale; snout fairly depressed, projecting beyond mouth, without lateral lobe; eyes are dorsolateral in position, not visible from outside of head; mouth small and inferior; lips are thick and fringed with a distinct inner fold to each lip, lobate or entire; a pair of small maxillary barbels concealed in lateral groove; no teeth on jaws; pharyngeal teeth in three rows; upper jaw not extending to front edge of eye; simple (unbranched) dorsal fin rays three or four, branched dorsal fin rays 12 to 14; dorsal fin inserted midway between snout tip and base of caudal fin; pectoral and pelvic fins laterally inserted; pectoral fin devoid of an osseous spine; caudal fin deeply forked; lower lip usually joined to isthmus by a narrow or broad bridge; pre-dorsal scale 12-16; lateral line distinct, complete and running along median line of the caudal peduncle; lateral line scales 40 to 44; lateral transverse scale rows six or six and a half between lateral line and pelvic fin base; snout not truncate, without any lateral lobe; colour bluish on back, silvery on flanks and belly (Khan and Jhingran 1975, FAO-*Labeo rohita*).

4.2.1.3. Life Cycle

In rohu the seeds are commonly produced by induced breeding (Hypophysation). The seeds hatches into hatchlings which develops into fry within 15-20 days, the fry Develops into fingerlings within 2-3 months which grows to a harvestable size of 1-1.5kg within 12-18months.

In its early life stages rohu prefer zooplankton, mainly composed of rotifers and cladocerans, with phytoplankton forming the emergency food. In the fingerling stage, there is a strong positive selection for all the zooplanktonic organisms and for some

smaller phytoplanktons like desmids, phytoflagellates and algal spores. On the other hand, adults show a strong positive selection for most of the phytoplankton. In the juvenile and adult stages rohu is essentially an herbivorous column feeder, preferring algae and submerged vegetation. Furthermore, the occurrence of decayed organic matter and sand and mud in its gut suggests its bottom feeding habit (FAO-*Labeo rohita*).

4.2.1.4. Culture of Rohu

Rohu is the most preferred species among the cultivated Indian major carps and the principal species reared in carp polyculture systems along with the other two Indian major carps *viz*.,catla, (*Catla catla)* and mrigal, (*Cirrhinus mrigala*). Due to its wider feeding niche, which extends from column to bottom, rohu is usually stocked at relatively higher levels than the other two species.

The compatibility of rohu with other carps like catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*) made it an ideal candidate for carp polyculture systems. Its high growth potential, coupled with high consumer preference, have established rohu as the most important freshwater species cultured in India, Bangladesh and other adjacent countries in the region.

In the Koleru lake area of Andhra Pradesh, the centre of commercial carp farming activity in India, the practice commonly involves the rearing of rohu and catla in two-species farming, with rohu constituting over 70 percent of the stock. Rohu also forms one of the important components in the sewage-fed carpculture system practiced in an area totaling over 4000 ha in West Bengal, India. In this form of culture, which includes multiple stocking and the multiple harvesting of fish larger than 300 g, primary treated sewage is provided to the fish ponds as the main input. Even without the provision of

supplementary feed, this system produces 2-3 tonnes/ha/yr; with supplementary feeding, this can be increased to 4-5 tonnes/ha/yr (FAO-*Labeorohita*).

4.2.1.5. Collection and Rearing

The test fish (50±5 gram weight) (Plate-6) was purchased from Sabari fish farm, Kallidaikurichi, Tirunelveli and transported to the laboratory in plastic containers containing oxygenated water. They were acclimated to the laboratory condition (Water temperature 29±1^{0}C, pH 7.2±0.1, dissolved oxygen 5.84±0.042 ml/l and Salinity 0.127±0.023 ppt) and fed with Rice bran and Groundnut oil cake *adlibitum* for 15 days. The aquarium water was changed daily in order to maintain the fishes in healthy state. The diseased and dead fishes were discarded immediately. Tap water was used for the present study in all the experiments in which the fishes were pre-acclimatized.

4.2.2. Feed Preparation

The dried powder of *Marsilea quadrifolia* was dissolved in ethanol in the ratio of 1:20, shaken well intermittently for 48 hours and filtered through filter paper. The filtrate was concentrated to a paste like consistency and stored in a refrigerator.

The β-Glucan (1-3/1-6 Glucan) purchased from Source Naturals.INC, P.O.Box 2118, Santa Cruz, CA 95062 (www.Source naturals.com) was used in this experiment.

Plate-6. *Labeo rohita*, the Experimental Fish

The control and balanced diet were prepared using the following ingredients.

Ingredients	Control	M-1	M-2	M-3	G-1	G-2	G-3
Rice Bran	10g	10g	10g	10g	10g	10g	10g
Wheat Bran	10g	10g	10g	10g	10g	10g	10g
Soya Flour	23g	23g	23g	23g	23g	23g	23g
Fish Meal	24g	24g	24g	24g	24g	24g	24g
Ground nut oil cake	23g	23g	23g	23g	23g	23g	23g
Tapioca flour	10	10	10	10	10	10	10
Vitamins & Minerals premix	2g	2g	2g	2g	2g	2g	2g
Extract/Powder	-	*Marsilea* 250mg (0.25%)	*Marsilea* 500mg (0.5%)	*Marsilea* 1000mg (1%)	β-glucan 250mg (0.25%)	β-glucan 500mg (0.5%)	β-glucan 1000mg (1%)

The above ingredients are mixed well with water and sterilized in pressure cooker for 30 minutes, cooled and required amount of the extracts/powder and vitamin and mineral premix were added, made into dove consistency by adding sunflower oil, prepared into the form of noodles which were then shade dried and broken into small appropriate sized pieces. They were packed in air tight containers and stored in the refrigerator.

4.2.3. Experimental Design

The fishes were divided into seven groups each with 50 fishes. Group-1 received control diet (C), Group-2 received 0.25% *Marsilea* extract incorporated diet (M-1),

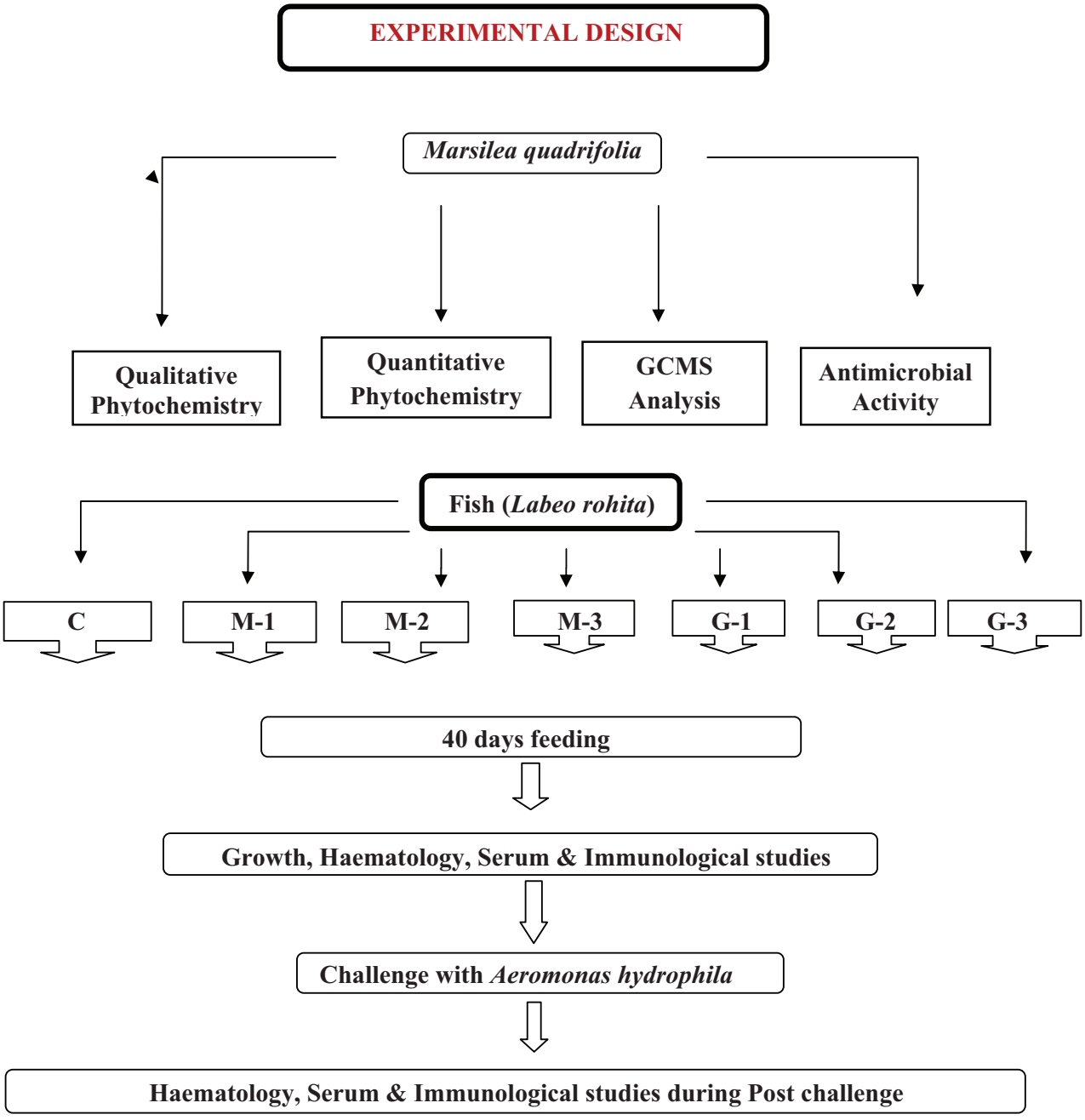
Group-3 was fed with 0.5% *Marsilea* extract incorporated diet (M-2), Group-4 received 1% *Marsilea* incorporated diet (M-3), Group-5 was fed with 0.25% β-glucan fortified diet, Group-6 received 0.5% β-glucan supplemented diet and Group-7 was fed with 1% β-glucan incorporated diet. The fishes were fed *adlibitum* daily twice (Morning and Evening) for 40 days. The fishes were weighed on day one and at the end of 40^{th} day. The whole experiment was run in triplicate.

4.2.4. Collection of Blood

At the end of 40 days feeding trial, 10 fishes from each group was collected very gently using a small dip net and transferred to separate plastic containers, anesthetized by dipping in water mixed with clove oil (one drop/10 liter of water). Immobilized fishes were collected for blood sampling. The blood was collected from caudal vein and or from the heart directly using 1ml insulin syringe.The blood was collected in non-heparinized Eppendorf tubes for the collection of the serum and in heparinized tubes for routine haematological studies.

4.2.4.1. Haematological Analysis

The total Erythrocyte counts, total leucocyte counts and differential leucocyte counts were performed by the method prescribed by Wilhelm Schaperclaus 1991. The total thrombocyte counts, Erythrocytic indices like Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were calculated as prescribed by Kanai L Mukherjee 2002.



C=Control diet, M-1 = 0.25% *Marsilea* extract, M-2 = 0.5% *Marsilea* extract and M-3 = 1% extract, G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan and G-3 = 1% β-Glucan

4.2.4.1.1. Erythrocyte Count

Blood is drawn in a dry erythrocyte pipette to the 0.5 graduation and then Hayem's solution (2.5g $Na_2So4.7H2O$, 1g NaCl, 0.25g $HgCl_2$ are dissolved in 100 ml of distilled water [pH = 7-7.4]) to the 101 mark. The pipette is then shaken for 1 minute. Immediately after shaking, the Neubauer counting chamber is filled with the mixture, free from air-bubbles; the liquid contained in the capillary portion of the pipette is rejected (dilution 1:200). The erythrocytes were counted in five group squares (1 group square = 16 small squares), the number of small squares being 80 in 1/400 mm^2. All cells lying inside the group squares and also the erythrocytes lying to the left and below the demarcation line are counted. Calculation of the number of erythrocytes is done according to the following equations.

$$EC/mm^3 = \frac{\text{cells counted}}{\text{Area x depth of chamber x dilution}}$$

$$EC/mm^3 = \frac{\text{Erythrocytes in 80 small squares}}{80 \text{ x } 1/400 \text{ x } 1/10 \text{ x } 1/200}$$

$$EC/mm^3 = \frac{\text{Total of the erythrocytes x 400 x 10 x 200}}{80}$$

4.2.4.1.2. Haemoglobin Estimation

Haemoglobin content was determined by Cyanmethaemoglobin method, using the reagent HEMOCOR-D. 0.02ml of blood was mixed with 5.00ml of Hemocor-D reagent in a test tube, mixed well and incubated at room temperature for a minimum of 3 minutes and the absorbance was read on colorimeter in 540nm wave length. The concentration was estimated using the following calculation.

Haemoglobin (g/dl) = Absorbance x 36.8

4.2.4.1.3. Haematocrit

Heparinized blood is filled to 3/4th in capillary haematocrit tubes (length 75mm and internal diameter 1mm approximately), the end is sealed with modeling clay and centrifuged at 13000 rpm for 5 minutes. Then the capillary tubes are placed in the haematocrit reader, the value upto the red cell column was read and expressed as percentage.

4.2.4.1.4. Leucocyte Count

The blood is drawn in to the erythrocyte pipette upto 0.5 mark, solution A (0.025g of Neutral Red,0.900g NaCl in 100 ml of distilled water [pH = 7-7.4]) is drawn in until the bulb is about half filled, and solution B (0.012g of crystal violet, 3.800g of Sodium citrate, 0.400 ml of formalin in 100 ml of distilled water [pH = 7-7.4]) is then drawn upto 101 mark, after shaking, the counting chamber is filled with the mixtures so as to be free from air bubbles and counting is done in the large squares which are present at the four angular points of the Neubauer counting chamber and are demarcated by triple lines (1mm^2). The number of leucocytes is calculated from the following equations.

$$LC/mm^3 = \frac{\text{Total number of cells counted in the four squares of } 1\text{mm}^2}{\text{Area x chamber height x blood dilution}}$$

$$LC/mm^3 = \frac{\text{Total number of cells counted in the four squares of } 1\text{mm}^2}{4 \text{ x } 1/10 \text{ x } 1/200}$$

$$LC/mm^3 = \frac{\text{Leucocytes in } 4\text{mm}^2 \; x \; 2000}{4}$$

$$LC/mm^3 = \text{Leucocytes in } 4\text{mm}^2 \text{ x } 500$$

4.2.4.1.5. Differential Leucocyte Count

A drop of fresh blood is placed on a degreased slide, smear is prepared, fixed with methanol and air dried. Then the smears are flooded with 20-30 drops of May-Grunwald solution for 3 minutes, adding an equal quantity of distilled water for 1 minute then decanted and stained with dilute Giemsa solution for 15-20 minutes. Then the smear is rinsed with excess distilled water, dried and observed under microscope. The different leucocytes like neutrophils, basophils, eosinophils, lymphocytes and monocytes were identified, counted and expressed in percentage.

4.2.4.1.6. Thrombocyte Count

The blood is drawn in to the erythrocyte pipette upto 0.5 mark and then Platelet diluting fluid (Sodium citrate – 3.8g, Formalin – 0.2ml, Brilliant cresyl blue – 0.1g, Distilled water – 100ml) upto 101 mark. The pipette is then shaken for 1 minute. Immediately after shaking, the Neubauer counting chamber is filled with the mixture, free from air-bubbles; the liquid contained in the capillary portion of the pipette is rejected (dilution 1:200). Thrombocytes present in the whole finely ruled area (red cells) using high power objective. The total number of cells counted is multiplied by 2,000 to get the number of thrombocytes per c.mm of blood.

4.2.4.1.7. Mean Corpuscular Volume (MCV)

To determine the average volume of a single red cell in cubic microns the following equation is used.

$$\mathrm{MCV}(\mu^3) = \frac{\text{Haematocrit (\%)} x\ 10}{\text{Red blood cells in millions}}$$

4.2.4.1.8. Mean Corpuscular Haemoglobin(MCH)

The average haemoglobin content of a single red cell in pico grams is calculated using the following formula.

$$\text{MCH(pg)} = \frac{\text{Haemoglobin (g/dl)} x\ 10}{\text{Red blood cells in millions}}$$

4.2.4.1.9. Mean Corpuscular Haemoglobin Concentration (MCHC)

It is an expression of the average haemoglobin concentration per unit volume (100) of haematocrit.

$$\text{MCHC (\%)} = \frac{\text{Haemoglobin (g/dl)} x\ 100}{\text{Haematocrit (\%)}}$$

4.2.4.2. Biochemical Analysis

4.2.4.2.1. Serum separation

The non heparinized blood was collected in a clot activator Eppendorf tubes and allowed to clot at room temperature, then the tube was centrifuged for 10 minutes at 3000rpm to separate the serum. The supernatant serum was carefully separated and stored in a refrigerator.

4.2.4.2.2. Serum total Protein Estimation

The total serum protein was estimated by Biuret Method using total protein test kit purchased from Jeev Diagnostics Private limited, Chennai. 0.010ml of serum was added to 1ml Biuret reagent, mixed well and let stand for 3 minutes and the optical density was read on Spectrophotometer at 545nm wave length.The standard was prepared by adding 0.010ml of total protein standard (provided by Jeev Diagnostics Private limited, Chennai along with the total protein test kit) with 1ml of Biuret reagent. The amount was calculated using following formula.

$$\frac{\text{Sample O. D}}{\text{Standard O. D}} \text{x } 5 = \text{gram Total Protein/dl}$$

4.2.4.2.3. Serum Albumin Estimation

The serum albumin content was estimated by BCG method using Albumin Test Kit purchased fromJeev Diagnostics Private Limited, Chennai. 0.010ml of serum was added to 1ml of BCG Reagent, mixed well and allowed to stand for 5 minutes at room temperature and the optical density was read on a Spectrophotometer at 630nm wave length. The standard was prepared by adding 0.010ml of Albumin standard (provided by Jeev Diagnostics Private limited, Chennai along with the Albumin test kit) with 1ml of BCG reagent. The amount was calculated using following formula.

$$\frac{\text{Sample O. D}}{\text{Standard O. D}} \text{x } 5 = \text{gram Albumin/dl}$$

4.2.4.2.4. Serum Globulin Estimation

The globulin concentration of the serum was estimated by subtracting serum albumin from serum total protein**.**

Serum Globulin = Total serum protein – serum albumin

4.2.4.2.5. Albumin Globulin ratio (A/G ratio)

Albumin globulin ratio was calculated by dividing the albumin concentration with globulin concentration.

$$\text{A/G ratio} = \frac{\text{Albumin}}{\text{Globulin}}$$

4.2.4.2.6. Serum Cholesterol

The serum Cholesterol content was estimated by CHOD-POD method using Cholesterol Test Kit purchased from Jeev Diagnostics Private Limited, Chennai. 0.010ml of serum was added to 1ml of Cholesterol Reagent, mixed well and allowed to stand for 10 minutes at room temperature and the optical density was read on a

Spectrophotometer at 505nm wave length. The standard was prepared by adding 0.010ml of cholesterol standard (provided by Jeev Diagnostics Private limited, Chennai along with the Albumin test kit) with 1ml of cholesterol reagent. The amount was calculated using following formula.

$$\frac{\text{Sample O. D}}{\text{Standard O. D}} \text{x } 200 = \text{mg Cholesterol/dl}$$

4.2.4.2.7. Blood Glucose

The blood glucose was estimated by GOD-POD method using Glucometer (Arkray, Japan). One drop of fresh blood was allowed to flood the Glucocard 01 strip and the reading was noted from the glucometer directly.

4.2.4.2.8. SDS-PAGE analysis

The serum proteins of all group fishes were electrophoretically separated by SDS-PAGE and the Electrophoretogram was analysed densitometrically.

The basic methodology adopted was as described by Laemmli *et al* (1970) with some modifications. Standardization of the technique was performed as the percentage of separating gel is a critical parameter in all electrophoretic separations of different proteins in the sample. Separating gels of 12.5%, 11.5% and 11% were tried to choose an ideal percentage, which gives a better electrophoretic separation. Finally, a separating gel of 11.5% concentration, prepared from a 30% stock of acrylamide and bisacrylamide monomers was selected along with a stacking gel of 6%. The concentration of serum samples to be loaded on the gel was also standardized to get an ideal resolution.

Reagents for electrophoresis:

A.**Stock acrylamide solution** (30%)

Acrylamide -29.1g

NN'-methylene bisacrylamide -0.8g

The mixture was dissolved in minimum quantity of water and made up to I00mI using distilled water. The mixture was filtered using Whatman No.1 filter paper and stored in amber coloured bottles in a refrigerator.

B. **Gel buffers**:

Separating Gel Buffer

1.5MTris -18.17g

SDS (Sodium dodecyl sulphate) –0.04g

The pH was adjusted to 8.8 using 2M HCl and the solution was made up to100mL using distilled water.

Stacking gel buffer

0.5M Tris - 6.05g

SDS - 0.04g

The pH was adjusted to 6.8 using 2M HCI and the solution was made upto I00 mL using distilled water.

Electrode buffer

Tris - 3g

Glycine - 14.4g

SDS - 1g

Adjust Tris pH to 8.6 with HCl and add SDS. Made upto l000mL using distilled water.

C. Polymerizing Agent

Ammonium persulphate (APS)

D.10% SDS solution

E. Composition of 11.5% gel

10% (freshly prepared) - 10g SDS in l00 mL distilled water

a. **Separating gel**

Acrylamide and NN' methylene bisacrylamide - 11.5mL

Separating gel buffer - 6mL

Water - 12.7mL

10 %SDS - 300 µl

TEMED - 30 µl

APS - 100 µl

b. Stacking gel

Acrylamide and NN'Bisacrylamide - 2mL

Stacking gel buffer - 2.5mL

Water - 5.4mL

10%SDS – l00 µl

TEMED (N,N,N',N'-Tetramethyl-ethylene diamine)- 10 µl

APS - 40 µl

The separating gel components were mixed gently and poured into the prepared cassette. Few drops of butanol were over layered to prevent meniscus formation and the gel was left undisturbed to set for 30 minutes. After polymerization of the separating gel, the overlaying butanol was removed and the cassette was washed with double distilled water and dried. The prepared stacking gel mixture was then poured over the separating gel. The comb was placed in the stacking gel and allowed to set for 30 minutes.

After the gel got solidified the comb was removed without distorting the shape of the well. The gel was carefully set on the electrophoretic apparatus after removing the

clips, bottom spacers etc. with the plate having the "U" shape cut facing the upper tank using clamps and screws provided. The electrode buffer was added to the tanks and care was taken to prevent entrapment of air bubbles at the bottom of the gel. The electrodes were then connected to the power pack.

F. **Sample buffer**

Stacks of sample buffer with SDS were prepared as follows:

Glycerol : 2mL

p-mercaptoethanol :ImL

Stacking gel buffer : 1.8mL

Bromophenolblue : 0.6mL (0.5%)

I0 %SDS :1mL

G. Staining solution (500 mL)

Coomassie Brilliant Blue R 250 - 0.75gm

Methanol - 230mL

Acetic acid - 40mL

Distilled water - 230mL

H. Destaining solution (500mL, freshly prepared)

Methanol - 25 mL

Acetic acid - 35 mL

Distilled water - 440 mL

I.Marker: Protein molecular weight marker was purchased from GENEI, Bangalore.

Sample application and electrophoresis

Serum stored at -20°C was brought to room temperature. 10 µl of the sample was mixed with 90 µl of distilled water. 50 µl of this mixture was then mixed with 50 µl of

sample buffer with SDS and boiled for 1minute. 10 µl of protein molecular weight marker was mixed with 60 µl of sample with 2% SDS and boiled for 1minute. The prepared samples were applied into the wells of the stacking gel and layered with running buffer in order to avoid disturbance to the sample. A constant voltage of 60 volts was applied until the dye front crossed the stacking gel and it was increased to 140 volts and electrophoresis was continued until the dye front reached the bottom of the gel.

Staining the gels

Immediately after the completion of electrophoresis, the gels were carefully separated from the trays, transfered into plasic trays and washed in tap water. After staining the gels for two hours in Coomassie Brilliant Blue R 250, the excess stains were washed off and the gels were immersed in destaining solution.

Determination of Molecular Weight

Molecular weight of standards used as molecular markers for SDS PAGE were 205000, 97400, 66000, 43000, 29000, 20100, 14300, 6500, 3000 daltons. Rf values of the standard markers were calculated by using the following formula

$$\text{Rf (Relative front)} = \frac{\text{Distance moved by the solute}}{\text{Distance moved by dye}}$$

The Rf values, molecular weight and the amount of the protein present in each protein fraction was analysed using densitometer.

4.2.4.3. Immunological Parameters

4.2.4.3.1. Agglutinating antibody titer

Agglutinating antibody titer was determined with a microagglutination assay using each antigen independently following the method of Klesius *et al* (2000) with some modifications. Briefly, *A. hydrophila* were grown on tryptic soy agar (HiMedia)

slants plus 0.1% glucose for 18 hrs at room temperature. Cells were suspended in 0.85% saline. For the 0 antigen, cells were killed with 1% (v/v) formalin and washed three times in saline. The killed bacteria were then centrifuged at 4000rpm for 15 min and the resulting cell pellets were resuspended and washed in phosphate buffered saline (PBS, pH 7.2). Following three washes, the bacteria were adjusted to an optical density of 1 at 540 nm in PBS.

Round bottom 96-well micro titer plates were initially plated with 50 µL of PBS. In each well, 50 µL of serum was added and mixed. From the first well (1:1), 50 µL of mixed serum was transferred to the 2nd well. This serial dilution was continued till the 25th well. The well without serum served as negative control. Later, a drop of inactivated *A. hydrophila* suspension (10^8 CFU/ml) was mixed with diluted serum in a glass slide and incubated at room temperature for 5min. The agglutination was observed under the dissection microscope. The last dilution of serum showing clear agglutination was taken as the end point for titer estimation.

4.2.4.3.2. Serum lysozyme Concentration

Serum lysozyme was determined by turbidometric assay by the method of Sankaran and Shanto (1972) with some modifications. Briefly, Serum (0.1 mL) was placed in test tubes and 0.9 mL of a 0.75 mg mL-1 *Micrococcus luteus* (MTCC, 106) suspension in phosphate buffered saline, pH 6.2 was added and mixed well. The absorbance was measured at 450 nm by a spectrophotometer for 10 min intervals after mixing with bacteria and rate of absorbance calculated. Lysozyme activities were calculated using hen egg white lysozyme (Sigma-Aldrich) as a standard. The unit of lysozyme presents in serum (µg/ml) was obtained from standard curve.

4.2.4.3.3. Serum bactericidal assay

Serum Bactericidal Activity was performed by the method prescribed by Kajita *et al* (1990). *Aeromonas hydrophila* was cultured in brain heart infusion (BHI) broth and the bacteria were pelletized by centrifuging the broth at 3000rpm at 4^{o}C for 15 min. The bacterial pellet was washed thrice with PBS by centrifuging at 10,000 rpm for 15 min. The final suspension of bacteria in PBS was adjusted to an optical density of 0.5 at 540 nm, which gives approximately 10^8 cells per ml. 100µl of this bacterial suspension and 900µl of fresh antiserum or control serum were mixed in sterile eppendorf tubes. A control was also maintained with bacterial suspension (100µl) and normal saline (900µl). The tubes were incubated at 25^{o}C for 60 min and subsequently all incubation mixtures were used to determine the CFU/ml by the spread plate method on BHI agar. The bactericidal activity of the serum was expressed as percentage of the CFU in comparison to PBS control group.

4.2.4.3.4. Myeloperoxidase assay

Myeloperoxidase content of serum was determined as described by Quadi and Rath (1997) and partially modified by Sahoo *et al* (2005). Briefly, 10µl of serum was diluted with 90 µl of Hanks balanced salt solution without Ca^{2+} and Mg^{2+}in 96 well microtitre plate to which 35 µl of 20mM 3, 3', 5, 3' tetramethylbezidine hydrochloride (geni, India) and 5mM H_2O_2 were added. After 2 min of incubation, 35 µl of 4M sulphuric acid was added to stop reaction. Optical density was read at 450 nm in a microtiter plate reader.

4.2.5. Statistical Analysis

The results obtained were statistically analysed using the following formulae

$$Mean = \frac{\sum x}{\mathrm{N}}$$

Where $\sum x$ = Total summation of the samples

N= Total Number of samples

$$\text{Standard Deviation } = \sqrt{\frac{\sum(x - \bar{x})^2}{\mathrm{N} - 1}}$$

where $\sum(x - \bar{x})^2$ = The total squared deviation of each score from mean

N = Total Number of samples

$$Percentage\ Change = \frac{\text{Experimental } - \text{ Control}}{\text{Control}} \text{x } 100$$

The results were analysed statistically by One way Anova (Post hoc –Duncan) using SPSS (16) software**.**

4.2.6. Growth Parameters

Growth Parameters were calculated using the following formulae

Mean body weight gain = Final weight (g) – Initial weight (g)

$$Percent\ Weight\ Gain = \frac{\text{weight gain (g)}}{\text{Initial weight (g)}} \text{x } 100$$

$$Specific\ Growth\ Rate\ (\%) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Time (days)}} \text{x } 100$$

4.3. RESULTS

Labeo rohita fishes were divided into 7 groups each in triplicate were fed with different feeds (C = control, M-1 = 0.25% *Marsilea* extract incorporated feed, M-2 = 0.5% *Marsilea* extract incorporated feed, M-3 = 1% *Marsilea* extract incorporated feed, G-1 = 0.25% Glucan incorporated feed, M-2 = 0.5% Glucan incorporated feed and G-3 = 1% Glucan incorporated feed) for 40 days. At the end of the experimental duration, growth, haematological, serum biochemical and immunological parameters were

analysed to evaluate the feed induced changes. The results were tabulated (Table - 5) and statistically treated.

Ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material (Wang 2010), nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol extraction (Cowan 1999). Thus ethanol was used as solvent to extract the phytochemicals from *Marsilea quadrifolia.*

4.3.1. *Marsilea* incorporated diet

4.3.1.1. Growth Parameters

Fishes fed with different concentrations of *Marsilea* extract incorporated diet exhibited significant to highly significant increase in Mean weight gain, % weight gain and specific growth rate. M-2 feed fed fishes recorded a maximum of 18.33% of specific growth rate. However M-3 feed fed fishes recorded a significant decline in specific growth rate.

Table-5.Effect of dietary *Marsilea* in growth parameters of *Labeo rohita* (Mean±SD)

Sl. No	Parameters	Control	M-1	M-2	M-3
1	Weight Gain	2.91^{b} ± 0.2	4.59^{c} ± 0.5	7.33^{d} ± 0.41	1.55^{a} ± 0.48
2	% Weight Gain	6.55^{b} ± 0.71	8.80^{c} ± 0.74	12.43^{d} ± 0.31	3.04^{a} ± 1.14
3	Specific Growth Rate (%)	7.28^{b} ± 0.5	11.48^{c} ± 1.25	18.33^{d} ± 1.02	3.89^{a} ± 1.21

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea.*

Means with same superscript in the same row is not statistically significant

4.3.1.2. Erythrocytic Parameters

Higher levels of total erythrocyte counts (TEC) were observed in M-1 and M-2 groups only, however it was significant only in M-2. But significantly high levels of haemoglobin were recorded in all groups; however it was significantly high in M-2 and M-3 groups only. Highly significant increase in Haematocrit (Ht) was observed only in M-1 group fishes. The erythrocytic indices like Mean corpuscular volume (MCV) has increased significantly in M-1group only and a significant decrease in M-2 was observed. Increased levels of Mean corpuscular haemoglobin (MCH) was recorded in M-2 and M-3 feeds. The Mean corpuscular haemoglobin concentration (MCHC) also significantly increased in M-2 and M-3 feed fed fishes (Table-6).

Table-6.Effect of dietary *Marsilea* in Erythrocytic parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	M-1	M-2	M-3
1	TEC (10^6)	1.69^{a} ± 0.04	1.78^{ab} ± 0.12	1.93^{b} ± 0.15	1.67^{a} ± 0.13
2	Haemoglobin (g%)	5.83^{a} ± 0.35	6.27^{ab} ± 0.7	7.43^{c} ± 0.58	6.6^{ab} ± 0.26
3	Haematocrit (Ht%)	15.66^{a} ± 0.57	19^{b} ± 1	15.66^{a} ± 1.15	15.66^{a} ± 0.58
4	MCV (µ3)	92.50^{b} ± 1.6	106.82^{c} ± 1.97	81.07^{a} ± 2.19	94.22^{b} ± 4.40
5	MCH (µgm)	34.43^{a} ± 1.28	35.14^{a} ± 1.66	38.45^{b} ± 0.5	39.69^{b} ± 1.92
6	MCHC (%)	37.22^{b} ± 1.34	32.91^{a} ± 1.96	47.43^{d} ± 0.69	42.12^{c} ± 0.33

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.

Means with same superscript in the same row is not statistically significant

4.3.1.3. Leucocytic Parameters

The thrombocyte population declined significantly only in M-1 group fishes, however significantly higher levels were observed in M-3 feed fed group fishes. The total leucocytes recorded a dose dependent increase in all the experimental groups, which were statistically significant in M-2 and M-3 groups. But in differential leucocyte counts, statistically insignificant increase in Neutrophil counts was recorded only in M-3 feed group fishes, but lymphocytes of M-2 recorded a significant increase. No significant changes were observed in basophil, eosinophil and monocyte populations (Table-7).

Table-7.Effect of dietary *Marsilea* in Leucocytic parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	M-1	M-2	M-3
1	Thrombocyte (10^5)	1.19^{ab} ± 0.009	1.07^{a} ± 0.07	1.17^{ab} ± 0.08	1.29^{b} ± 0.07
2	TLC (10^4)	3.87^{a} ± 0.12	3.98^{a} ± 0.14	4.29^{b} ± 0.11	4.21^{b} ± 0.06
3	Neutrophils (%)	30.33^{ab} ± 2.51	31^{ab} ± 5	28.33^{ab} ± 4.5	35.66^{b} ± 5.03
4	Eosinophils (%)	2.33^{ab} ± 0.57	2.33^{ab} ± 0.57	1.66^{a} ± 1.15	3.67^{b} ± 1.15
5	Basophils (%)	3^{a} ± 1	2.33^{a} ± 0.57	1.66^{a} ± 1.15	3^{a} ± 1
6	Lymphocytes (%)	59.66^{b} ± 2.51	58.67^{ab} ± 3.21	63.33^{b} ± 1.15	53^{a} ± 2.64
7	Monocytes (%)	4.66^{a} ± 1.52	5.66^{ab} ± 1.15	4.33^{a} ± 1.53	4.67^{a} ± 1.15

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.

Means with same superscript in the same row is not statistically significant

4.3.1.4. Serum Biochemical Parameters

Serum Protein and Globulin levels elevated significantly in dose dependent manner in all *Marsilea* diet fed groups when compared to control. Significant increase in Albumin level was observed only in M-2 group. Highly significant decline in Albumin/Globulin ratio was observed in M-1 and M-3 feed fed fishes indicating higher level of globulin. The cholesterol level was higher in all experimental groups but not statistically significant. The blood glucose level elevated significantly only in M-1 and declined insignificantly in M-2 and M-3 groups (Table-8).

Table-8.Effect of dietary *Marsilea* in Serum biochemical parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	M-1	M-2	M-3
1	Protein (g/dl)	5.13^{a} ± 0.15	5.7^{b} ± 0.1	6.2^{c} ± 0.26	6.93^{d} ± 0.35
2	Albumin (g/dl)	2.20^{ab} ± 0.13	2.06^{a} ± 0.08	2.53^{c} ± 0.15	2.43^{bc} ± 0.15
3	Globulin (g/dl)	2.93^{a} ± 0.02	3.64^{b} ± 0.03	3.67^{b} ± 0.11	4.5^{c} ± 0.2
4	Albumin/Globulin ratio	0.74^{c} ± 0.04	0.56^{a} ± 0.02	0.68^{b} ± 0.02	0.53^{a} ± 0.01
5	Cholesterol (mg/dl)	128^{a} ± 2.64	131^{a} ± 10.53	134.3^{a} ± 6.03	135^{a} ± 9.54
6	Blood Glucose (mg/dl)	93^{ab} ± 7	109.3^{b} ± 6.02	82.6^{a} ± 6.51	81^{a} ± 6.56

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.

Means with same superscript in the same row is not statistically significant

4.3.1.5. Immunological Parameters

The agglutination titer analyses in *Marsilea* diet fed fishes, it was observed that even sixteen times of serum dilution has the potential to agglutinate the bacteria which was two times more dilution when compared with their control counterparts. Similarly the serum lysozyme concentration has increased significantly in all *Marsilea* fed fishes than control fishes which may be the reasons for the significant improvement in the serum bactericidal activity. The myeloperoxidase enzyme activity was also higher in all *Marsilea* diet fish groups indicating an improvement in pathogen resisting potential (Table-9).

Table-9.Effect of dietary *Marsilea* in Immunological parameters of *Labeo rohita*(Mean±SD)

Sl.No	Parameters	Control	M-1	M-2	M-3
1	Agglutination titre	8.67^{a} ± 1.15	10.67^{a} ± 1.17	14.67^{b} ± 1.21	16.33^{bc} ± 1.53
2	Lysozyme Assay (µg/ml)	1.05^{a} ± 0.05	1.63^{b} ± 0.12	2.97^{c} ± 0.13	3.30^{d} ± 0.17
3	Serum Bactericidal Activity (1-cfu/control (%))	14.33^{a} ± 1.52	27.66^{b} ± 1.78	31.67^{c} ± 1.64	48.33^{d} ± 1.53
4	Myeloperoxidase Activity (OD)	0.65^{a} ± 0.09	1.0^{b} ± 0.05	0.98^{b} ± 0.13	1.02^{b} ± 0.06

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.
Means with same superscript in the same row is not statistically significant

Thus incorporation of *Marsilea*in feed had not only enhanced growth but also improved haemoglobin, MCH, MCHC, Platelets, total leucocytes (in particular

lymphocytes), serum protein and globulin levels. The elevations were at optimum levels at M-2 (0.5% *Marsilea* extract diet) feed group. On the other hand decrease in blood glucose can be attributed to the hypoglycemic effect of *Marsilea* and further it was also observed that dietary incorporation of *Marsilea* at 1% level inhibited the growth of fish. Significant to highly significant increase in the immunological parameters like agglutination titre, lysozyme assay, serum bactericidal activity, and myeloperoxidase activity was observed in almost all groups of *Marsilea* diet fed fishes and higher values were noted at M-3 dose of *Marsilea*.

4.3.2. β-Glucan incorporated diet:

4.3.2.1. Growth Parameters

Highly significant elevation in mean weight gain, % weight gain and specific growth rate was observed in fishes fed with G-1 and G-2 feed and a decline in growth was observed in G-3 group when compared with control. Higher growth promoting potential was observed in G-1 group (Table-10).

Table-10.Effect of dietary β-glucan in growth parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	G-1	G-2	G-3
1	Weight Gain	2.91^{a} ± 0.2	7.36^{c} ± 0.45	5.15^{b} ± 0.05	3.07^{a} ± 0.08
2	% Weight Gain	6.55^{a} ± 0.71	15.25^{c} ± 1.69	10.21^{b} ± 0.36	5.70^{a} ± 0.28
3	Specific Growth Rate (%)	7.28^{a} ± 0.5	18.39^{c} ± 1.15	12.88^{b} ± 0.12	7.69^{a} ± 0.21

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.

Means with same superscript in the same row is not statistically significant

4.3.2.2. Erythrocytic Parameters

Decline in total erythrocyte count (TEC) with increase in glucan concentration was recorded, however the levels were higher in G-1 and G-2 groups when compared with control counterparts. In contrast to total erythrocytes, haemoglobin recorded a highly significant increase in all glucan fed groups and was highest at G-2. Similarly Haematocrit (Ht) level remained high in all the experimental groups especially significantly higher in G-1 group, this can be attributed to increased levels of erythrocytes, platelets and leucocytes in the same group (Table-11).

Table-11. Effect of dietary β-glucan in Erythrocytic parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	G-1	G-2	G-3
1	TEC (10^6)	1.69^{a} ± 0.04	1.82^{ab} ± 0.11	1.71^{ab} ± 0.09	1.69^{a} ± 0.11
2	Haemoglobin (g%)	5.83^{a} ± 0.35	7.06^{ab} ± 0.21	8.26^{c} ± 0.32	7.53^{b} ± 0.21
3	Haematocrit (%)	15.66^{a} ± 0.57	24.33^{c} ± 1.15	18.66^{bc} ± 0.57	17.67^{ab} ± 1.52
4	MCV (μ3)	92.50^{a} ± 1.6	133.28^{c} ± 2.67	104.85^{b} ± 0.89	104.23^{b} ± 2.57
5	MCH (μgm)	34.43^{a} ± 1.28	38.73^{b} ± 1.4	48.19^{d} ± 1.37	44.56^{c} ± 1.71
6	MCHC (%)	37.22^{b} ± 1.34	29.06^{a} ± 1.01	45.95^{d} ± 1.05	42.79^{c} ± 2.59

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.
Means with same superscript in the same row is not statistically significant

The erythrocytic Indices like Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH) and Mean corpuscular haemoglobin concentration

(MCHC) increased significantly, indicating the accommodation of more haemoglobin per erythrocyte.

4.3.2.3. Leucocytic Parameters

Increase in the population of thrombocytes was not much statistically significant in the glucan fed fishes, and the total leucocyte counts were significantly higher only in G-1 group. In differential leucocyte counts, neutrophils of G-1 exhibited a significant decrease and monocytes increased significantly. The lymphocyte populations were remarkably higher in G-1 group but not statistically significant. No significant changes were observed in eosinophil and basophil populations (Table-12).

Table-12.Effect of dietary β-glucan in Leucocytic parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	G-1	G-2	G-3
1	Thrombocyte (10^5)	1.19^{ab} ± 0.009	1.19^{ab} ± 0.06	1.27^{b} ± 0.11	1.28^{b} ± 0.11
2	TLC (10^4)	3.87^{a} ± 0.12	4.42^{b} ± 0.17	3.91^{a} ± 0.07	3.83^{a} ± 0.09
3	Neutrophils (%)	30.33^{ab} ± 2.51	24.33^{a} ± 3.51	30.66^{ab} ± 5.03	35.67^{b} ± 3.05
4	Eosinophils (%)	2.33^{ab} ± 0.57	3^{abc} ± 1	2.33^{ab} ± 1.15	4.33^{c} ± 0.58
5	Basophils (%)	3^{a} ± 1	1.66^{a} ± 0.47	1.66^{a} ± 0.47	2.3^{a} ± 1.15
6	Lymphocytes (%)	59.66^{b} ± 2.51	63.66^{b} ± 3.78	59.66^{b} ± 4.93	53^{a} ± 2
7	Monocytes (%)	4.66^{a} ± 1.52	7.33^{c} ± 0.15	5.66^{ab} ± 1.15	4.67^{a} ± 1.52

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.

Means with same superscript in the same row is not statistically significant

4.3.2.4. Serum Biochemical Parameters

The serum protein, Albumin and globulin levels increased significantly in all the glucan feed fed fishes and the increase was dose dependent in protein and globulin levels. Significant to highly significant decline in albumin/globulin ratio in all glucan feed fed fishes explains an elevated globulin levels when compared to their control counterparts. No significant alterations were observed in cholesterol and blood glucose levels (Table-13).

Table-13. Effect of dietary β-glucan in Serum Biochemical parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	G-1	G-2	G-3
1	Protein (g/dl)	5.13^{a} ± 0.15	7.06^{b} ± 0.25	7.96^{c} ± 0.35	8.1^{c} ± 0.2
2	Albumin (g/dl)	2.20^{ab} ± 0.13	2.71^{bc} ± 0.2	2.75^{c} ± 0.13	2.02^{a} ± 0.19
3	Globulin (g/dl)	2.93^{a} ± 0.02	4.35^{b} ± 0.05	5.21^{c} ± 0.23	6.07^{d} ± 0.02
4	Albumin/Globulin ratio	0.74^{d} ± 0.04	0.62^{c} ± 0.04	0.52^{b} ± 0.01	0.33^{a} ± 0.03
5	Cholesterol (mg/dl)	128^{a} ± 2.64	132.6^{a} ± 12.01	132.66^{a} ± 7.5	129^{a} ± 0.7
6	Blood Glucose (mg/dl)	93^{ab} ± 7	99.3^{bc} ± 8.08	89^{ab} ± 8.54	84^{a} ± 8

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.

Means with same superscript in the same row is not statistically significant

4.3.2.5. Immunological Parameters

The agglutination titer performed with the serum of control and glucan feed fed fishes demonstrated that the serum of glucan fed fishes had the potential to agglutinate with the bacteria even at 26 times dilution which is an indication of highly improved immune response. Similarly, the serum bactericidal activity and myeloperoxidase activity has increased significantly in all glucan fed fish groups. The concentration of lysozyme has increased tremendously in all the glucan fed fish groups indicating glucan's potential in improving the immune response (Table-14). However, the improvement in immunological parameters were significant only in G-1 and G-2.

Table-14.Effect of dietary β-glucan in Immunological parameters of *Labeo rohita* (Mean±SD)

Sl.No	Parameters	Control	G-1	G-2	G-3
1	Agglutination titre	8.67^{a} ± 1.15	18.00^{b} ± 2.00	26.00^{c} ± 2.00	15.33^{d} ± 1.15
2	Serum Bactericidal Activity (1-cfu/control (%))	14.33^{a} ± 1.52	64.33^{c} ± 1.63	71.33^{d} ± 2.08	48.00^{b} ± 2.00
3	Lysozyme Assay (µg/ml)	1.05^{a} ± 0.05	5.14^{c} ± 0.06	6.56^{d} ± 0.14	3.3^{b} ± 0.12
4	Myeloperoxidase Activity (OD)	0.65^{a} ± 0.09	1.16^{b} ± 0.13	1.54^{c} ± 0.14	1.04^{b} ± 0.07

G-1 = 0.25%β-Glucan, G-2 = 0.5%β-Glucan, G-3 = 1%β-Glucan.

Means with same superscript in the same row is not statistically significant

β-Glucan is a known Immunostimulant with vast literature. In the present work also it was observed that incorporation of β-Glucan in fish feed has significantly elevated the growth, haemoglobin, packed cell volume, MCV, MCH, MCHC, total leucocytes,

serum protein, albumin, globulin, especially at 0.25% level. It was also recorded that incorporation of glucan has significantly increased the agglutination titre, serum bactericidal activity, Lysozyme concentration and myeloperoxidase activity.

4.3.3. *Marsilea* Versus Glucan

When the potential of *Marsilea* and Glucan were compared with control feeds, it was observed that *Marsilea* also possess high growth promoting potential like Glucan. The maximum specific growth rate observed at 0.5% level in *Marsilea* is obtained at 0.25% level of Glucan itself (Figure-1).

It was observed that both *Marsilea* is more potent in improving the levels of total erythrocytes and Glucan improved the concentration of haemoglobin than *Marsilea* (Figure-2 and 3). However the *Marsilea* is equally potent to Glucan in improving total leucocyte counts (Figure-4) and thrombocyte counts. Biochemical analysis of the serum of *L.rohita* fed with *Marsilea* and Glucan were found to be potent in improving serum protein and globulin levels (Figure-5 and 6), however the increase was marginally higher in Glucan fed fishes. It was also observed that the glucose levels were lower in *Marsilea* fed fishes than Glucan, which canbe attributed to its hypoglycemic (Glucose reducing potential) property (Table-15 and 16).

Table-15. Comparison of Erythrocytic and Leucocytic Parameters in *L.rohita* fed with *Marsilea* and β-Glucan diets (Mean±SD).

Sl. No	Parameters	Control	M-1	M-2	M-3	G-1	G-2	G-3
1	TEC (10^6)	1.69^{a} ± 0.04	1.78^{ab} ± 0.12	1.93^{b} ± 0.15	1.67^{a} ± 0.13	1.82^{ab} ± 0.11	1.71^{ab} ± 0.09	1.69^{a} ± 0.11
2	Haemoglobin (g%)	5.83^{a} ± 0.35	6.27^{ab} ± 0.7	7.43^{c} ± 0.58	6.6^{ab} ± 0.26	7.06^{bc} ± 0.21	8.26^{d} ± 0.32	7.53^{c} ± 0.21
3	Haematocrit (Ht%)	15.66^{a} ± 0.57	19^{c} ± 1	15.66^{a} ± 1.15	15.66^{a} ± 0.58	24.33^{d} ± 1.15	18.66^{bc} ± 0.57	17.67^{ab} ± 1.52
4	Thrombocyte (10^5)	1.19^{ab} ± 0.009	1.07^{a} ± 0.07	1.17^{ab} ± 0.08	1.29^{b} ± 0.07	1.19^{ab} ± 0.06	1.27^{b} ± 0.11	1.28^{b} ± 0.11
5	TLC (10^4)	3.87^{a} ± 0.12	3.98^{a} ± 0.14	4.29^{b} ± 0.11	4.21^{b} ± 0.06	4.42^{b} ± 0.17	3.91^{a} ± 0.07	3.83^{a} ± 0.09
6	Neutrophils (%)	30.33^{ab} ± 2.51	31^{ab} ± 5	28.33^{ab} ± 4.5	35.66^{b} ± 5.03	24.33^{a} ± 3.51	30.66^{ab} ± 5.03	35.67^{b} ± 3.05
7	Eosinophils (%)	2.33^{ab} ± 0.57	2.33^{ab} ± 0.57	1.66^{a} ± 1.15	3.67^{bc} ± 1.15	3^{abc} ± 1	2.33^{ab} ± 1.15	4.33^{c} ± 0.58
8	Basophils (%)	3^{a} ± 1	2.33^{a} ± 0.57	1.66^{a} ± 1.15	3^{a} ± 1	1.66^{a} ± 0.47	1.66^{a} ± 0.47	2.3^{a} ± 1.15
9	Lymphocytes (%)	59.66^{b} ± 2.51	58.67^{ab} ± 3.21	63.33^{b} ± 1.15	53^{a} ± 2.64	63.66^{b} ± 3.78	59.66^{b} ± 4.93	53^{a} ± 2
10	Monocytes (%)	4.66^{a} ± 1.52	5.66^{ab} ± 1.15	4.33^{a} ± 1.53	4.67^{a} ± 1.15	7.33^{c} ± 0.15	5.66^{ab} ± 1.15	4.67^{a} ± 1.52

Means with same superscript in the same row is not statistically significant

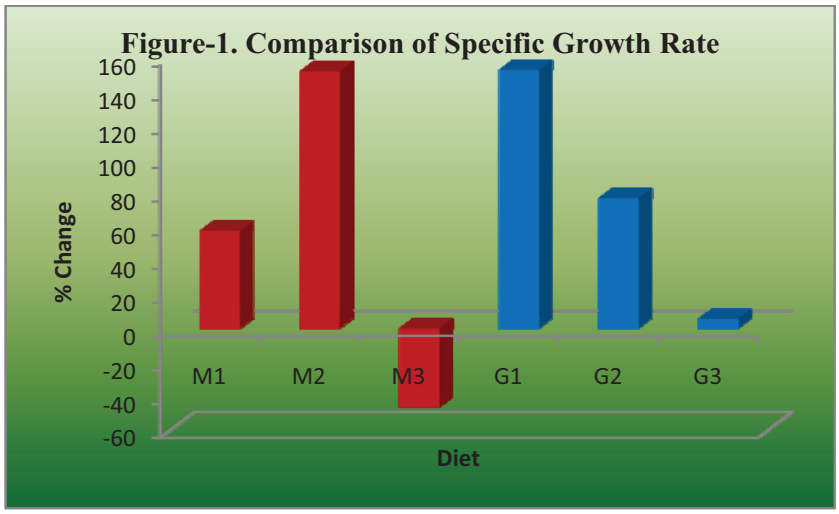
C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*

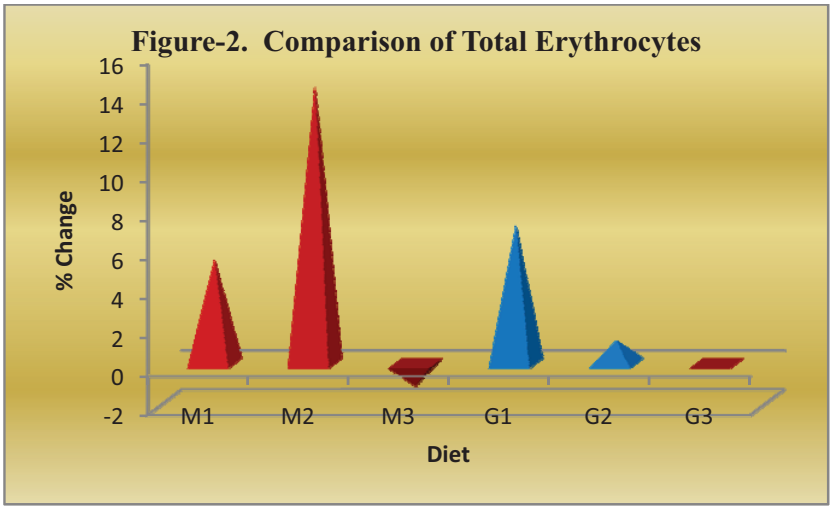
Table-16. Comparison of Serum biochemical and Immunological Parameters in *L.rohita* fed with *Marsilea* and β-Glucan diets (Mean±SD).

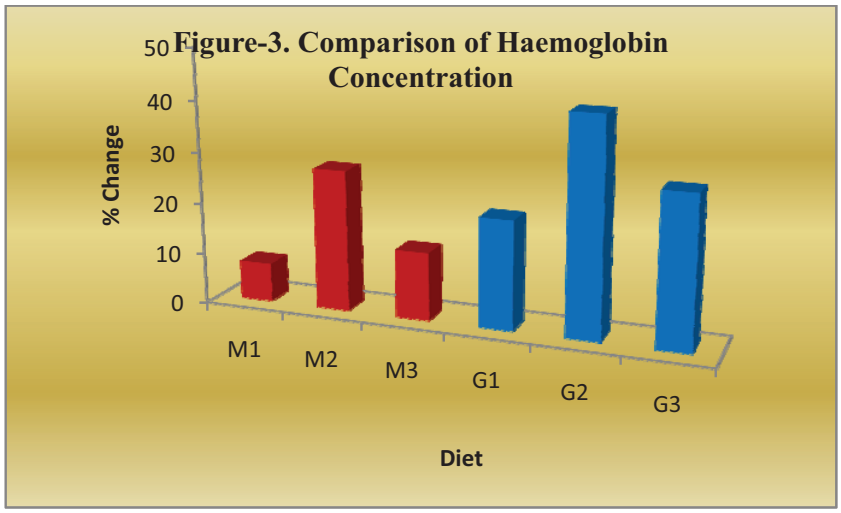
Sl. No	Parameters	Control	M-1	M-2	M-3	G-1	G-2	G-3
1	Protein (g/dl)	5.13^{a} ± 0.15	5.7^{b} ± 0.1	6.2^{c} ± 0.26	6.93^{d} ± 0.35	7.06^{d} ± 0.25	7.96^{e} ± 0.35	8.1^{e} ± 0.2
2	Albumin (g/dl)	2.20^{ab} ± 0.13	2.06^{a} ± 0.08	2.53^{cd} ± 0.15	2.43^{bc} ± 0.15	2.71^{cd} ± 0.2	2.75^{d} ± 0.13	2.02^{a} ± 0.19
3	Globulin (g/dl)	2.93^{a} ± 0.02	3.64^{b} ± 0.03	3.67^{b} ± 0.11	4.5^{c} ± 0.2	4.35^{c} ± 0.05	5.21^{d} ± 0.23	6.07^{e} ± 0.02
4	Albumin/Globulin ratio	0.74^{e} ± 0.04	0.56^{b} ± 0.02	0.68^{d} ± 0.02	0.53^{b} ± 0.01	0.62^{c} ± 0.04	0.52^{b} ± 0.01	0.33^{a} ± 0.03
5	Cholesterol (mg/dl)	128^{a} ± 2.64	131^{a} ± 10.53	134.3^{a} ± 6.03	135^{a} ± 9.54	132.6^{a} ± 12.01	132.66^{a} ± 7.5	129^{a} ± 0.7
6	Blood Glucose (mg/dl)	93^{ab} ± 7	109.3^{c} ± 6.02	82.6^{a} ± 6.51	81^{a} ± 6.56	99.3^{bc} ± 8.08	89^{ab} ± 8.54	84^{a} ± 8
7	Agglutination titre	8.67^{a} ± 1.15	10.67^{a} ± 1.17	14.67^{b} ± 1.21	16.33^{bc} ± 1.53	18.00^{c} ± 2.00	26.00^{d} ± 2.00	15.33^{bc} ± 1.15
8	Lysozyme Assay (µg/ml)	1.05^{a} ± 0.05	1.63^{b} ± 0.12	2.97^{c} ± 0.13	3.30^{d} ± 0.17	5.14^{e} ± 0.06	6.56^{f} ± 0.14	3.3^{d} ± 0.12
9	Serum Bactericidal Activity (cfu/control)	14.33^{a} ± 1.52	27.66^{b} ± 1.78	31.67^{c} ± 1.64	48.33^{d} ± 1.53	64.33^{e} ± 1.63	71.33^{f} ± 2.08	48.00^{d} ± 2.00
10	Myeloperoxidase Activity (OD)	0.65^{a} ± 0.09	1.0^{b} ± 0.05	0.98^{b} ± 0.13	1.02^{b} ± 0.06	1.16^{b} ± 0.13	1.54^{c} ± 0.14	1.04^{b} ± 0.07

Means with same superscript in the same row is not statistically significant

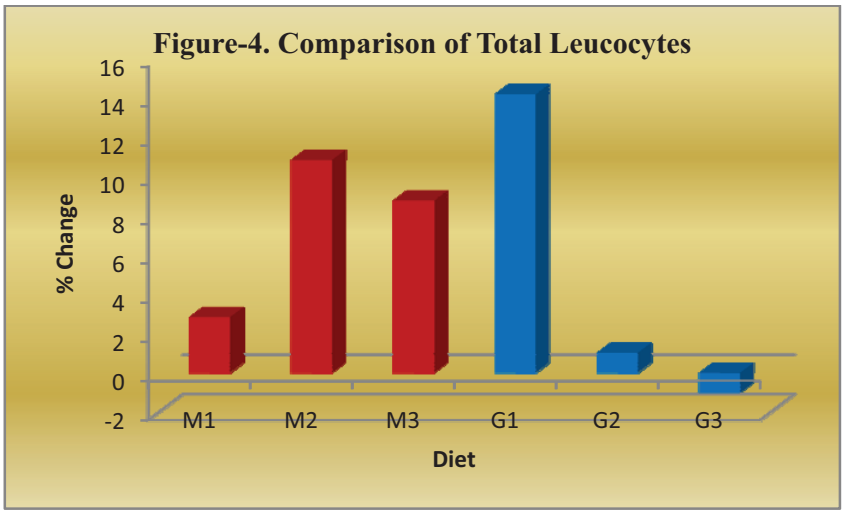
C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*

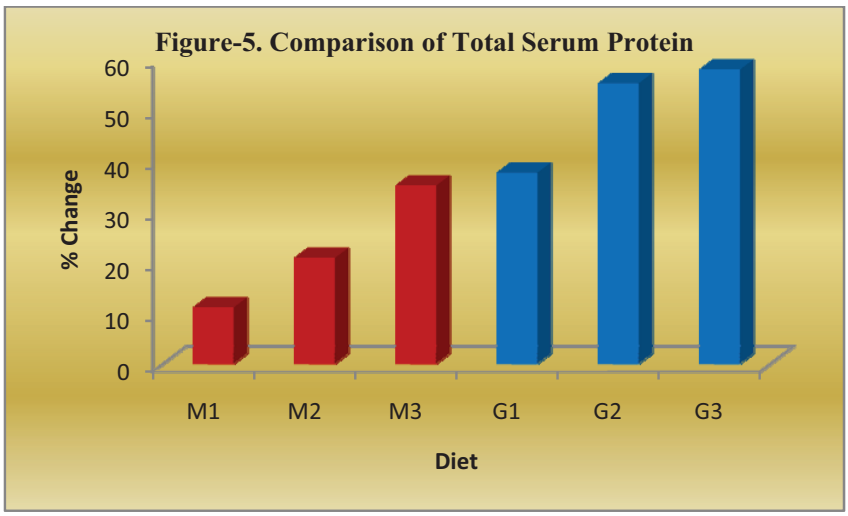


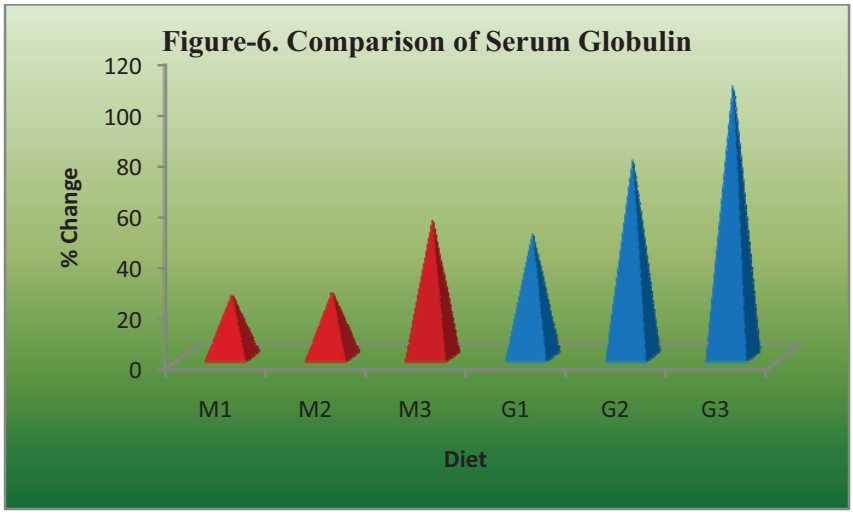




C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*







C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*

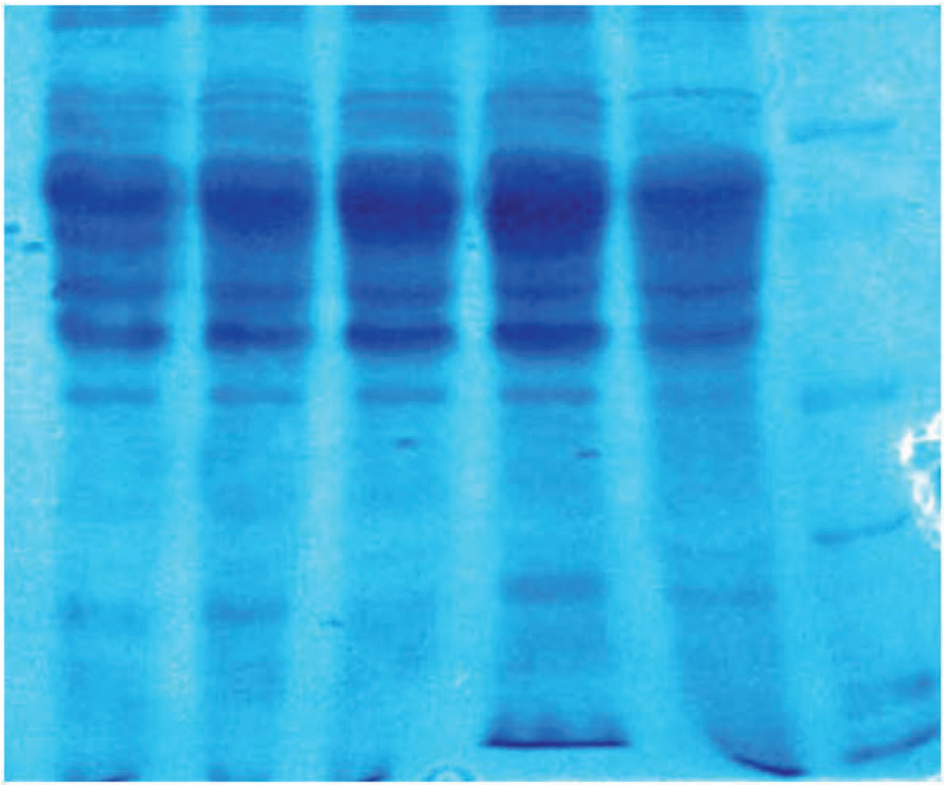
4.3.3.1. SDS-PAGE Analysis

As significant haematological and serum biochemical changes were observed in M-2, M-3, G-1 and G-2 groups the serum of these groups were electrophoretically analysed along with control group for the presence of different protein fractions. The electrophoretic separation of serum by SDS-PAGE revealed the presence of only three protein fractions in control group, where as in M-2 group 5 protein fractions, in M-3 4 protein fractions, in G-1 6 protein fractions and in G-2 4 protein fractions with different molecular weights were observed. It was clearly observed that protein fraction with high molecular weight (100 and above) and with low molecular weight (below 40) was present in both control and in all the test groups, but new protein fractions with intermediate molecular weight were observed in all the experimental diet groups, ie, in M-2 three new protein fractions (80.297, 59.731 and 48.074), in M-3 two new protein fractions (71.658 and 55.646), in G-1four new protein fractions (70.235, 55.169, 45.974 and 40.451) and in G-2 two new protein fractions (70.941 and 52.455) were observed. Probably the new protein fractions are may be of globulin fractions. This clearly indicates the feed (*Marsilea* and Glucan) has the potential in improving the serum protein fractions. Further it was also clear that *Marsilea* at 0.5% (M-2 group) has the potential in inducing the production of different proteins as like the well known immunostimulant β-Glucan (Plate-7 and Plate-8, Table-17).

When the Immunological parameters like agglutination titer, serum bactericidal activity, lysozyme concentration and myeloperoxidase activity of *Marsilea* fed fishes was compared with the known immunostimulant Glucan it was observed that *Marsilea* also have the potential to enhance the non specific immune response (Figure – 7, 8, 9 and 10).

Plate -7.SDS-PAGEof Serum of different feed groups of *Labeorohita.*

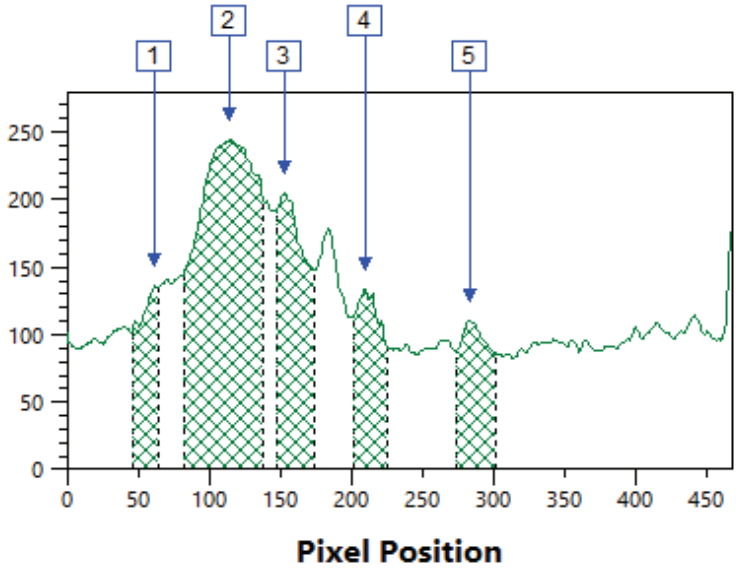
Marker M2 G1 M3 G2 Control



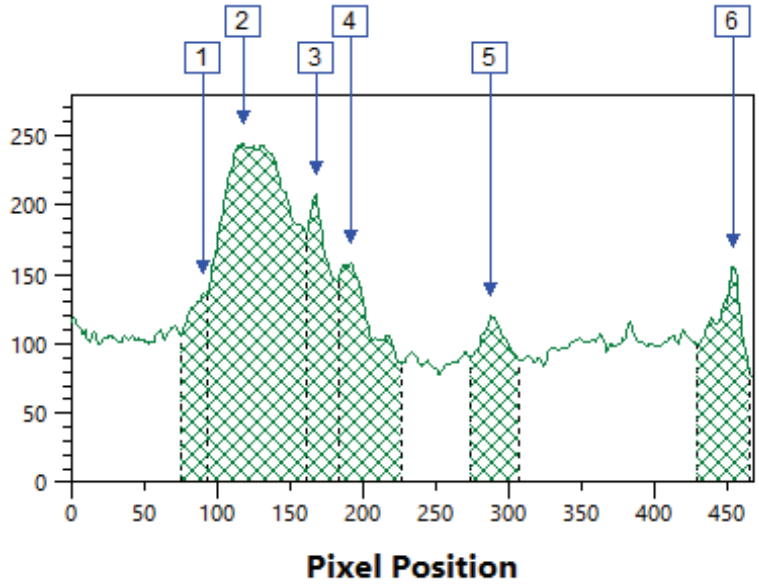
C=Control diet, M-2= 0.5% *Marsilea* diet, M-3= 1% *Marsilea* diet, G-1=0.25% Glucan diet, G-2=0.5% Glucan diet

Plate – 8.DensitometricGraph of Serum of different feed groups of *Labeorohita*

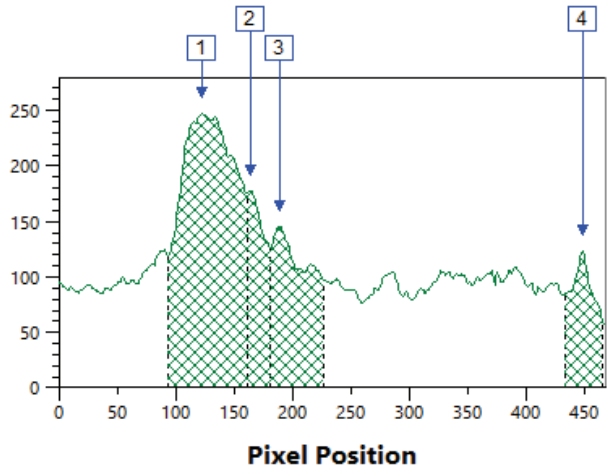
M-2 Diet



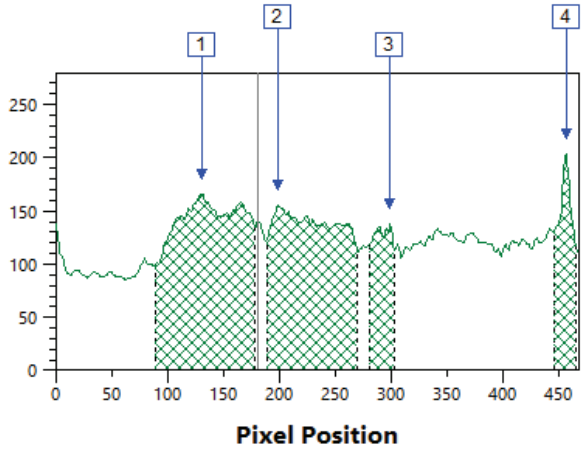
G-1 diet



M-3 diet



G-2 diet



Control diet

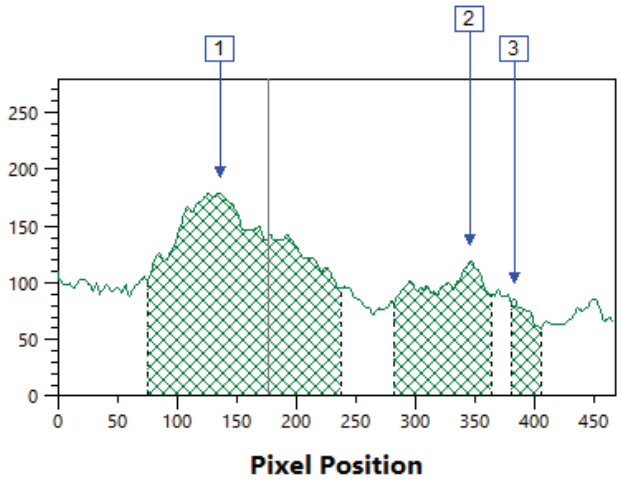


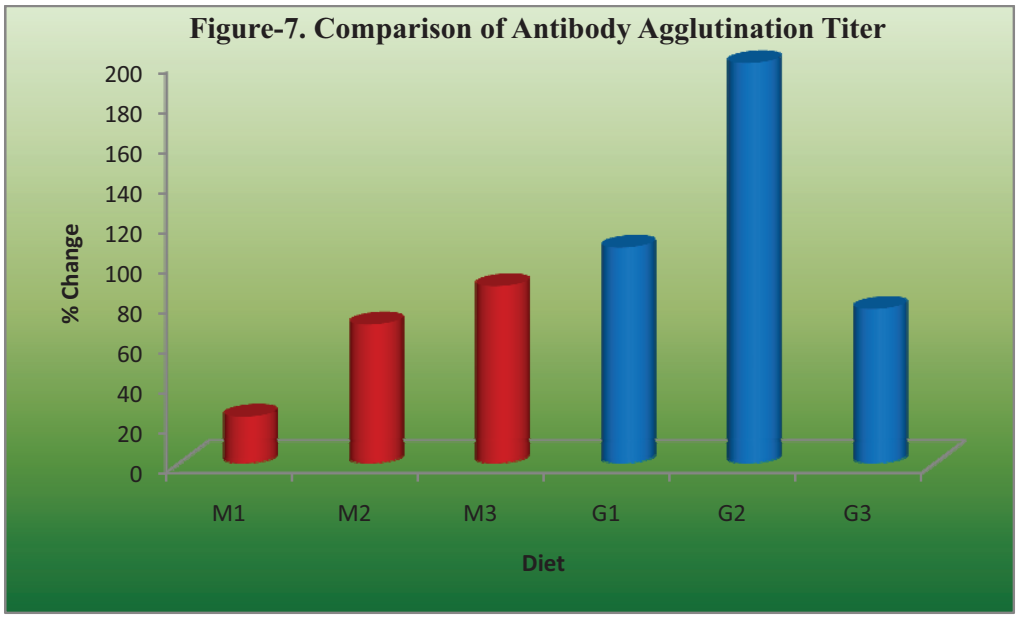
Table 17. Densitometric Profile of serum proteins of different diet group fishes.

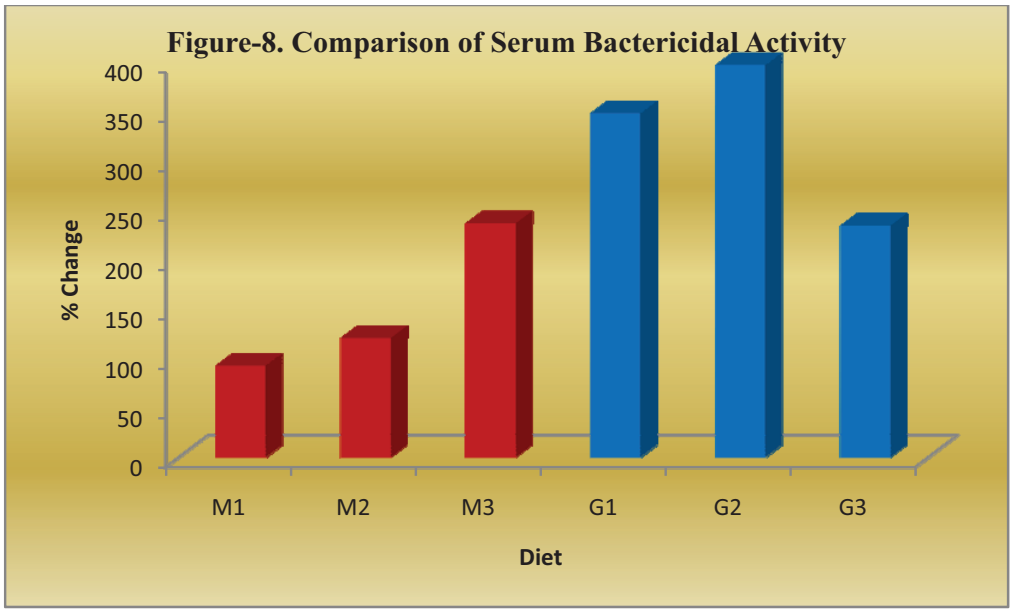
Band No	Marker		M2		G1		M3		G2		Control	
	M.W	R.F	M.W	R.F	M.W	R.F	M.W	R.F	M.W	R.F	M.W	R.F
1	205	0.173	133.542	0.239	116.757	0.262	104.952	0.281	103.737	0.284	106.186	0.279
2	97	0.267	80.297	0.333	70.235	0.36	71.658	0.356	70.941	0.358	30.343	0.638
3	66	0.486	59.731	0.397	55.169	0.416	55.646	0.414	52.455	0.429	28.841	0.746
4	43	0.55	48.074	0.452	45.974	0.465	31.639	0.612	30.962	0.625		
5	20	0.704	31.522	0.614	40.451	0.505						
6	14	0.878			37.283	0.535						

M-2 = 0.5% Marsilea diet, G-1 = .025% β-Glucan diet, M-3 = 1% Marsilea diet, G-2 = 0.5% β-Glucan diet. M.W=Molecular weight, RF= Relative front

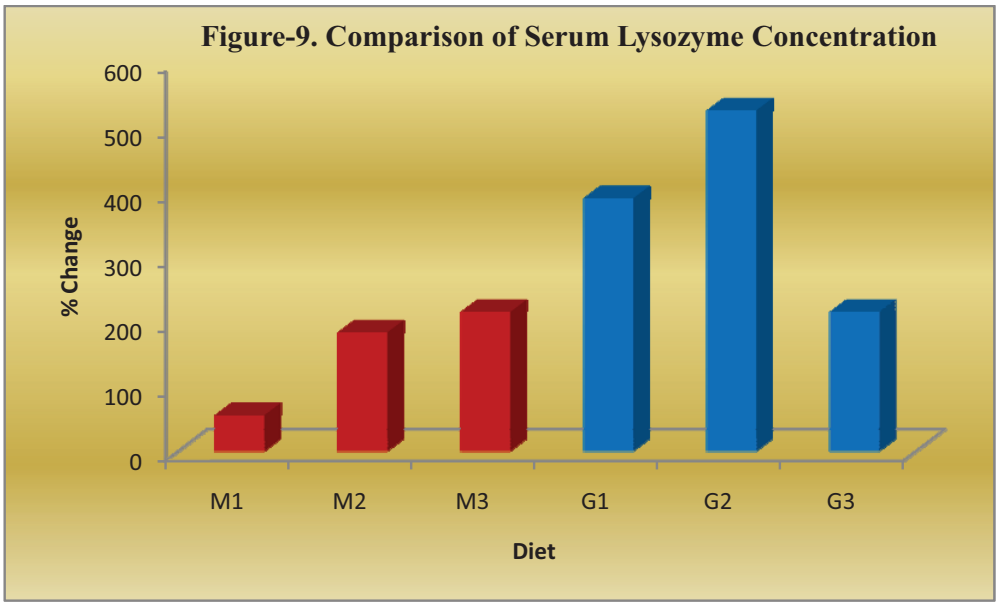
It was also observed that optimum improvement in different parameters at 0.25% level of glucan is attained at 0.5% level of *Marsilea*. Thus dietary incorporation of *Marsilea* also improves growth, haematological, serum biochemical and immune parameters as like Glucan. The Figures-1 to 10 clearly depicts the enhancement (in % Change) in *Marsilea* and Glucan diet fed fishes when compared to their control counterparts.

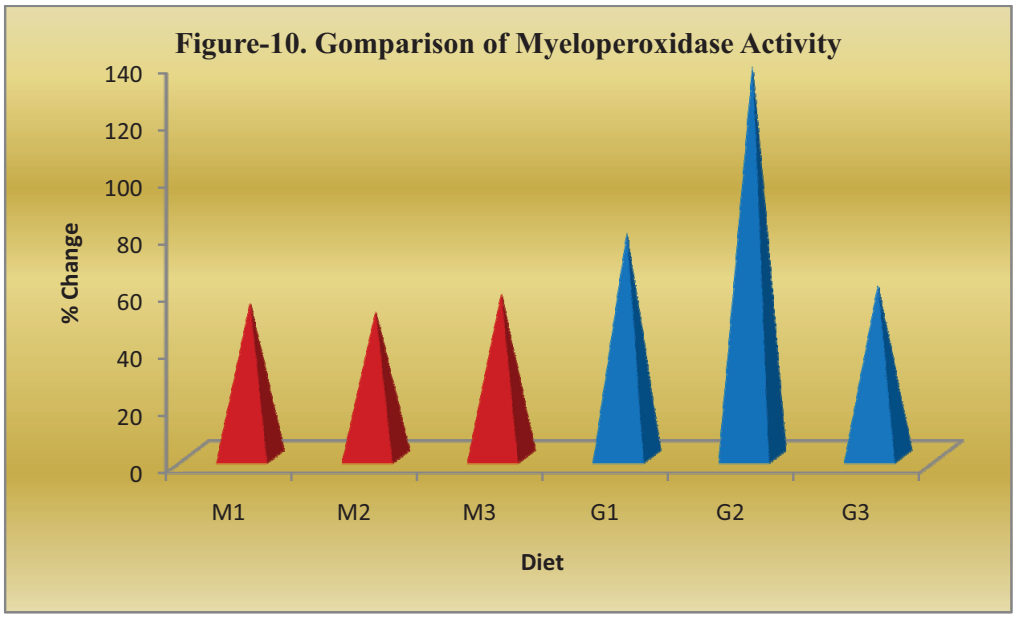
In the present study it was observed that incorporation of *Marsilea* in the fish feed improved specific growth rate, serum protein, globulin, haemoglobin, MCH, MCHC, total platelets, total leucocytes and lymphocytes and a similar effect produced by the well known Immunostimulant β-glucan.





C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*





C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*

4.4. DISCUSSION

Several herbs were tested for their growth-promoting activities in aquatic animals (Citarasu *et al* 2002 and Sivaram *et al* 2004). Aly *et al* (2008) observed a significant increase in the body weight gain and specific growth rates in Tilapia fed with *Echinacea* supplemented diet at a rate of 0.25ppt for 6 months. The improved fish growth and feed utilization may be because of the palatability or attractiveness of the diets, which in turn cause increased feed intake, further the plant may inhibit the pathogens present in the digestive tract, may enhance the population of beneficial microorganisms, and/or may enhance the microbial enzyme activity that consequently improves the feed digestibility and nutrient absorption (Mohsen Abdel-Tawwab *et al* 2010). Lin *et al* (2006) reported that traditional Chinese medicinal herb (CMH) may influence digestive processes by enhancing enzyme activity and improving digestibility of nutrients and thus may increase the diet evacuation rate as herb levels increased. Goda (2008) found that supplementing diets with *ginsana, Panax ginseng* significantly improved Nile tilapia growth and feed utilization.

The percentage volume of erythrocytes, in fish blood is an indication of the health status. It can be helpful in detecting any abnormal changes through the use of Immunostimulants. Reduced haematocrit values may indicate that fish are not eating properly or are suffering from infections (Blaxhall 1972). Hematocrit, haemoglobin and the erythrocytic concentration values indicate the oxygen carrying capacity and serve as indices of the aerobic capabilities of the fish. Leucocytes and thrombocytes are considered as important parameters to evaluate both the fish's state of health and their immune system (Tavares-Dias *et al* 2004).

The present study reveals an increase in cellular immunological indicators such as erythrocytes, leucocytes and thrombocytes in the experimental fish may be due to the

increase in the levels of immunity which in turn could be due to the action of the extract present in the diets.

There is a close relationship between the level of protein synthesis in liver tissue and plasma protein pools, total protein levels in plasma may be elevated due to the increased levels of protein synthesis in liver tissue. The increase in plasma protein results when anabolic processes exceed catabolic ones, and reserve protein is produced in greater quantity to meet increased metabolic requirements of the fish (Helmy *et al* 1974).

Serum protein includes various humoral elements of the non-specific immune system and increase in serum total protein, globulin and albumin are likely to be a result of the enhancement of the non-specific immune response of fishes (Citarasu *et al* 2006). Serum albumin not only maintains osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues but also acts as plasma carrier protein to transport steroid hormones, hemin, fatty acids and also compounds like drugs (Asadi *et al* 2012, Wikipedia-serum_albumin). Improved levels of albumin in *Marsilea* fed fishes might also improve the transport of different chemical compounds needed for immunity to the target sites.

Suheyla Karatas *et al* (2003) fed Rainbow trout (*Oncorhynchus mykiss*) with dietary Mistletoe (*Viscum album*), Nettle (*Urtica dioica*) and Ginger (*Zingiber officinale*) for 21 days and observed no significant change in specific growth rate; however the total plasma protein levels were elevated. Dugenci *et al* (2003) reported that plasma total protein level in rainbow trout increased significantly after feeding the fish with various herbal extracts.

The lipids are the usual economic form of biomolecules used by fish to stock energy and can be stored in many different organs (Guijarro *et al* 2003).Improved levels of cholesterol observed in the present investigation implies improved reserve energy.

Goda (2008) found a similar observation in Nile Tilapia that erythrocytes, haematocrit and haemoglobin increased significantly with increasing dietary *ginsana* levels compared to control fish. The present study agrees with the results obtained by Hrubec *et al* (2000) for Tilapia (*Oreochromis* hybrid) and Dobsikova *et al* (2006) for common carp, *Cyprinus carpio.* Aly *et al* (2008) observed a significant increase in the total leucocyte count in Tilapia, fed with *Echinacea* supplemented diet at a rate of 0.25 ppt for 6 months. Rainbow trout (*Oncorhynchus mykiss*) fed with dietary *Nigella sativa* (Black cumin seeds) for 21 days exhibited improved levels of hematocrit, leucocrit and serum protein levels, however no significant change in leucocyte levels were observed (Dorucu *et al* 2009).

Asadi *et al* (2012) observed a significant increase in haemoglobin level, MCHC, serum globulin, total protein and serum lysozyme levels in Rainbow trout when fed with dietary *Nasturtium nasturtium* (Water cress) for 21 days which is in accordance with the present findings. *Oreochromis mossambicus* fed with dietary *Andrographis paniculata* for 45 days exhibited significant rise in specific growth rate, erythrocytes, haemoglobin, MCV, MCHC, leucocytes and thrombocyte populations (Prasad and Mukthiraj 2011). Immanuel *et al* (2009) fed Tilapia with herbal diet (*Cynodon dactylon, Aegle marmelos, Withania somnifera* & *Zinger officinale*) for 45 days observed an increase in specific growth rate, PCV, total protein, albumin, globulin and leucocrit levels (highest in Zinger diet), however serum cholesterol decreased significantly and no significant change in blood glucose was observed.

Aly and Mohamed (2010) incorporated *Echinacea purpurea* and Garlic (*Allium sativum*) separately in feed and fed to Nile Tilapia for 3 months and after 4 months observed that haematocrit, leucocytes, lymphocytes and specific growth ratehas increased significantly in both feeds. Mohsen Abdel Tawwab (2010) fed Nile Tilapia with dietary Green Tea for 12 weeks and observed a significant increase in specific growth rate, erythrocytes, total leucocytes, lymphocytes, serum protein, albumin, globulin, glucose, lipid and a decrease in monocytes and granulocytes. Chinnasamy Arulvasu *et al* (2013) observed improved levels of specific growth rate, erythrocytes, haemoglobin, total leucocytes, total serum protein and serum bactericidal activity in *Catla catla* fed with dietary *Zingiber officinale.* Specific growth rate has improved in *Catla catla* fed with dietary *Cynodon dactylon* for 45 days (Kaleeswaran *et al* 2010).

Hajibeglou Abasali & Sudagar Mohamad (2010) fed common carp (*Catla catla*) with dietary *Ocimum basilicum, Cinnamomum zeylanicum, Juglans regis and Mentha piperita* (mixed in equal proportion) fed for 45 days and observed a significant increase in serum protein, globulin, albumin, erythrocytes, total leucocytes and haemoglobin levels but adecrease in glucose levels. Mohamad H. Ahmad *et al* (2011) fed Nile Tilapia with Dietary Cinnamon (*Cinnamomum zeylonicum*) for 90 days and observed significant improvement in specific growth rate, erythrocytes, haemoglobin, PCV and serum total protein levels. Aly *et al* (2008) observed a non significant increase in haematocrit, total leucocytes and specific growth rate in *Oreochromis niloticus* fed with Garlic incorporated diet for 2 months. Prasad and priyanka (2011) fed cat fish (*Pangasianodon hypothalamus*) with dietary *Garcinia gummi-gutta* for 45 days, observed a significant decrease in specific growth rate, blood glucose, total cholesterol, triglycerides and low density lipids but significant increase in erythrocytes, haemoglobin, PCV, MCHC, total leucocytes, thrombocytes, total serum protein and high density lipid.

Rahim *et al* (2014) classified the SDS-PAGE serum proteins in grass carp (*Ctenopharyngodon idella*) into six protein fractions as pre-albumin, albumin, alpha-1, alpha-2, beta and gamma globulins from low to heavy molecular weight, the new protein fractions with moderate molecular weight appeared in the experimental diets may be of globulins indicating immunostimulant feed induced enhancement in the humoral immunity.

Elham Awad *et al* 2013 fed rainbow trout (*Oncorhynchus mykiss*) for 14 days with different concentrations of black Cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) separately and observed that both cumin seed oil and nettle extract has increased the lysozyme concentration, antiprotease activity, total protein, myeloperoxidase activity, bactericidal activity and Immunoglobulin G titers. Haghighi *et al* (2014) fed *Aloe vera* extract to *Onchorhynchus mykiss* for 10 weeks and observed that the serum total protein, albumin, globulin, respiratory burst activity, phagocytic activity and serum lysozyme activity has significantly increased. Ortuno (2002) observed a significant increase in myeloperoxidase activity when gilthead seabream fed with E.alba leaf extract. Behra *et al* (2011) recorded increase in respiratory burst, myeloperoxidase, haemagglutination, haemolytic and bacterial agglutination when *L.rohita* was injected with curcumin.

From the present study it can be inferred that as like potent Immunostimulant β-glucan, *Marsilea quadrifolia* also have the potential in improving the specific growth rate may be due to its ability to improve the palatability, digestibility, enhancing the beneficial microorganisms' population there by enhancing the microbial enzyme activity and improved rate of absorption. The extracts of *Marsilea* may also activate the liver, there by bringing an elevated levels of serum protein, albumin and globulin levels which in turn are responsible for the improved levels of disease resistance, which are further

strengthened by the improved levels of haemoglobin, total leucocytes and total thrombocytes. Further, improved levels of serum cholesterol assure steady supply of stored energy. When compared to Glucan diet fed fishes, significant elevations in agglutination titers, lysozyme concentration, serum bactericidal activity and myeloperoxidase activity in *Marsilea* incorporated diet fed fishes also indicates strengthened humoral and cellular immunity. Thus it can be concluded that *Marsilea* is also equally a potent growth promoter and a potential Immunostimulant to fish.

CHAPTER - 5

Assessment of immunepotential of marsilea quadrifolia in labeo rohita challenged with aeromonas hydrophila

Sivagurunathan A, Immunodiagnostic studies in a chosen fresh water fish administered with a medicinal Aquatic fern *Marsilea quadrifolia,* Ph.D. Thesis, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India.

CHAPTER – 5

ASSESSMENT OF IMMUNEPOTENTIAL OF *MARSILEA QUADRIFOLIA* IN *LABEO ROHITA* CHALLENGED WITH *AEROMONAS HYDROPHILA*

5.1. INTRODUCTION

India is a large depository of medicinal plants but studies on the effects of a number of medicinal plant extracts on immunostimulation and growth promotion of cultured fishes are fragmentary (Prasad and Mukthiraj 2011).

Fishes not only play an important role in the demand of food for humans but also they are widely used for various biological experiments (Pandey *et al* 2012). Fishes, particularly *Danio rerio* (Zebra fish) have emerged as major model organism for biomedical research, such as in developmental genetics, neurophysiology, oncology and biomedicine (Pandey *et al* 2012, Pandey 2011). A number of experiments and the use of drugs have been performed in fish. The antibacterial, antiparasitic and anaesthetic drugs, besides the pharmacokinetic and pharmacodynamic parameters have been well experimented on the fish. Drugs, e.g,. tetracyclines, pencillins, macrolides, quinolones, sulfonamides, immunostimulants, anticancer agents, herbal drugs, vaccines etc. have been successfully experimented on fish. Thus, fish can be used as model organism in the experimental studies (Pandey 2011).

Although herbal remedies have been with us for human therapy for millennia, there has been relatively little research on the medicinal plants to be used against fish diseases. Herbal drugs can be used not only as remedies but even more so, as growth promoters, stress resistance boosters and preventatives of infections. Hence, herbal drugs

in disease management are gaining success, because they are cost effective, eco-friendly and have minimal side effects. A large portion of the world population, especially in developing countries depends on the traditional system of medicine for a variety of diseases. Several hundred genera are used medicinally, as plants are vital sources for potent and powerful drugs. Plants are rich in a wide variety of secondary metabolites of phytochemical constituents such as tannins, alkaloids and flavonoids, which act against different diseases (Pandey and Madhuri 2010, Ravikumar *et al* 2010). Herbs have also been used in other countries for control of shrimp and fish diseases, and successful results have been reported in Mexico, India, Thailand and Japan. Traditional herbal medicines seem to be the potential Immunostimulator. Thus, the use of medicinal plants is an alternative to antibiotics in fish health management, as there is an increasing risk of developing resistance to antibiotics (Yin *et al* 2008) besides accumulation of tissue residues, environmental pollution and immunosuppression (Ellis 1988). Jeney *et al* (2009) opined that the herbal extracts can be used in fish culture as alternatives to vaccines, antibiotics or chemotherapeutic agents.

Hematological parameters are closely related to the response of the animal to the environment, an indication that the environment where fishes live could exert some influence on the hematological characteristics (Gabriel *et al* 2004). These indices have been employed in effectively monitoring the responses of fishes to the stressors and thus their health status under such adverse conditions. They can provide substantial diagnostic information once reference values are established under standardized conditions (Rey Vazquez and Guerrero 2007). It is also possible for early diagnosis of illnesses in case of evaluating hematological data, particularly blood parameters (Rimsh and Adamova, 1973). Hematological parameters are among one of the important tools for fish disease diagnosis (Ruane *et al* 2000, Ranzani-Paiva *et al* 2005, Ghiraldelli *et al* 2006).

Knowledge of hematology is very important since it deals with the morphology, physiology and the biochemistry of blood. By analyzing blood cell characteristics, disease status can be identified (Anderson, 2003).

Haematology, including erythrocyte count, haemoglobin concentration, haematocrit and leucocyte count has provided valuable information for fishery biologists in the assessment of fish health (Banaee *et al* 2008). Haematological and biochemical tests are important tools that could be used in fish health assessment. Blood is a pathophysiological reflector of the whole body and therefore, blood parameters are important in diagnosing the status of fish health (Decie and Lewis 1991) particularly when some additives used in the feed and also demonstrates a stable physiological reflection of the whole body. The determinants of white blood cell count (total and differential counts) are considered to be an important parameter of fish health status.

Albumin/globulin ratio is a measurable humoral component at the non-specific defenses. Leucocytes are one of the main parts of the cellular immunity system and fluctuation of them is increasingly used as indicators of stress response in fish (Stoskopf 1993). In response to stressors in the aquatic environment, an overall increase could mean infection or response to stressors (Adams 2002). The mechanisms of innate resistance and adaptive immunity are interdependent. Cellular interactions through cytokines, antibodies, complement and their corresponding surface receptors represent communication elements in the cross talk between the two types of pathogenic resistance (Haniffa *et al* 2011).

Innate immunity essentially serves as the host's first line of defence against invasion of pathogens whereas adaptive/acquired/specific immunity plays a vital role in protection against recurrent infections by generating memory cells (cell-mediated immunity) and specific soluble and membrane-bound receptors (humoral immunity) such

as T-cell receptors and immunoglobulin (Ig) that allow for the fast and efficient elimination of the specific fish pathogens. In both types of immunity, cells and molecules play an important role (Ahilan *et al* 2010, Ellis 2001, Swain 2006). The important molecules viz., lysozyme, myeloperoxidases, superoxides, acute-phase proteins, interferons, complement, properdin, lysins and agglutinins, are some of the important innate immune parameters and have often been used as indicators of aquatic stress response and disease resistance (Shailesh Saurabh and Sahoo 2008). Measurement of myeloperoxidase activity is an indicator of phagocytic and neutrophil activities in non-specific immune response (Weeks and Warinner 1986), the presence of protective proteins in fish blood can be evaluated by serum bactericidal activity and this is an important tool to analyze the innate immune system (Biller-Takahashi *et al* 2013).

In fish, lysozyme, an enzyme with antibiotic properties that is released by leucocytes (mainly the phagocytes like neutrophils and macrophages), has a broader activity than mammalian lysozyme and has been frequently used as an indicator of nonspecific immune functions, which is of primary importance in combating infections in fish (Ellis 1999, Biller-Takahashi *et al* 2012, Demers and Bayne 1997) and the intensity of antibody formation being directly proportional to the activity of the lysozyme, Such an increase in the enzyme level can reflect changes in the white cell population during the development of the immune response. Thus, infection, which brings about changes in the numbers of leucocytes, may affect lysozyme concentration, and estimation of lysozyme may be of diagnostic value to determine the disease status of fish. (Shailesh Saurabh and Sahoo 2008).

Many studies have proved that herbal additives enhanced the growth of fishes and protected from diseases. Many plant compounds have been found to have non-specific immunostimulating effects in humans and animals of which more than a dozen

have been evaluated in fish and shrimp (Pandey and Madhuri 2010, Ye *et al* 2011, Kamilya *et al* 2008, Selvaraj *et al* 2005). It was suggested that plant constituents might directly activate innate defence mechanisms by acting on receptors and trigger gene activation, which might result in production of anti-microbial molecules (Bricknell and Dalmo 2005). The non-specific immune system of fish is highly advanced and in some respects may be superior to the mammalian (Watts *et al* 2001).

Diseases caused by *Aeromonas hydrophila* bacterium are some of the most widespread in freshwater fish culture. Septicaemia caused by motile aeromonids is a ubiquitous problem that affects fishes found in warm, cool and cold freshwater around the world. *A.hydrophila* has been associated with diseases in fishes like carp, eels, milkfish, channel catfish, Tilapia and ayu. This microorganism can also be an opportunist in stress related diseases in salmonids. *A.hydrophila* infection causes tail rot, motile Aeromonas septicemia (MAS) and epizootic ulcerative syndrome (EUS) as a primary pathogen. EUS is a globally distributed disease and has become an epidemic affecting a wide variety of wild and cultured fish species in south-east Asia, including India (Castro *et al* 2008).

As it is evident that there is a continuous urge to find novel chemical compounds that too plant based to combat the constantly changing pathogens. The present work was designed to evaluate the Immunostimulant potential of *Marsilea quadrifolia,* an aquatic medicinal fern against *Aeromonas hydrophila* infection. Many authors reported the growth stimulating potential of different plants and their role in relative improvement in survival after infection. The present work is aimed to evaluate the immune response of the fish fed with *Marsilea quadrifolia* incorporated diet and post challenged with *Aeromonas hydrophila* through standard immunological parameters. Further health enhancement in *Labeo rohita* fed by *M.quadrifolia* is assessed by comparing these

immunological parameters with a well known synthetic Immunostimulant β-Glucan administered experimentally.

5.2. MATERIALS AND METHODS

The experimental fishes comprised of three groups of *Labeo rohita* fed with control diet, *Marsilea quadrifolia* incorporated diet (M-1 = 0.25% *Marsilea*, M-2 = 0.5% *Marsilea* and M-3 = 1% *Marsilea*) and β-Glucan incorporated diet (G-1 = 0.25% Glucan, G-2 = 0.5% Glucan and G-3 = 1% Glucan). Fishes which were previously fed with these diets for 40 days were recruited for post challenge studies with *Aeromonas hydrophila*. This chapter includes estimation of sub lethal dose (LC_{50}) and inoculation in *Labeo rohita* and health assessment through haematological serological and immunological parameters as described in the previous chapter.

5.2.1. *Aeromonas hydrophila* Culture

Pure strains of Fresh *Aeromonas hydrophila* culture was kindly donated by CARE (Centre for Aquatic Research and Extension), St.Xavier's College (Autonomous), Palayamkottai, TamilNadu, India. The bacterium was grown in nutrient broth for 24 hours by incubating at 36^0C. The culture was centrifuged at 3000rpm for 5minutes and the pellet was resuspended in physiological saline. Serial dilutions like 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared.

5.2.2. LC_{50} Analysis

Six groups of seven fishes each were selected for LC_{50} studies. The first group served as control and was injected with 0.1ml of saline, the next group received 0.1ml of *Aeromonas hydrophila* from 10^{-1} dilution, the third group of fishes were injected (Intra peritoneally) with 0.1ml of *Aeromonas hydrophila* from 10^{-2} dilution, similarly the other groups were injected with 10^{-3}, 10^{-4} and 10^{-5} dilutions of *Aeromonas hydrophila* culture.

The mortality was recorded after 24hours, 48 hours and 72 hours. Probit Analysis was performed using SPSS (16) package.

5.2.3. Experimental Design

After 40 days of feeding trial, 30 fishes from each group (C, M-1, M-2, M-3, G-1, G-2 and G-3) were separated. The calculated LC_{50} dose of *Aeromonas hydrophila* for 72 hours was chosen as the test dose and injected (0.1ml) intraperitoneally for all selected fishes. 10 fishes from each group were separated after 1 day of bacterial challenge, anesthetized and the blood was collected using 1ml Insulin syringe from caudal vein and/or directly from the heart. Similarly, the blood was collected from another set of 10 fishes on 3rd day and 5th day after bacterial challenge. The experiement was run in triplicate.

The haemtological parameters like Total erythrocytes counts (TEC), Haemoglobin content (Hb), haematocrit (Ht), Total leucocytes count (TLC), differential leucocytes count (DLC), erythrocytic indices like MCV, MCH and MCHC, serum protein, albumin, globulin, albumin globulin ratio, serum cholesterol, blood glucose, electrophoretic separation of serum proteins, immunological parameters like agglutination antibody titre, lysozyme assay, serum bactericidal activity and myeloperoxidase activity were analysed by methods as described in the previous chapter. The results were tabulated and statistically treated (One way Anova-Duncan Post hoc multiple comparison) using SPSS (16) package.

5.3. RESULTS

5.3.1. LC_{50} Bioassay

Lethal concentration of *Aeromonas hydrophila* to *Labeo rohita* was evaluated. Fishes were divided into six groups each with seven fishes. The first group was injected

with 0.1ml of 10^{-1} dilution of *Aeromonas hydrophila* suspension, the second group received 0.1ml onf 10^{-2} dilution, the third, fourth and fifth groups received 10^{-3}, 10^{-4} and 10^{-5} dilutions of *Aeromonas hydrophila* suspension respectively, and the sixth group received 0.1ml of physiological saline. The mortalities were recorded for 24, 48 and 72 hours. Through Probit analysis using SPSS Software (16) package the LC_{50} value was calculated (Table-18).

The 72hour LC_{50} dose of *Aeromonas hydrophila* to *Labeo rohita* was calculated as 10^{-3} dilution and its colony forming unit/ml was ascertained as 1 x $10^{7.}$ This dose of *Aeromonas hydrophila* was selected as the test dose for the challenge studies.

Table – 18. Probit Analysis of *Labeo rohita* against *Aeromonas hydrophila*

Bacterial Dilution	No. of fishes Introduced	Mortality		
		24 hours	48 hours	72 hours
10^{-1}	7	4	4	5
10^{-2}	7	3	3	4
10^{-3}	7	1	3	4
10^{-4}	7	1	2	3
10^{-5}	7	1	1	1
Control	7	0	0	0
95% confidence limits for Bacterial dilution		0.06	0.019	0.001
95% confidence limits for log Bacterial dilution		-1.225	-1.73	-2.873

Labeo rohita pre administered with dietary *Marsilea* and Glucan for 40 days were infected with *Aeromonas hydrophila* ($1x10^7$cfu [10^{-3} dilution]) intraperitoneally. Haematological, serological and immunological studies were carried out on 1st, 3rd and

5th day of post challenge. The results obtained were tabulated, statistically treated and compared with their respective unchallenged counterparts.

5.3.2. *Marsilea* diet

5.3.2.1. Erythrocytic Parameters

In post challenged fishes the total erythrocyte counts declined significantly with increase in duration in C and M-1 groups, however in M-3 group highly significant increase was observed on 1st day and upto3rd day it remained elevated and declined marginally, in M-2 group declining trend was observed. It was also noticed that in all groups the total erythrocyte counts exhibited a declining trend on 5th day after challenge with *A.hydrophila*.

The haemoglobin concentration exhibited a declining trend in both C, M-1 and M-2 groups but significant elevation was observed in M-3 group, further it was clearly observed that the haemoglobin concentration of M-2 and M-3 diets were significantly higher on 1st day of post challenge. However the concentration of haemoglobin remained high in M-3 group fishes when compared to other groups. Significant decrease in Haematocrit (Ht) percentage was observed in both C and M-1 groups but in M-2 and M-3 groups it has elevated significantly on 1st day and the decrease was only marginal (Table – 19). Decrease in Haematocrit in C and M-1 groups may be due to decrease in RBC and Increase in Haematocrit in M-2 M-3 may be due to increase in RBC and /or decrease in plasma levels.

Table – 19. Erythrocytic parameters of *Labeo rohita* fed with *M.quadrifolia* and post challenged with *Aeromonas hydrophila* (Mean±SD)

Sl. No	Parameter	Duration (days)	Control	M-1	M-2	M-3
			M±SD	M±SD	M±SD	M±SD
1	TEC (10^6)	0	1.69±0.04^{b}	1.78±0.12^{d}	1.93±0.15^{bc}	1.67±0.13^{a}
		1	1.58±0.05^{b}	1.61±0.1^{bc}	2.03±0.15^{c}	2.36±0.17^{c}
		3	1.07±0.10^{a}	1.41±0.08^{ab}	1.72±0.1^{b}	1.99±0.13^{b}
		5	0.9±0.2^{a}	1.26±0.12^{a}	1.41±0.06^{a}	1.61±0.11^{a}
2	Haemoglobin (g%)	0	5.83±0.35^{b}	6.27±0.7^{b}	7.43±0.58^{bc}	6.6±0.26^{a}
		1	5.2±0.26^{a}	5.43±0.61^{ab}	8.06±0.25^{c}	9.63±0.37^{c}
		3	5.1±0.1^{a}	5.27±0.15^{a}	6.57±0.25^{ab}	7.16±0.25^{b}
		5	4.8±0.2^{a}	5.1±0.26^{a}	6.03±0.7^{a}	6.5±0.2^{a}
3	Haematocrit (%)	0	15.66±0.57^{d}	19±1^{c}	15.66±1.15^{ab}	15.66±0.58^{a}
		1	14.67±0.58^{c}	17.33±0.58^{b}	22.33±1.53^{c}	27.67±1.52^{c}
		3	11.0±0.28^{b}	15.33±0.57^{a}	18.33±1.52^{b}	19.67±3.21^{b}
		5	9.33±0.57^{a}	14±1^{a}	15.33±1.53^{a}	16.67±0.58^{ab}

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.

Means with same superscript in the same column is not statistically significant

The erythrocytic indices have indicated a general increase in the volume of erythrocytes (Mean corpuscular volume-MCV). The MCH (Mean corpuscular haemoglobin) level have increased significantly only in post challenged control group fishes, but all other experimental groups exhibited a significant increase only on 5th day of post challenge, it was also clearly observed that the changes are not that much drastic as in control. MCHC (Mean corpuscular haemoglobin concentration) exhibited an increasing trend in all post challenged groups. However in control and M-1 groups the

increase was significantly higher than the unchallenged and in M-2 and M-3 the values were lower than the unchallenged one (Table – 20).

Table – 20. Erythrocytic Indices of *Labeo rohita* fed with *M.quadrifolia* and post challenged with *Aeromonas hydrophila* (Mean±SD)

Sl. No	Parameter	Duration (days)	control	M-1	M-2	M-3
			M±SD	M±SD	M±SD	M±SD
1	MCV (μ^3)	0	92.50±1.6^a	106.82±1.97^a	81.07±2.19^a	94.22±4.40^a
		1	92.61±1.12^a	107.58±3.77^a	109.96±5.44^b	117.0±2.11^b
		3	103.14±10.24^a	108.86±3.40^a	106.48±2.55^b	98.42±10.35^a
		5	106.49±19.65^a	111.56±4.22^a	108.3±6.04^b	103.25±4.35^a
2	MCH (µgm)	0	34.43±1.28^a	35.14±1.66^{ab}	38.45±0.5^a	39.69±1.92^b
		1	32.83±0.7^a	33.61±1.66^a	39.78±2.54^{ab}	40.77±1.46^b
		3	47.75±3.82^b	37.41±1.8^{bc}	38.21±0.91^{ab}	36.06±1.19^a
		5	54.83±10.21^b	40.61±2.01^c	42.6±3.25^b	40.27±1.39^b
3	MCHC (%)	0	37.22±1.34^a	32.91±1.96^a	47.43±0.69^c	42.12±0.33^b
		1	35.44±0.51^a	31.29±2.53^{ab}	36.18±1.39^a	34.84±0.63^a
		3	46.36±0.91^b	34.37±1.47^{ab}	35.90±1.65^a	37.01±5.29^{ab}
		5	51.48±1.69^c	36.46±0.98^b	39.30±1.40^b	39.0±.0.67^{ab}

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.

Means with same superscript in the same column is not statistically significant

5.3.2.2. Leucocytic Parameters

Thrombocyte counts of *A.hydrophila* challenged groups exhibited a highly significant increase on 1st day when compared with unchallenged counterparts and declined steeply on 3rd and started improving on 5th day in all the groups, however its population remained high in all *Marsilea* diet groups, especially in M-2 group. Total leucocyte counts exhibited a highly significant decline in C, M-1 and in M-3 groups and

insignificant decline in M-2 group fishes. Though there was a decrease, leucocyte population was significantly high in M-2 and M-3 groups. In differential leucocyte counts, declining trend in neutrophil population was observed in all the groups, further the neutrophils declined in all the post challenged fishes compared with their respective unchallenged ones. However the decline was highly significant in M-3 group. It was also noticed that the population of neutrophils were lesser in *Marsilea* diet fed, post challenged fishes than their control counterparts. Eosinophils and basophils exhibited fluctuating changes in all groups but significant decline of eosinophil was noticed in M-3 group. Lymphocyte populations increased significantly in post challenged M-2 and M-3 diet fed fishes and the elevation was highly significant in M-3 diet whereas only fluctuating changes were observed in control feed fed fishes. The monocyte populations were significantly higher on 1st day after challenge in both control and experimental groups thereafter declined gradually. Further it was also observed that the population was significantly higher only in M-1 and M-2 groups only (Table – 21).

5.3.2.3. Serum Biochemistry

Serum protein, albumin and globulin levels of post challenged fishes of control and M-1 groups exhibited a declining trend with increase in time, but highly significant increase were observed in both M-2 and M-3 groups (Table-22). Highest value of protein, albumin and globulin (13.46 ± 0.42, 4.6±0.2 and 8.86±0.2g/dl) was recorded on 3rd day in M-2 feed fed fishes. No significant change was observed in A/G ratio between pre challenged and post challenged in control group and significant increase was observed in post challenged fishes of M-1 and M-3, whereas highly significant decrease in M-2 group was observed indicating a steady increase in globulin concentration. Serum cholesterol levels in all the groups (control, M-1, M-2 and M-3) exhibited an increasing trend upto 3rd day of post challenge when compared to unchallenged and declined

significantly on 5th day; however elevated serum cholesterol level was recorded in M-2 group.

Table – 21. Leucocytic Parameters of *Labeo rohita* fed with *M.quadrifolia* and post challenged with *Aeromonas hydrophila* (Mean±SD)

Sl. No	Parameter	Duration (days)	control	M-1	M-2	M-3
			M±SD	M±SD	M±SD	M±SD
1	Thrombocyte (10^5)	0	1.19±0.09^{c}	1.07±0.07^{c}	1.17±0.08^{c}	1.29±0.07^{c}
		1	2.76±0.15^{d}	2.96±0.15^{d}	1.87±0.12^{d}	4.4±0.26^{d}
		3	0.31±0.06^{a}	0.35±0.04^{a}	0.41±0.06^{a}	0.29±0.02^{a}
		5	0.62±0.11^{b}	0.75±0.03^{b}	0.81±0.06^{b}	0.89±0.05^{b}
2	TLC (10^4)	0	3.87±0.12^{c}	3.98±0.14^{c}	4.29±0.11^{b}	4.21±0.06^{b}
		1	3.71±0.08^{c}	3.59±0.08^{b}	4.27±0.12^{b}	4.08±0.11^{b}
		3	3.28±0.14^{b}	3.3±0.2^{a}	4.07±0.11^{ab}	3.65±0.14^{a}
		5	2.79±0.11^{a}	3.4±0.08^{ab}	4.03±0.12^{a}	3.76±0.15^{a}
3	Neutrophils (%)	0	30.33±2.51^{a}	31±5^{b}	28.33±4.5^{a}	35.66±5.03^{b}
		1	31.66±2.51^{a}	27.33±4.5^{ab}	20.66±5.03^{a}	16±3.6^{a}
		3	28.66±4.04^{a}	20.33±4.5^{a}	22.33±4.5^{a}	20.33±3.21^{a}
		5	34.33±4.04^{a}	24±4.58^{ab}	23.66±4.04^{a}	19.66±2.51^{a}
4	Eosinophils (%)	0	2.33±0.57^{a}	2.33±0.57^{a}	1.66±1.15^{a}	3.67±1.15^{b}
		1	2±1^{a}	3±1^{a}	2.66±1.15^{a}	4.33±0.57^{b}
		3	2.66±1.52^{a}	2.3±1.15^{a}	2.33±0.57^{a}	1.66±0.47^{a}
		5	2.66±1.15^{a}	1.66±1.15^{a}	1.33±0.57^{a}	1.66±0.47^{a}
5	Basophils (%)	0	3±1^{a}	2.33±0.57^{a}	1.66±1.15^{a}	3±1^{a}
		1	3±1^{a}	2.33±1.52^{a}	2.33±0.57^{a}	2.66±1.52^{a}
		3	2.66±1.52^{a}	2.66±0.58^{a}	1.66±0.47^{a}	2.33±1.15^{a}
		5	2±1^{a}	1.66±1.15^{a}	1.66±0.47^{a}	1.66±1.15^{a}
6	Lymphocytes (%)	0	59.66±2.51^{a}	58.67±3.21^{a}	63.33±1.15^{a}	53±2.64^{a}
		1	55.66±2.51^{a}	59±2^{a}	65.66±3.21^{a}	72±1.73^{b}
		3	59.33±2.08^{a}	68±4.35^{b}	68.33±4.72^{a}	69.33±3.78^{b}
		5	57±2.64^{a}	67±4.35^{b}	68.66±4.93^{a}	71±1.73^{b}
7	Monocytes (%)	0	4.66±1.52^{ab}	5.66±1.15^{a}	4.33±1.53^{a}	4.67±1.15^{a}
		1	7.66±0.57^{c}	8.33±0.57^{b}	8.66±1.15^{b}	5.33±1.52^{a}
		3	6.66±1.52^{bc}	6.66±1.15^{ab}	5.33±0.57^{a}	6.33±0.57^{a}
		5	4±1^{a}	5.67±1.55^{a}	4.66±0.58^{a}	6±1^{a}

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.

Means with same superscript in the same column is not statistically significant

Table – 22. Serum Biochemical Parameters of *Labeo rohita* fed with *M.quadrifolia* and post challenged with *Aeromonas hydrophila* (Mean±SD)

Sl. No	Parameter	Duration (days)	control	M-1	M-2	M-3
			M±SD	M±SD	M±SD	M±SD
1	Protein (g/dl)	0	5.13±0.15^{d}	5.7±0.1^{b}	6.2±0.26^{a}	6.93±0.35^{a}
		1	4.67±0.05^{c}	5.03±0.21^{a}	8.9±0.21^{b}	10.7±0.25^{b}
		3	4.23±0.21^{b}	4.9±0.2^{a}	13.46±0.42^{d}	10.73±0.25^{b}
		5	3.23±0.20^{a}	5.03±0.2^{a}	11.13±0.30^{c}	7.0±0.45^{a}
2	Albumin (g/dl)	0	2.20±0.13^{c}	2.06±0.08^{b}	2.53±0.15^{a}	2.43±0.15^{a}
		1	1.91±0.17^{b}	1.84±0.16^{a}	3.35±0.18^{b}	3.36±0.23^{b}
		3	1.75±0.07^{b}	1.87±0.07^{ab}	4.6±0.2^{d}	3.86±0.21^{c}
		5	1.37±0.08^{a}	1.78±0.07^{a}	3.83±0.31^{c}	2.46±0.20^{a}
3	Globulin (g/dl)	0	2.93±0.02^{c}	3.64±0.03^{c}	3.67±0.11^{a}	4.5±0.2^{a}
		1	2.75±0.13^{c}	3.18±0.09^{ab}	5.61±0.1^{b}	7.33±0.25^{c}
		3	2.48±0.14^{b}	3.01±0.11^{a}	8.86±0.2^{d}	6.86±0.05^{b}
		5	1.85±0.12^{a}	3.25±0.13^{b}	7.3±0.2^{c}	4.53±0.25^{a}
4	Albumin/Globulin ratio	0	0.74±0.04^{a}	0.56±0.02^{ab}	0.68±0.02^{c}	0.53±0.01^{b}
		1	0.69±0.1^{a}	0.57±0.05^{ab}	0.59±0.04^{b}	0.46±0.02^{a}
		3	0.7±0.02^{a}	0.62±0.01^{b}	0.52±0.01^{a}	0.56±0.03^{b}
		5	0.74±0.01^{a}	0.54±0.01^{a}	0.52±0.05^{a}	0.54±0.01^{b}
5	Cholesterol (mg/dl)	0	128±2.64^{b}	131±10.53^{a}	134.3±6.03^{a}	135±9.54^{ab}
		1	135.6±4.04^{bc}	159±9.53^{b}	167.3±5.50^{c}	141.3±10.59^{b}
		3	141.3±3.51^{c}	190±6.24^{c}	177.3±7.51^{c}	173±11.13^{c}
		5	106±8.54^{a}	125.67±9.45^{a}	147±7.54^{b}	118±7.55^{a}
6	Blood Glucose (mg/dl)	0	93±7^{b}	109.3±6.02^{b}	82.6±6.51^{a}	81±6.56^{a}
		1	109±6.55^{c}	101±5.56^{b}	131.3±6.11^{b}	106.6±8.02^{b}
		3	80.3±5.51^{b}	84.3±5.03^{a}	172.3±7.51^{c}	209.6±13.05^{d}
		5	62±8^{a}	81.3±6.11^{a}	137.3±6.65^{b}	162.6±8.02^{c}

M-1 = 0.25%*Marsilea*, M-2 = 0.5%*Marsilea*, M-3 = 1%*Marsilea*.

Means with same superscript in the same column is not statistically significant

The blood glucose level in the control group increased significantly on 1st day and then declined significantly on 3rd and 5th day, exhibiting erratic up and down fluctuations. Whereas in M-1 feed fed fishes steady decrease was recorded. However in M-2 and M-3

groups the blood sugar levels remained significantly high and the peak value was on 3rd day.

5.3.2.4. Immunological Parameters

The serum agglutination titer of challenged fishes of control as well as experimental groups has increased significantly when compared to their unchallenged counter parts; the increase was noted till the 3rd day of exposure and subsequently declined on the 5th day. It was also noted that highly significant increase in agglutination titer was observed in fishes fed with M-3 diet denoting that even at 23.33 times of dilution, the serum had the ability to agglutinate the antigen. A two fold increase was observed when compared to control feed fed fishes (Table-23).

The lysozymal concentration in the serum has increased in all challenged groups, the increase in the concentration was recorded till the 3rd day of post challenge and on the 5th day it declined significantly in the control feed fed fishes; however the decline was only marginal in all the *Marsilea* feed fed fishes. Further it was also observed that the concentration of serum lysozyme has increased significantly in a dose dependent manner in the *Marsilea* diet groups.

The presence of protective proteins in fish blood can be evaluated by serum bactericidal activity and this is an important tool to analyze the innate immune system. In the present experiment the serum bactericidal activity has increased significantly in *Marsilea* fed post challenged fishes than their control counterparts, especially in M-2 and M-3 feed groups. It was observed that the increase was almost two times, indicating feed induced enhancement in the non specific immune response.

Table – 23. Immunological Parameters of *Labeo rohita* fed with *M.quadrifolia* and post challenged with *Aeromonas hydrophila* (Mean±SD)

Parameter	Duration (days)	control	M-1	M-2	M-3
		M±SD	M±SD	M±SD	M±SD
Agglutination Titre (Reciprocal of dilution)	0	8.67±1.15^{ab}	10.67±1.17^{a}	14.67±1.21^{ab}	16.33±1.53^{a}
	1	11.33±1.41^{bc}	11.33±1.43^{a}	14.67±1.32^{ab}	17.33±1.15^{a}
	3	12.00±2.00^{c}	13.00±0.71^{a}	17.33±1.15^{b}	23.33±1.61^{b}
	5	8.00±1.23^{a}	11.67±1.53^{a}	12.00±2.00^{a}	16.33±1.51^{a}
Lysozymal Assay (µg/ml)	0	1.05±0.05^{a}	1.63±0.12^{a}	2.97±0.13^{a}	3.30±0.17^{a}
	1	2.29±0.17^{b}	2.55±0.07^{c}	3.56±0.09^{c}	4.11±0.06^{b}
	3	2.17±0.04^{b}	3.20±0.10^{d}	3.83±0.11^{b}	5.19±0.58^{d}
	5	0.96±0.06^{a}	2.15±0.07^{b}	3.23±0.06^{d}	4.96±0.11^{c}
Serum Bactericidal Activity (1-cfu/control (%))	0	14.33±1.52^{a}	27.66±1.53^{a}	31.67±1.48^{a}	48.33±1.53^{a}
	1	18.66±0.57^{b}	31.67±1.52^{c}	40.66±1.15^{c}	58.33±1.48^{b}
	3	20.33±1.53^{b}	34.67±1.15^{c}	42.33±1.55^{c}	59.34±1.52^{b}
	5	19.67±2.51^{b}	30.33±1.62^{b}	37.66±1.52^{b}	50.66±1.15^{a}
Myelo peroxidase Activity(OD at 450nm)	0	0.65± 0.09^{a}	1.00±0.05^{a}	0.98±0.13^{a}	1.02±0.06^{a}
	1	0.81±0.04^{b}	1.16±0.05^{b}	1.13±0.11^{ab}	1.38±0.09^{b}
	3	1.02±0.05^{d}	1.23±0.06^{b}	1.31±0.13^{b}	1.44±0.04^{b}
	5	0.85±0.07^{b}	1.23±0.04^{b}	1.24±0.06^{b}	1.35±0.03^{b}

M-1 = 0.25% *Marsilea*, M-2 = 0.5% *Marsilea*, M-3 = 1% *Marsilea*.

Means with same superscript in the same column is not statistically significant

The myeloperoxidase activity has also increased significantly in all groups of challenged fishes, however the increase was dose dependent with more potent activity being recorded in M-3 diet fed fishes. It was also clearly noted that elevation in immunological responses was evident upto 3rd day of pathogenic challenge and on the 5th

day marginal decline was observed in *Marsilea* diet fed fishes and a steep decline was observed in fishes fed with control diet. Thus incorporation of *Marsilea* in diet can enhance and sustain the immune response.

5.3.3. Glucan diet

5.3.3.1. Erythrocytic Parameters

A general declining trend with increase in time in total erythrocyte counts (TEC) was observed in both control and experimental post challenged groups when compared with their unchallenged counterparts; however the decline was highly significant in control group, significant in G-1 group and marginal in G-2 group but increased significantly in G-3 group. The haemoglobin concentration has also recorded similar declining trend but the decline was minimal in G-1 group, significant decline was observed in G-2 and G-3 groups, however the lowest value (6.33±0.21**)** of haemoglobin recorded in G-3 was higher than the control group. The percentage of haematocrit (Ht) decreased significantly in control and G-1 groups, on the other hand significant increase was observed in G-3 group over their control counterparts. When compared to their unchallenged counterparts, though declining trend was observed for total erythrocytes and haemoglobin concentration, the values were higher in all groups of Glucan diet (Table – 24).

The Mean Corpuscular Volume (MCV) of erythrocytes has increased significantly upto 5th day in challenged fishes in G-2 group only, whereas declining trend was observed in G-1 group of challenged fishes over their unchallenged counterparts. The Mean Corpuscular Haemoglobin (MCH) levels increased gradually in all the post challenged groups (significant on 5th day in G-2 group) except G-3 where a significant decrease was observed. However it was highly. But the Mean Corpuscular Haemoglobin

Concentration (MCHC) recorded a significant increase in C and G-1 group only and in G-2 and G-3 group the haemoglobin concentration declined significantly when compared with their unchallenged counterparts (Table – 25).

Table – 24. Erythrocytic Parameters of *Labeo rohita* fed with β-Glucan and post challenged with *Aeromonas hydrophila* (Mean±SD)

Sl. No	Parameter	Duration (days)	control	G-1	G-2	G-3
			M±SD	M±SD	M±SD	M±SD
1	TEC (10^6)	0	1.69±0.04^b	1.82±0.11^c	1.71±0.09^b	1.69±0.11^a
		1	1.58±0.05^b	1.71±0.09^c	1.72±0.09^b	2.09±0.09^b
		3	1.07±0.10^a	1.43±0.08^b	1.27±0.13^a	1.78±0.12^a
		5	0.9±0.2^a	1.18±0.09^a	1.18±0.11^a	1.59±0.13^a
2	Haemoglobin (g%)	0	5.83±0.35^b	7.06±0.21^b	8.26±0.32^b	7.53±0.21^b
		1	5.2±0.26^a	6.36±0.25^a	8.03±0.4^b	8.46±0.15^c
		3	5.1±0.1^a	6.13±0.11^a	6.6±0.26^a	6.5±0.3^a
		5	4.8±0.2^a	6.0±0.2^a	6.53±0.30^a	6.33±0.21^a
3	Haematocrit (%)	0	15.66±0.57^d	24.33±1.15^d	18.66±0.57^{ab}	17.67±1.52^a
		1	14.67±0.58^c	19.33±1.15^c	19.3±1^b	20.67±1.15^b
		3	11.0±0.28^b	17±0.48^b	16.66±0.57^a	18.66±1.15^{ab}
		5	9.33±0.57^a	13.66±0.57^a	18±1^{ab}	17.33±1.15^a

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.

Means with same superscript in the same column is not statistically significant

Table – 25. Erythrocytic Indices of *Labeo rohita* fed with β-Glucan and post challenged with *Aeromonas hydrophila* (Mean±SD)

Sl. No	Parameter	Duration (days)	Control	G-1	G-2	G-3
			M±SD	M±SD	M±SD	M±SD
1	MCV (μ3)	0	92.50±1.6^{a}	133.28±2.67^{b}	104.85±0.89^{a}	104.23±2.57^{a}
		1	92.61±1.12^{a}	113.28±3.77^{a}	112.06±2.94^{a}	98.72±3.05^{a}
		3	103.14±10.24^{a}	119.19±7.62^{a}	131.26±10.08^{b}	104.98±8.92^{a}
		5	106.49±19.65^{a}	116.04±4.66^{a}	152.74±11.49^{c}	109.16±4.05^{a}
2	MCH (μgm)	0	34.43±1.28^{a}	38.73±1.4^{a}	48.19±1.37^{ab}	44.56±1.71^{c}
		1	32.83±0.7^{a}	37.32±0.56^{a}	46.53±0.96^{a}	40.47±1.02^{b}
		3	47.75±3.82^{b}	42.96±1.94^{b}	51.95±3.60^{bc}	36.49±0.95^{a}
		5	54.83±10.21^{b}	50.97±2.39^{c}	55.4±2.91^{c}	39.95±2.13^{b}
3	MCHC (%)	0	37.22±1.34^{a}	29.06±1.01^{a}	45.95±1.05^{c}	42.79±2.59^{b}
		1	35.44±0.51^{a}	32.96±0.94^{b}	41.54±1.37^{b}	41.03±1.75^{b}
		3	46.36±0.91^{b}	36.07±0.68^{c}	39.59±0.35^{b}	34.91±2.67^{a}
		5	51.48±1.69^{c}	43.91±0.93^{d}	36.32±1.52^{a}	36.59±1.35^{a}

G-1 = 0.25%β-Glucan, G-2 = 0.5%β-Glucan, G-3 = 1%β-Glucan.

Means with same superscript in the same column is not statistically significant

5.3.3.2. Leucocytic Parameters

Like control feed fed fishes, the thrombocyte population increased significantly only on the 1st day of post challenge and there after declined sharply in all the glucan fed fishes. It is also observed that the thrombocyte population after declining on 3rd day started increasing on 5th day. Eventhough an increase was observed, it was significant especially in G-3 group. The Total Leucocyte Counts (TLC) of post challenged control fishes decreased with increase in days after exposure to pathogen, in G-1 and G-2 the decline was significant upto 3rd day of post challenge only and on the 5th day a sharp increase was observed. On the other hand in G-3 group fishes the total leucocyte count

declined significantly only on 5th day. In differential leucocyte counts the neutrophil populations were significantly lower when compared to unchallenged counterparts in glucan fed group and also to control group. The neutrophil population started increasing on 5th day and the values were higher in G-2 and G-3 groups. Not much change was observed in basophil and monocyte populations in post challenged groups except G-3 where a significant decline of eosinophil was noticed. The lymphocyte population increased significantly in all the post challenged fishes fed with glucan feed. It was also noticed that the monocyte populations were significantly higher in G-1 group than the other groups that too on 3rd day of post challenge (Table – 26).

5.3.3.3. Serum Biochemistry

The serum protein levels of post challenged fishes fed with glucan diet compared with their respective unchallenged fishes exhibited an increasing trend in G-1 and G-2 groups, G-3 group recorded a declining trend, but the levels of protein in all glucan diet fed fishes were significantly higher than the control counterparts (Table-27). The serum albumin levels recorded an increasing trend with increase in the duration of exposure, which was highly significant in G-3 group. The globulin levels in control and in G-3 group declined significantly in post challenged groups when compared with their unchallenged counterparts, but in G-1 and G-2 groups the globulin levels has decreased significantly on 1st day only and started increasing from 3rd day. However the globulin levels were higher only in G-1 group compared with other groups. The albumin/globulin ratio decreased significantly on 3rd day only in G-1 group but elevated levels were recorded in G-2 and G-3 groups (indicating an increase in albumin concentration). The cholesterol levels in post challenged fishes of control and experimental groups gradually

Table – 26. Leucocytic Parameters of *Labeo rohita* fed with β-Glucan and post challenged with *Aeromonas hydrophila* (Mean±SD)

Sl. No	Parameter	Duration (days)	control	G-1	G-2	G-3
			M±SD	M±SD	M±SD	M±SD
1	Thrombocyte (10^5)	0	1.19±0.09^{c}	1.19±0.06^{c}	1.27±0.11^{c}	1.28±0.11^{b}
		1	2.76±0.15^{d}	2.87±0.11^{d}	2.22±0.08^{d}	1.59±0.09^{c}
		3	0.31±0.06^{a}	0.23±0.03^{a}	0.35±0.04^{a}	0.58±0.07^{a}
		5	0.62±0.11^{b}	0.8±0.07^{b}	0.99±0.05^{b}	1.49±0.06^{c}
2	TLC (10^4)	0	3.87±0.12^{c}	4.42±0.17^{c}	3.91±0.07^{b}	3.83±0.09^{b}
		1	3.71±0.08^{c}	4.08±0.13^{b}	3.67±0.11^{b}	3.84±0.15^{b}
		3	3.28±0.14^{b}	3.62±0.11^{a}	3.24±0.09^{a}	3.68±0.08^{b}
		5	2.79±0.11^{a}	3.87±0.12^{ab}	3.69±0.08^{b}	3.23±0.15^{a}
3	Neutrophils (%)	0	30.33±2.51^{a}	24.33±3.51^{b}	30.66±5.03^{b}	35.67±3.05^{c}
		1	31.66±2.51^{a}	12.66±3.05^{a}	20.33±2.51^{a}	20±2.65^{ab}
		3	28.66±4.04^{a}	11±3.6^{a}	15.66±4.04^{a}	15.66±3.05^{a}
		5	34.33±4.04^{a}	14.33±4.04^{a}	18.66±4.04^{a}	21.66±3.05^{b}
4	Eosinophils (%)	0	2.33±0.57^{a}	3±1^{a}	2.33±1.15^{ab}	4.33±0.58^{c}
		1	2±1^{a}	1.66±0.47^{a}	1.66±0.47^{a}	1.33±0.57^{a}
		3	2.66±1.52^{a}	4.66±0.58^{b}	2.33±0.57^{ab}	2.33±0.57^{ab}
		5	2.66±1.15^{a}	2.33±0.57^{a}	3.66±0.57^{b}	2.67±0.57^{b}
5	Basophils (%)	0	3±1^{a}	1.66±0.47^{a}	1.66±0.47ab	2.33±1.15^{ab}
		1	3±1^{a}	1.66±0.47^{a}	1.33±0.57^{a}	1.66±0.47^{ab}
		3	2.66±1.52^{a}	2.33±1.15^{a}	2.33±0.57^{ab}	2.67±0.57^{b}
		5	2±1^{a}	2±1^{a}	2.67±0.57^{b}	1±0^{a}
6	Lymphocytes (%)	0	59.66±2.51^{a}	63.66±3.78^{a}	59.66±4.93^{a}	53±2^{a}
		1	55.66±2.51^{a}	77.33±2.08^{b}	74±4.36^{b}	69±2.64^{b}
		3	59.33±2.08^{a}	70.33±5.03^{ab}	69.33±3.21^{b}	71.66±1.52^{b}
		5	57±2.64^{a}	76±4.58^{b}	67.33±2.51^{b}	67±4.58^{b}
7	Monocytes (%)	0	4.66±1.52^{ab}	7.33±0.15^{a}	5.66±1.15^{ab}	4.67±1.52^{a}
		1	7.66±0.57^{c}	6.33±1.52^{a}	3±2^{a}	6.25±0.58^{b}
		3	6.66±1.52^{bc}	11.66±1.52^{b}	7±1.41^{b}	7.66±1.52^{b}
		5	4±1^{a}	5.33±1.52^{a}	7.67±1.53^{b}	7.66±1.52^{b}

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.

Means with same superscript in the same column is not statistically significant

Table – 27. Serum biochemical Parameters of *Labeo rohita* fed with β-Glucan and post challenged with *Aeromonas hydrophila*. (Mean±SD)

Sl. No	Parameter	Duration (days)	control	G-1	G-2	G-3
			M±SD	M±SD	M±SD	M±SD
1	Protein (g/dl)	0	5.13±0.15^{d}	7.06±0.25^{a}	7.96±0.35^{b}	8.1±0.2^{c}
		1	4.67±0.05^{c}	7.26±0.25^{a}	6.66±0.15^{a}	7.7±0.2^{b}
		3	4.23±0.21^{b}	8.33±0.15^{b}	7.5±0.26^{b}	7.13±0.21^{a}
		5	3.23±0.20^{a}	8.43±0.21^{b}	7.6±0.2^{b}	7.26±0.21^{a}
2	Albumin (g/dl)	0	2.20±0.13^{c}	2.71±0.2^{a}	2.75±0.13^{b}	2.02±0.19^{a}
		1	1.91±0.17^{b}	3.1±0.18^{b}	2.32±0.07^{a}	2.42±0.19^{a}
		3	1.75±0.07^{b}	2.65±0.15^{a}	2.91±0.09^{b}	3.36±0.21^{b}
		5	1.37±0.08^{a}	3.15±0.14^{b}	2.90±0.21^{b}	2.96±0.37^{b}
3	Globulin (g/dl)	0	2.93±0.02^{c}	4.35±0.05^{b}	5.21±0.23^{c}	6.07±0.02^{d}
		1	2.75±0.13^{a}	4.16±0.10^{a}	4.34±0.08^{a}	5.27±0.01^{c}
		3	2.48±0.14^{b}	5.68±0.02^{d}	4.58±0.18^{ab}	3.77±0.10^{a}
		5	1.85±0.12^{a}	5.3±0.07^{c}	4.69±0.12^{b}	4.3±0.17^{b}
4	Albumin/ Globulin ratio	0	0.74±0.04^{a}	0.62±0.04^{b}	0.52±0.01^{a}	0.33±0.03^{a}
		1	0.69±0.1^{a}	0.74±0.03^{c}	0.53±0.01^{a}	0.45±0.04^{a}
		3	0.7±0.02^{a}	0.46±0.03^{a}	0.63±0.02^{b}	0.89±0.07^{c}
		5	0.74±0.01^{a}	0.59±0.02^{b}	0.62±0.05^{b}	0.69±0.12^{b}
5	Cholesterol (mg/dl)	0	128±2.64^{b}	132.6±12.01^{ab}	132.66±7.50^{a}	129±7^{b}
		1	135.6±4.04^{bc}	139.3±10.06^{b}	148.3±6.50^{a}	161.3±7.51^{c}
		3	141.3±3.51^{c}	166.3±10.59^{c}	170.6±10.01^{b}	187.6±8.02^{d}
		5	106±8.54^{a}	115±12.12^{a}	140.3±10.02^{a}	105.3±7.02^{a}
6	Blood Glucose (mg/dl)	0	93±7^{b}	99.3±8.08^{b}	89±8.54^{a}	84±8^{a}
		1	109±6.55^{c}	82±6.55^{a}	90±8.18^{a}	85±9.53^{a}
		3	80.3±5.51^{b}	128.6±7.50^{c}	164.3±10.26^{b}	137±11.53^{b}
		5	62±8^{a}	163±9.16^{d}	189±8.54^{c}	148.3±9.50^{b}

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.

Means with same superscript in the same column is not statistically significant

elevated upto 3rd day and declined sharply on 5th day. Higher concentration of serum cholesterol was recorded in G-3 feed fed fishes. The blood glucose levels in all glucan fed post challenged fishes were lower on 1st day after challenge and thereafter increased significantly on 3rd and 5th day. Higher glucose levels were recorded on G-2 group fishes. However, in control feed fed fishes there was a significant increase only on 1st day and declined significantly on 3rd and 5th day.

5.3.3.4. Immunological Parameters

Declining trend in the serum agglutinating antibody titre was observed in the challenged fishes of control group when compared to their unchallenged counterparts, whereas the level of agglutinating antibodies increased significantly in glucan fed post challenged fishe (Table-28). The serum of G-2 diet fishes recorded that even at 32times dilution the serum had the ability to agglutinate the antigen which was a 3fold increase when compared with the control fishes. It was also observed that the agglutinating ability increased upto 3rd day irrespective of the group and declined on the 5th day. The serum lysozyme concentration also increased significantly in post challenged fishes of all the groups, however the increase was highly significant in G-2 group. In control group post challenged fishes the serum lysozyme concentration has increased significantly on 1st and 3rd day of post challenge and declined significantly on 5th day, whereas elevated levels of serum lysozyme was observed in glucan fed fishes especially in G-2 group. The serum bactericidal activity has elevated marginally in post challenged fishes of control group, it is interesting to note that the serum bactericidal activity of unchallenged glucan fed fishes was itself 3-4 times higher than the control fishes and these values has further improved in challenged fishes. Higher serum bactericidal activity was recorded in G-2 group. Similarly the myeloperoxidase enzyme production has also increased significantly in post challenged fishes of all groups and it was especially higher in G-2 group fishes.

Table – 28. Immunological Parameters of *Labeo rohita* fed with β-Glucan and challenged with *Aeromonas hydrophila* (Mean±SD)

Parameter	Duration (days)	control	G-1	G-2	G-3
		M±SD	M±SD	M±SD	M±SD
Agglutination Titre (Reciprocal of dilution)	0	8.67±1.15^{ab}	18.00±2.00^{a}	26.00±2.00^{a}	15.34±1.15^{a}
	1	11.33±1.41^{bc}	23.33±1.15^{b}	29.33±1.15^{b}	16.66±1.32^{ab}
	3	12.00±2.00^{c}	28.66±1.36^{c}	32.66±1.62^{c}	26.00±2.00^{c}
	5	8.00±1.23^{a}	21.33±1.53^{b}	28.66±1.38^{b}	18.66±1.65^{b}
Lysozymal Assay (µg/ml)	0	1.05±0.05^{a}	5.14±0.06^{a}	6.56±0.14^{a}	3.30±0.12^{a}
	1	2.29±0.17^{b}	6.47±0.09^{b}	8.18±0.11^{b}	3.97±0.13^{b}
	3	2.17±0.04^{b}	7.43±0.10^{c}	8.79±0.08^{c}	5.24±0.06^{d}
	5	0.96±0.06^{a}	7.44±0.05^{c}	8.65±0.08^{c}	4.84±0.16^{c}
Serum Bactericidal Activity (1 - cfu/control (%))	0	14.33±1.52^{a}	64.33±1.52^{a}	71.33±2.08^{a}	48.00±2.00^{a}
	1	18.66±0.57^{b}	65.33±1.15^{a}	76.66±1.15^{b}	56.67±2.08^{b}
	3	20.33±1.53^{b}	69.00±1.00^{b}	81.66±2.11^{c}	57.67±1.52^{b}
	5	19.67±2.51^{b}	71.33±1.52^{b}	79.33±1.52^{bc}	55.33±1.52^{b}
Myelo peroxidase Activity (OD at 450nm)	0	0.65± 0.09^{a}	1.16±0.13^{a}	1.54±0.14^{a}	1.04±0.07^{a}
	1	0.81±0.04^{b}	1.55±0.07^{b}	1.84±0.08^{c}	1.38±0.14^{b}
	3	1.02±0.05^{c}	1.45±0.06^{b}	1.78±0.07^{b}	1.44±0.06^{b}
	5	0.85±0.07^{b}	1.54±0.13^{b}	1.61±0.08^{ab}	1.41±0.08^{b}

G-1 = 0.25% β-Glucan, G-2 = 0.5% β-Glucan, G-3 = 1% β-Glucan.

Means with same superscript in the same column is not statistically significant

5.3.4. Evaluation of *Marsilea quadrifolia* as a potent Immunostimulant by comparison with β-Glucan

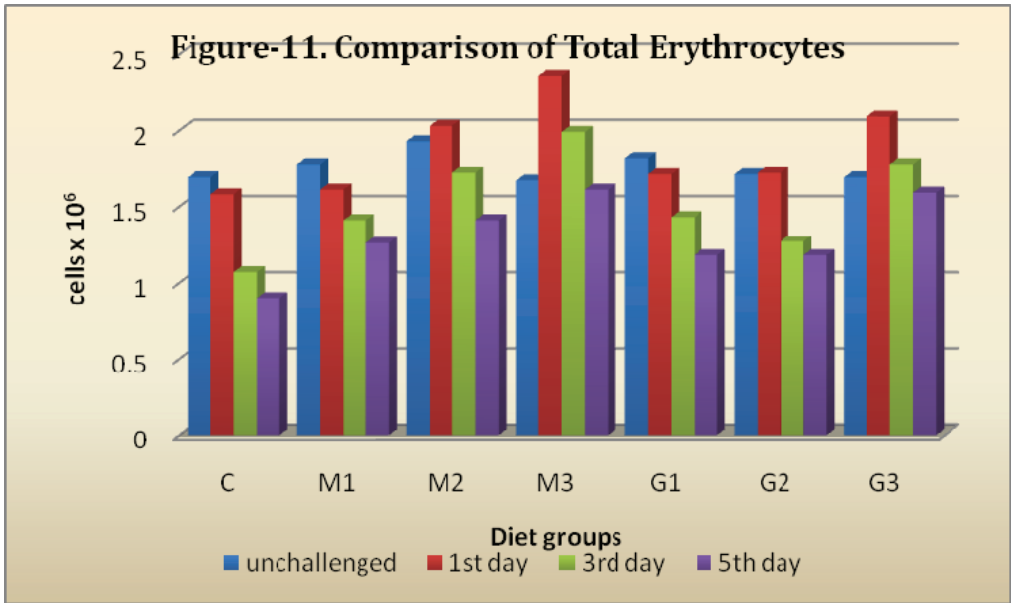
In this section the results of post challenged fishes of different groups ie, *Marsilea* incorporated group (M-1, M-2 and M-3) and Glucan supplemented diet group (G-1, G-2 and G-3) were compared with control feed fed fishes challenged with *Aeromonas hydrophila*.

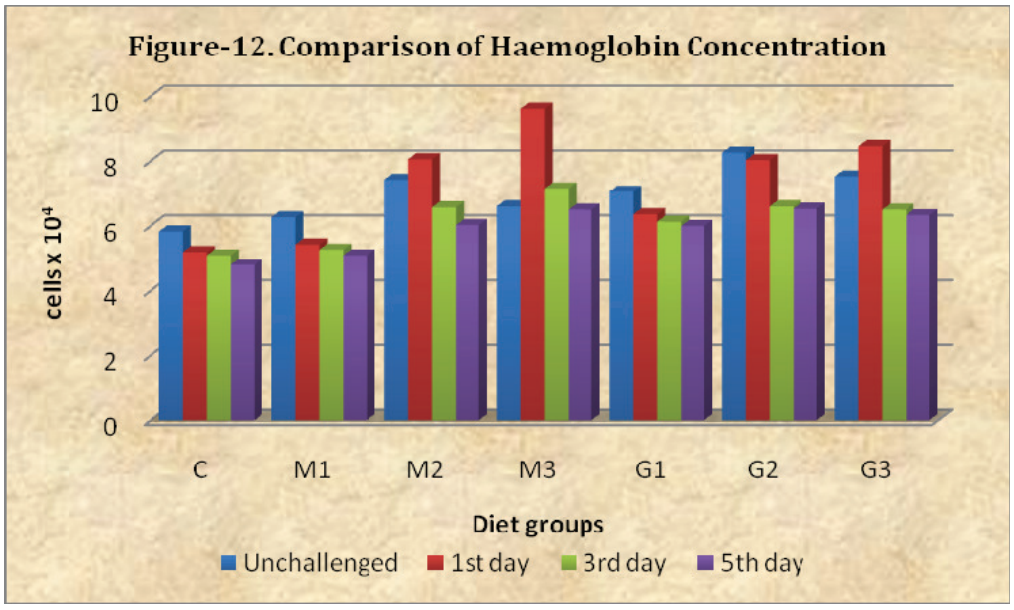
The total erythrocyte counts were significantly higher in all experimental groups than the control group. The values were significantly high in M-3 and G-3 and no significant change between *Marsilea* and Glucan diet groups were observed (Figure-11), however they follow a general declining trend. Eventhough the haemoglobin levels in all the groups exhibited a declining trend (Figure-12), the haemoglobin content remained high in all the experimental groups (*Marsilea* - 9.63±0.37 g% and Glucan – 8.46±0.15 g%) except the control (5.2±0.26 g%). This may evidently lead to anaemia in control diseased fish, where the possibility of recovery is minimal. The haematocrit pecentage was significantly high in all experimental groups, the haematocrit values has increased significantly in post challenged fishes in M-2, M-3 and G-3 groups and no significant difference was observed between *Marsilea* and Glucan diet groups.

The mean corpuscular volume (MCV) was significantly high in G-2 group and in *Marsilea* diet groups no significant difference was observed when compared to control. The mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) values declined in both *Marsilea* and Glucan diet groups than control, however the MCH values were not much low in Glucan diet groups. But no significant difference was noticed in MCHC in *Labeo rohita* fed with *Marsilea* and Glucan diets.

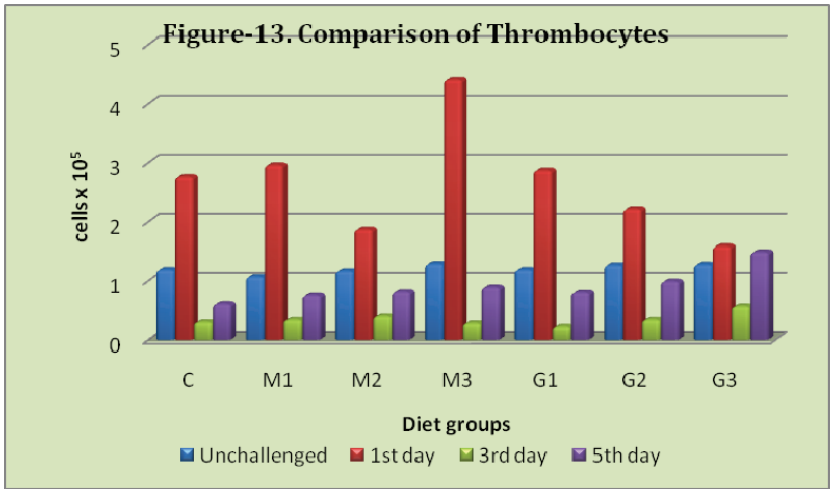
The thrombocyte counts were significantly high in all experimental feed groups (especially on 1st day of post challenge) over control and no significant difference was observed between Glucan and *Marsilea* diet except G-3 diet (Figure-13). The total leucocytes were also higher in both *Marsilea* and Glucan feed fed fishes, however declining trend was observed in all post challenged fishes and the decline was steep in control group and marginal in other groups (Figure-14). In differential leucocyte counts, the neutrophil counts were lower in all experimental fishes, however the population of neutrophils was marginally higher in *Marsilea* diet fed fishes than Glucan feed fed fishes. The lymphocyte population was significantly high in both *Marsilea* and Glucan feed fed groups and significantly high in Glucan diets (Figure-15). No much difference was recorded in monocyte population when compared with control feed group, however the monocyte population was higher in Glucan feed than *Marsilea* diet group.

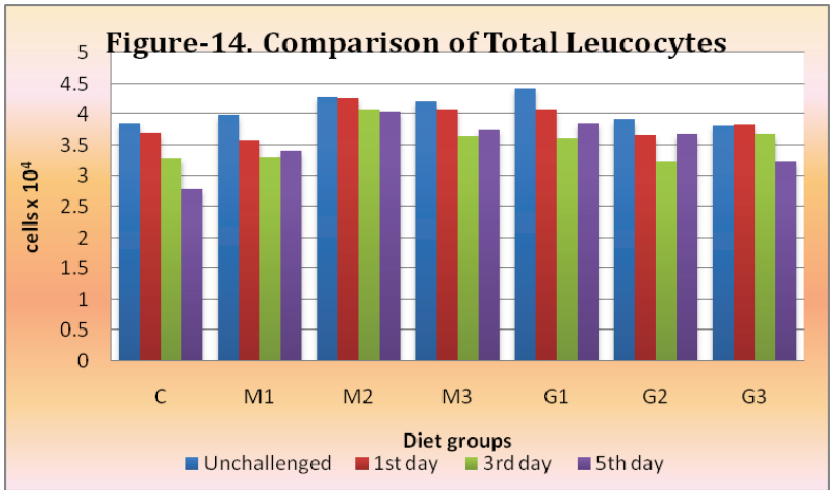
The figures-11-15 compares the postchallenged with their respective unchallenged counterparts clearly explains a decline in certain parameters like total erythrocyte count in the experimental fish a dose related response to infection. Similar physiological response was also observed in the immunostimulant supplemented fishes but the changes were not drastic as in control groups and also the population of different blood cells were always higher than the control group. Similarly increase in some blood cells like lymphocytes which was observed only in immunostimulant supplemented groups evidenced early activation of the immune system. In short, more was the circulating blood cells, higher was the immune response, as fishes primarily rely on non specific immune response. It was also observed that the elevations or decline were significant only upto 3rd day after challenge and on 5th day the responses are indicated recovery.

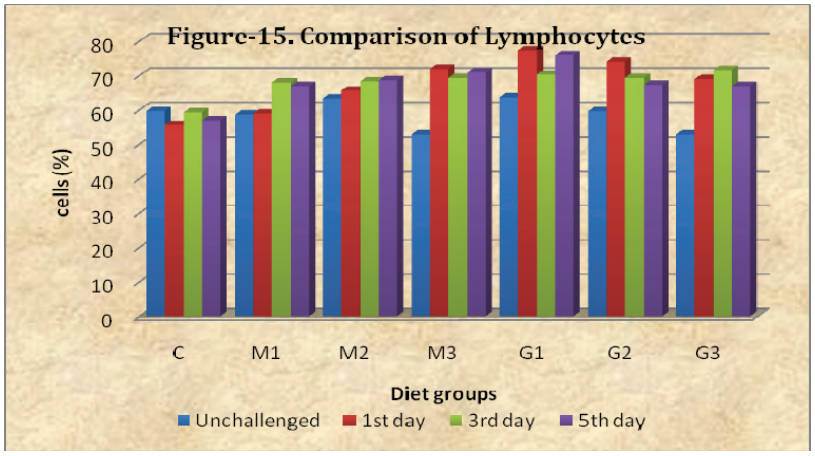




C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*



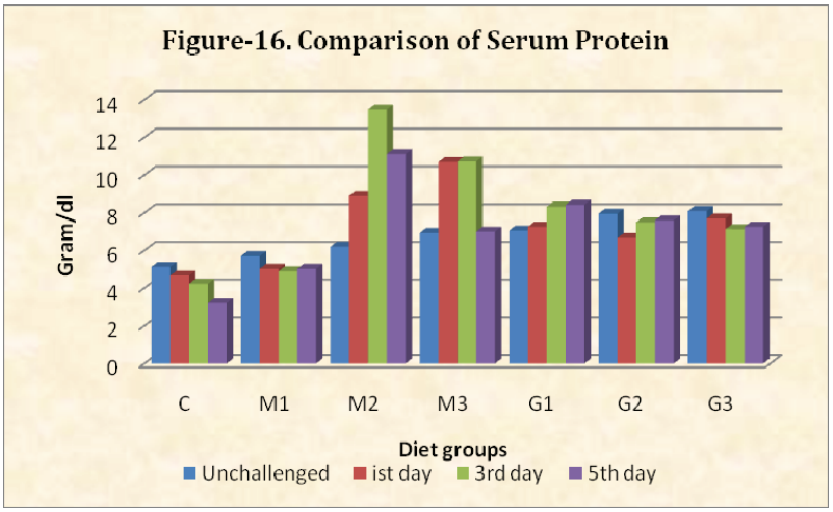


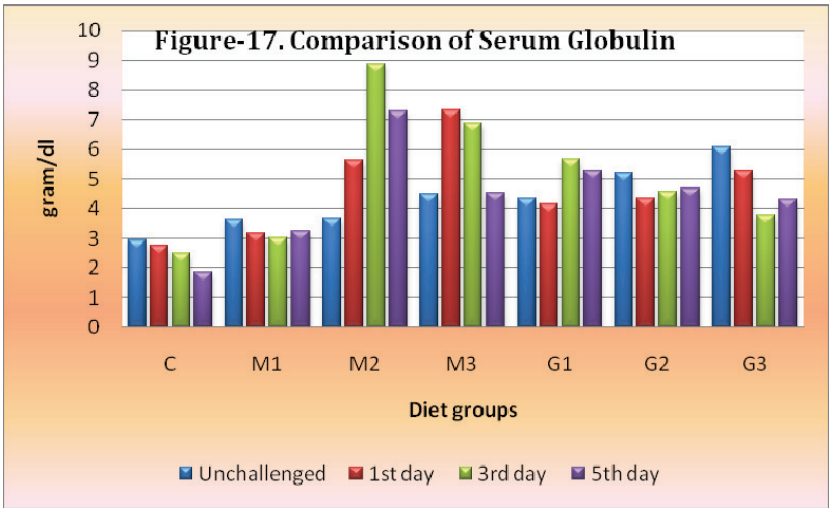


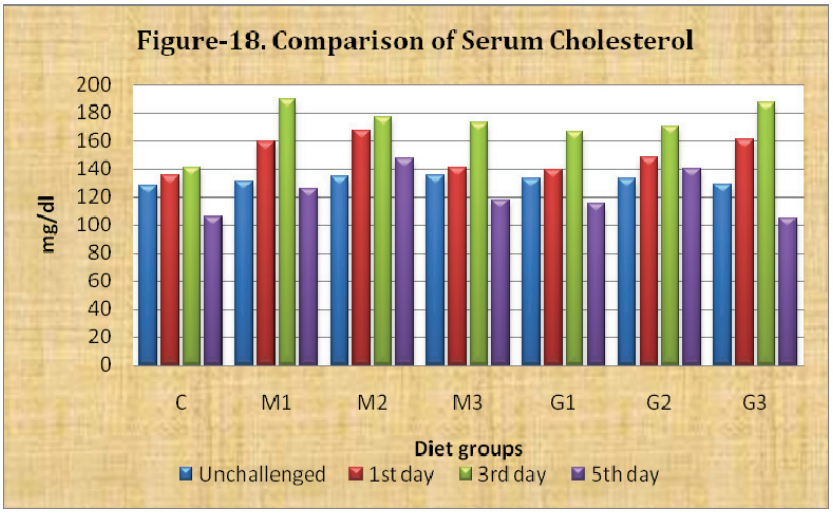
C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*

When the changes between the different feed groups of challenged fishes were compared it was observed that the serum protein, albumin, globulin, cholesterol and glucose levels were significantly higher in both *Marsilea* and glucan fed groups compared to control groups (Figure-16 and 17). However it was significantly higher in *Marsilea* groups (especially in M-2 group) than Glucan feed groups. The albumin/globulin ratio was significantly low in both *Marsilea* and Glucan diets groups when compared to control challenged fishes. However the decrease was significant in G-1 and M-3 feed group denoting a higher proportion of globulin level in serum. The cholesterol levels were significantly higher in M-2 and G-2 groups than the control challenged fishes (Figure-18). However there was no significant difference between *Marsilea* and Glucan diet groups. The blood glucose levels were higher in M-2, M-3, G-1 and G-2 groups, however the levels were significantly higher in *Marsilea* diet groups than Glucan diet groups.

The serum of challenged fishes of selected feed groups of 3rd day of challenge was subjected to electrophoretic separation and then densitometerically analysed. As most parameters recorded a peak value at 3rd day of post challenge the electrophoretic separation was carried out on this serum. It was observed that in post challenged fishes of control, G-2 and M-3 groups 8 different protein fractions with different molecular weight, where as 7 different protein fractions with different molecular weight were recorded in M-2 and G-1 groups. When compared to control challenged fishes the globulin fraction was dense in M-2, G-1 and G-2 groups. It was also clearly noted that the 8th protein fraction appeared was of low molecular weight probably maybe of pre-albumin fraction (Plate-10 and 11).



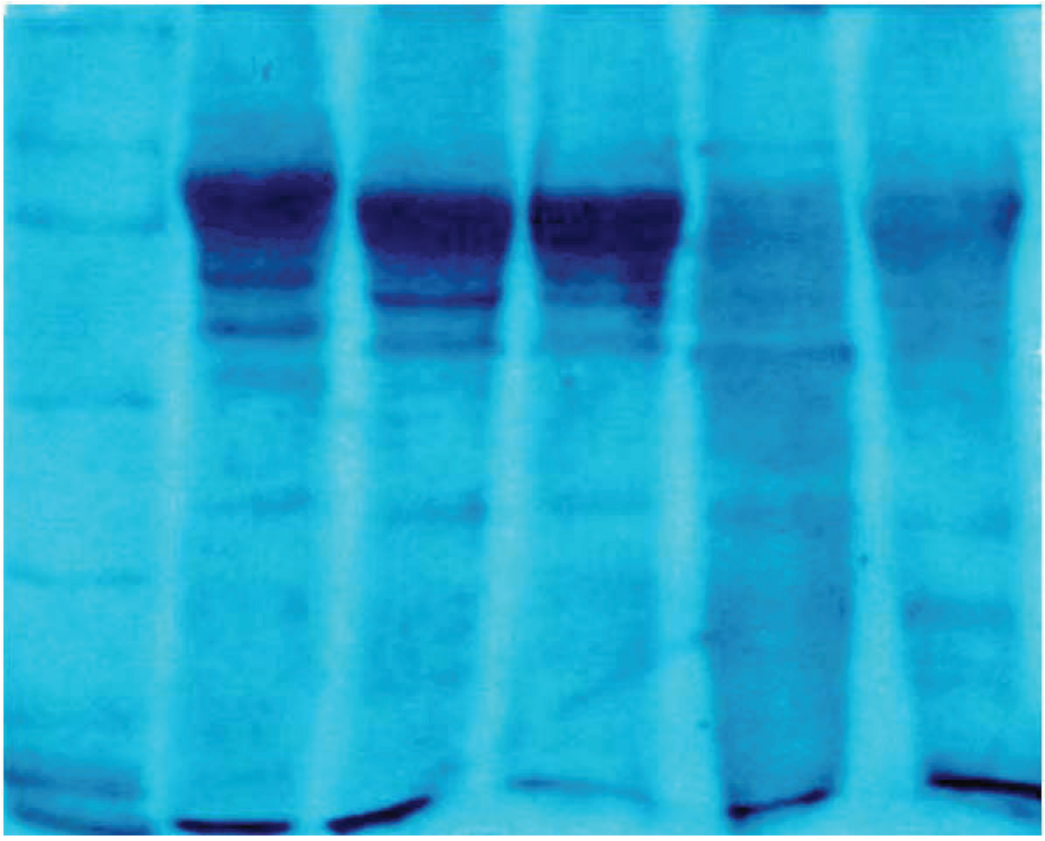




C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*

Plate-9. Comparison of serum Proteins by SDS-PAGE in different feed groups of *Labeo rohita* after 3rd day of challenge with *A.hydrophila*

C **M2** **G1** **G2** **M3** **MARKER**

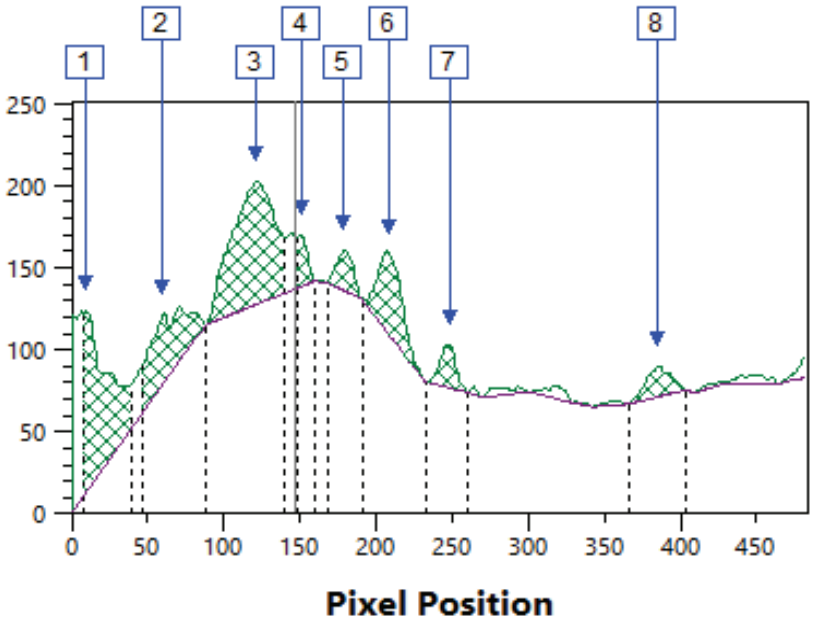


C= Control diet, M-2=0.5% *Marsilea* diet, M-3=1% *Marsilea* diet,

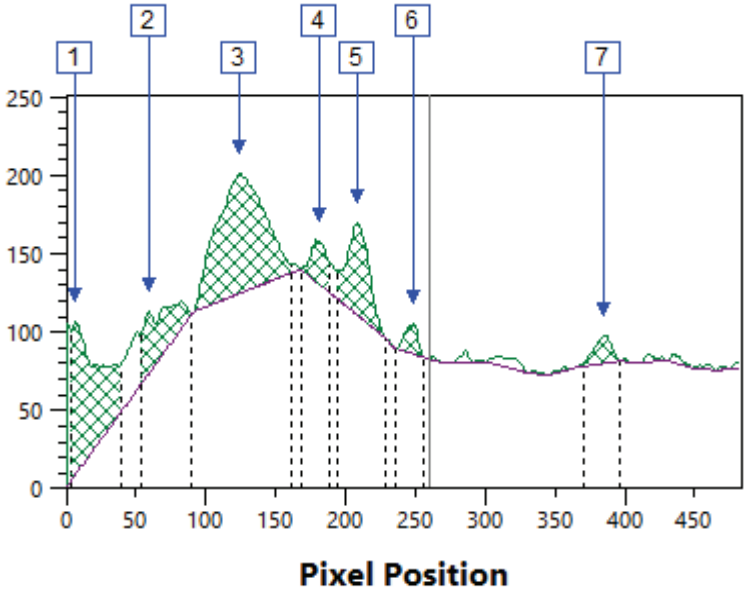
G-1= 0.25%Glucan diet, G-2=0.5% Glucan diet

Plate-10. Densitometric Graph of Serum Proteins in different feed groups of *Labeo rohita* after 3rd day of challenge with *A.hydrophila*

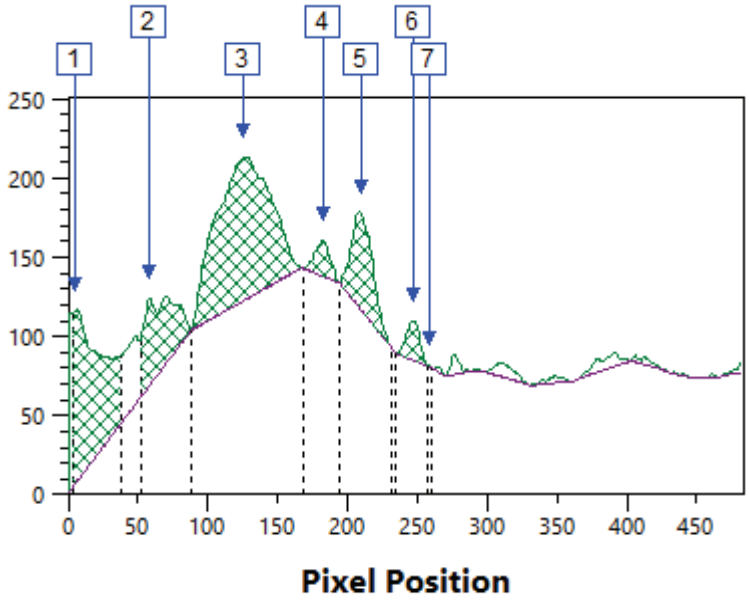
Control diet



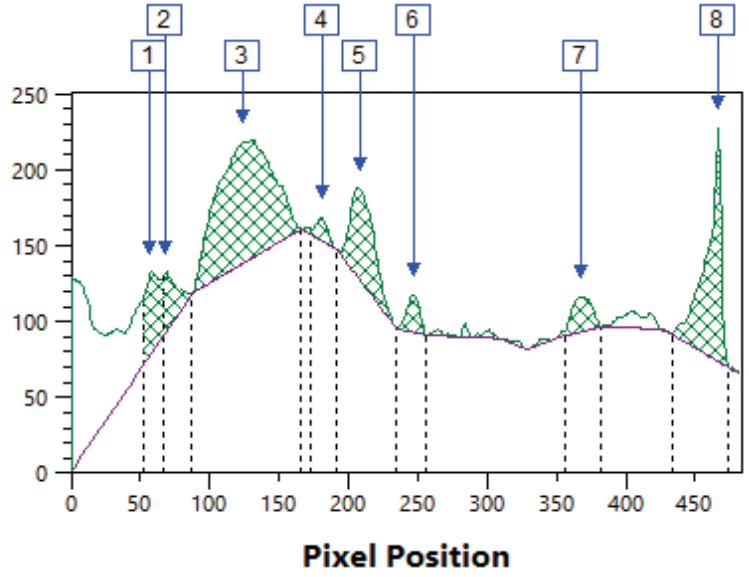
M2 (0.5% Marsilea diet)



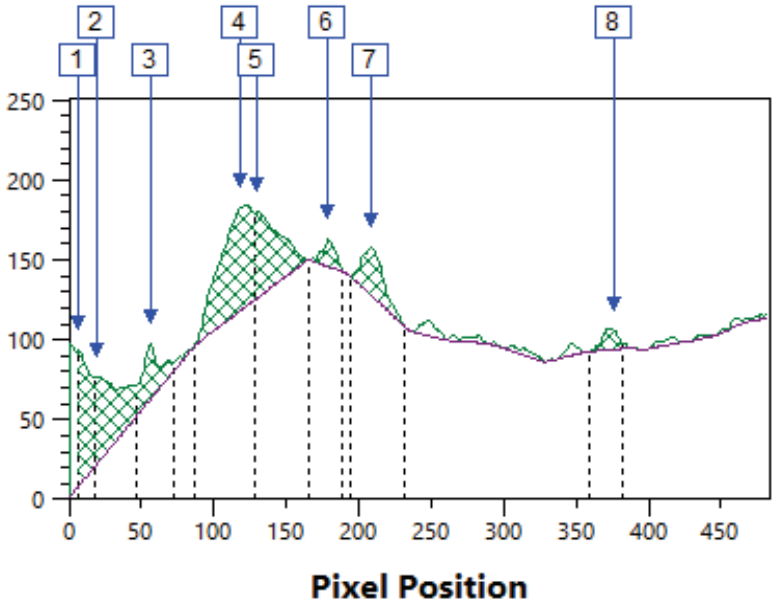
G 1 (0.25% Glucan diet)



G 2 (0.5% Glucan diet)



M 3 (1% Marsilea diet)



Marker

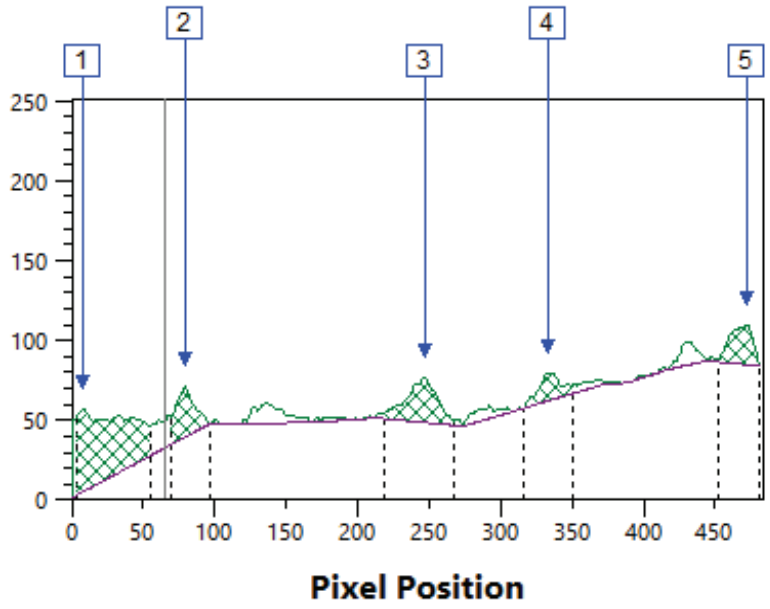


Table-29. Densitometric Profile of serum Proteins (3rd day of Post challenge) of fishes belonging to different diet groups challenged with *A.hydrophila*.

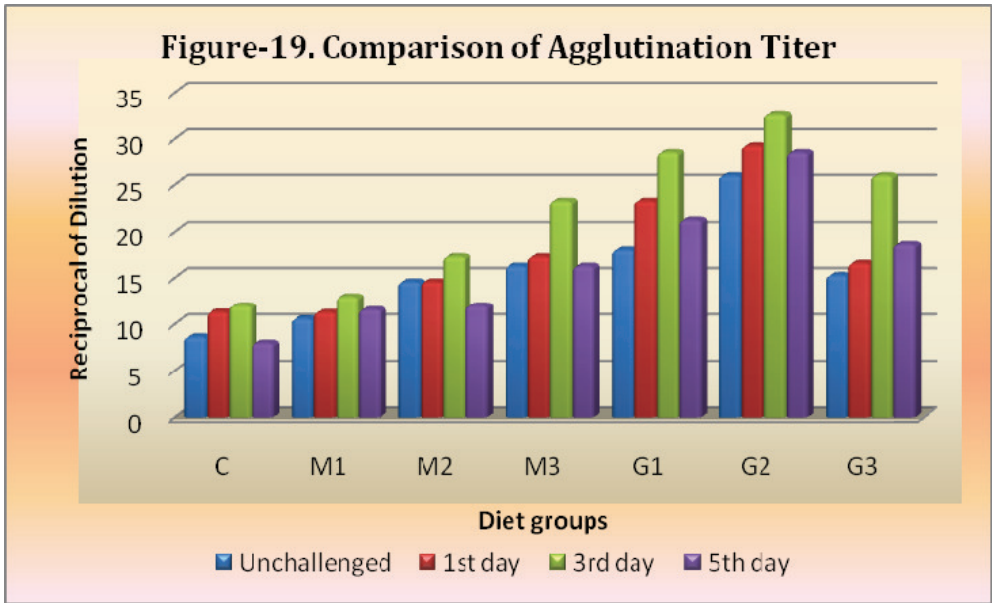
Band No	Control		M-2		G-1		G-2		M-3		Marker	
	M.W	R.F	M.W	R.F	M.W	R.F	M.W	R.F	M.W	R.F	M.W	R.F
1	246.539	0.117	268.693	0.1	277.557	0.094	249.195	0.115	254.601	0.11	205	0.158
2	221.644	0.138	246.539	0.117	246.539	0.117	221.644	0.138	228.805	0.131	97	0.277
3	128.123	0.25	125.68	0.254	123.293	0.258	126.894	0.252	128.123	0.25	66	0.508
4	104.182	0.296	76.895	0.369	75.041	0.375	76.27	0.371	76.895	.369	43	0.69
5	77.528	0.367	61.458	0.429	61.906	0.427	62.36	0.425	61.906	0.427	20	0.892
6	62.36	0.425	47.528	0.51	47.528	0.51	47.811	0.508	47.528	0.51	14	0.975
7	47.811	0.508	28.445	0.798	35.059	0.642	39.296	0.585	31.072	0.719		
8	28.445	0.798					29.543	0.76	29.214	0.771		

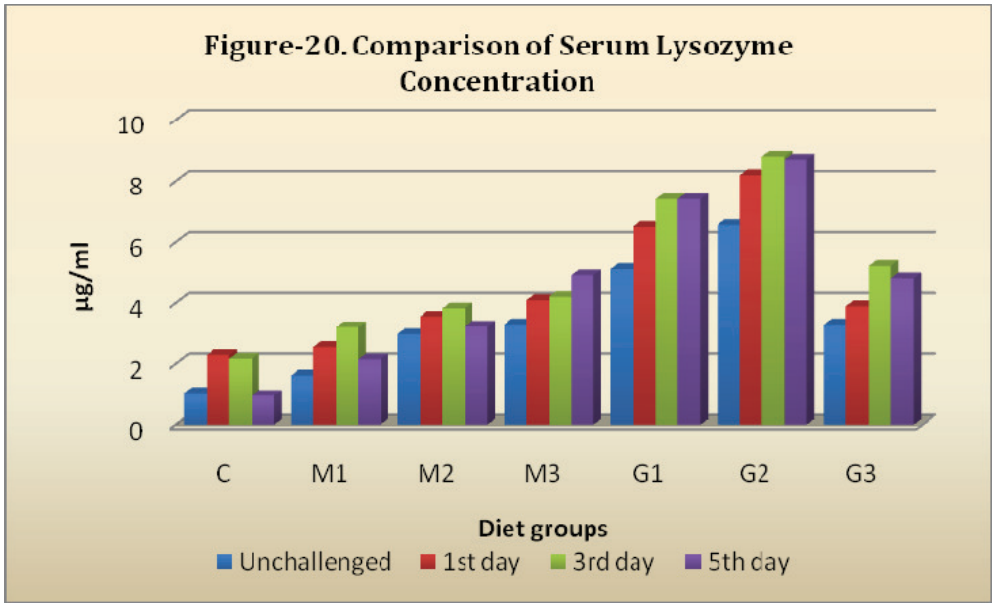
C= Control diet, M-2=0.5% Marsilea diet, M-3=1% Marsilea diet, G-1= 0.25%Glucan diet, G-2=0.5% Glucan diet

M.W=Molecular weight, RF= Relative front

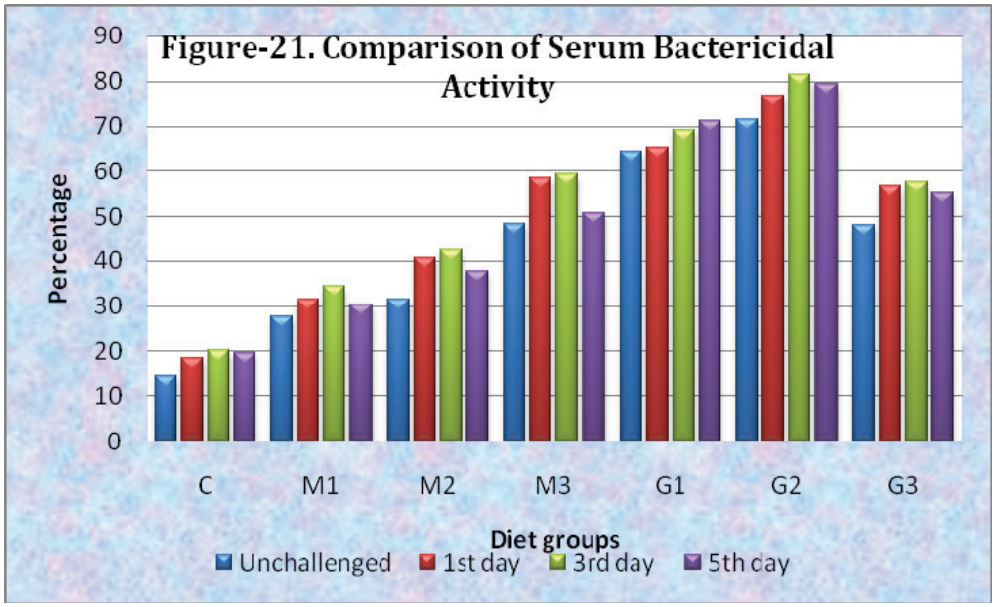
When the serum agglutination titer of different experimental groups were compared with control group it was observed that the serum agglutination titer increased significantly in post challenged groups of all the experimental groups, serum at dilution of even 32 times was able to agglutinate in G-2 diet whereas it was maximally upto 23 times in M-3 diet (Figure-19). The serum lysozyme concentration exhibited a declining trend in post challenged control group fish, whereas it remained elevated in post challenged fishes of all groups in glucan fed fishes and in M-3, In M-1 and M-2 the increase was only upto 3rd day and declined on 5th day (Figure-20). It was observed that the serum bactericidal activity significantly increased in post challenged fishes of all groups including control, it was especially high in G-1, G-2 and M-3 group (Figure-21). The myeloperoxidase activity also exhibited an increasing trend upto 3rd day and then declined marginally in all experimental groups, however the activity was higher in G-2 group when compared to *Marsilea* groups (Figure-22).

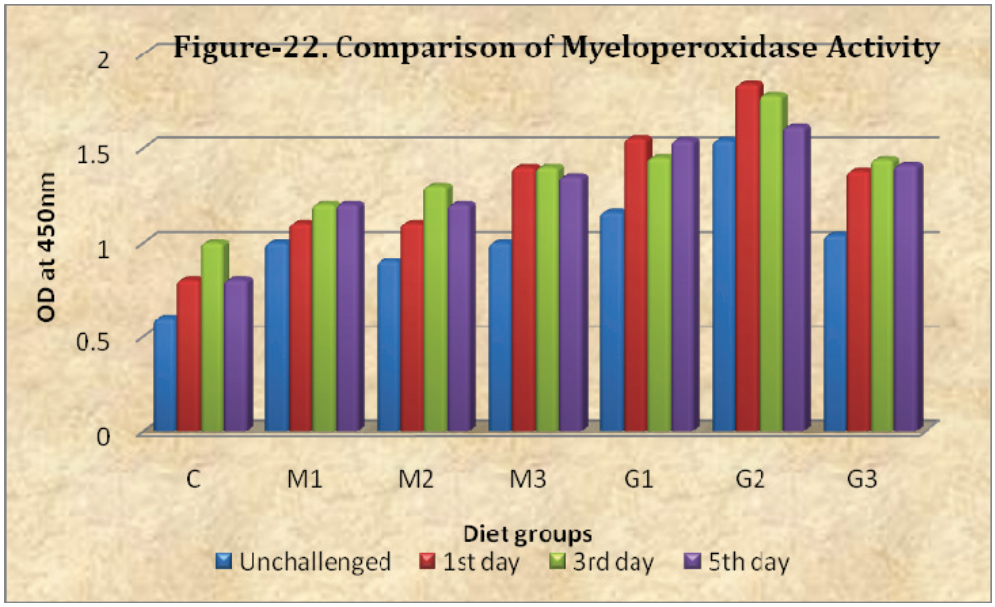
Positive results obtained in the above investigation clearly indicate that the chosen medicinal herb *Marsilea quadrifolia* has immunostimulatory and immunomodulatory qualities as that of a known Immunostimulant β-Glucan. Both *Marsilea* and Glucan were potent in improving the different haematological parameters which maybe one among the reason for the enhanced levels of serum proteins and globulins which in turn may be responsible for the elevated immunological responses like improved agglutination titre, lysozyme concentration, serum bactericidal activity and also myeloperoxidase activity. The electrophoretogram has also documented the presence of new type of low molecular weight protein which may be of immunoglobulin group to support the immunostimulant induced enhancement of specific and non-specific immune response. Thus the medicinal herb *Marsilea* may be considered to possess the Immunostimulant potential to improve the health status of fish.





C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*





C- Control; M1-0.25% *Marsilea* ; M2-0.5% *Marsilea* ; M3-1% *Marsilea* ; G1-0.25% *Marsilea* ; G2-0.5% *Marsilea* ; G3-1% *Marsilea*

5.4. DISCUSSION

Previous haematological studies on nutritional effects, infectious diseases and pollutants indicated that erythrocytes are a major and reliable indicator of various sources of stress (Rehulka 1989, 2000 and 2002). Moreover, erythrocytes transport haemoglobin that, in turn, transports oxygen, and the amount of oxygen received by tissue depends on the maturity of erythrocytes and amount of haemoglobin. Decrease in the haemoglobin concentration explains the inability of the fish to withstand and resist the infection induced stress as observed in control feed fed fishes.

Erythropoiesis is the primary physiological response ie, maintaining/providing the energy producing mechanism which is vital to keep any cell including immune cell in viable state thus strengthening the first line of defense (Annalakshmi *et al* 2013).

The increase in the values of erythrocytes may be due to feed additive triggered erythropoiesis and also decrease in the rate of oxidant induced haemolysis due to the presence of the antioxidants present in the plant extract (Sheeja *et al* 2006). This may be one of the reasons for the enhanced values of total erythrocytes in the present investigation also. Reduced haematocrit may indicate that fish are not eating or are suffering infections (Blaxhall 1972). However, increased levels of haematocrit were observed in both *Marsilea* and Glucan diet fed fishes. The blood indices such as MCV, MCH and MCHC are particularly important for the diagnosis of anemia in most animals (Coleseh 1986). The maintenance of constant level or increase of MCHC can be attributed to improvement of fish health (Suresh and Amolkumar 2009). Similar increase was observed in both glucan and *Marsilea* diet fed fishes in the present experiment also. In the present study an increase in TEC and Hb content was observed in *M.quadrifolia* administered fishes.

A gradual enhancement of erythrocyte and haemoglobin content was also observed with increased supplementation of levan in the diet, indicating an improvement in the health status of the fish (Gupta *et al* 2008). Sivagurunathan *et al* (2012) observed a significant increase in total erythrocytes, haemoglobin, total leucocytes, monocytes, lymphocytes, serum protein and albumin levels in *Cirrhinus mrigala* after five days of *Pseudomonas aeruginosa* infection in dietary *Nelumbo nucifera* fed fishes. Dietary Amla fed for 40 days to Tilapia improved the specific growth rate and feed conversion ratio, when they are infected with *Pseudomonas aeruginosa* the total erythrocytes, haemoglobin, total leucocytes, lymphocytes, neutrophils and serum total protein level has increased significantly (Sivagurunathan *et al* 2012). Fazlolahzadeh *et al* (2011) observed a significant increase in total erythrocytes, total leucocytes, lymphocytes and no significant difference was observed in haemoglobin, haematocrit values and a significant decrease in mean corpuscular volume (MCV) in Rainbow trout fed with dietary garlic and exposed to temperature stress. Rainbow trout infected with *Pseudomonas putida* exhibited a decline in erythrocytes, haematocrit, haemoglobin and increase in MCV and total leucocytes (Bektas and Ayik 2009). Thus the results of the present investigation agree with the above findings.

Dietary ethanolic extract of Propalis for 4 weeks to *Oreochromis niloticus* has improved specific growth rate, haematocrit, monocytes, lymphocytes and neutrophils than crude propalis administration (Azza and Abd-El-Rhman 2009). According to Martins *et al* (2004) the reduction in total erythrocyte count, haemoglobin value and haematocrit in the infected fishes occurs as a result of the parasitic infestation that often leads to anaemia. Furthermore, the parasites simply acts as a stressor and during primary stages of stress the haematocrit is altered due to the release of catecholamine, which can mobilize erythrocytes from spleen or induce erythrocytes to swell as a result of fluid

entry into the intracellular compartment (Chiocchia and Motais 1989). The drastic reduction in the erythrocyte counts in control infected fishes in this present experiment can be attributed to pathogen induced effect; further enhancement in erythrocyte counts in imunostimulant supplemented groups may be due to increased mobilization of erythrocytes from spleen.

According to Ruane *et al* (2000), fish infected by parasites significantly changed their haematological parameters. Leucocyte counts can be applied as a measure of general immune response. The increased leucocyte counts and shift values towards the myeloid line (especially a high number of myelocytes and metamyelocytes) reflects infection or inflammation. Leucocytosis is a condition characterized by an elevated number of white cells in the blood. Leucocytosis due to neutrophilia and slight lymphocytosis are characteristic of an acute infection (Ranzani-Paiva *et al* 2004).

Thrombocytes have been described as the most abundant blood cells after erythrocytes (Rey Vazquez and Guerrero 2007). The thrombocytes in fish is nucleated and functionally analogous to mammalian platelets, they have phagocytic ability and participate in defense mechanisms. (Stosik 1993, Stosik and Deptula 1992). Piscine thrombocytes represent a link between innate and adaptive immunity (Passantino *et al* 2005) and express surface and intracellular molecules that are involved in the immune function. It is also already agreed that the fish thrombocytes are blood phagocytes that form one of the protective barriers besides involving in blood coagulation process (Prasad and Priyanka 2011; Tavares-Dias and Moraes 2004, Prasad and Charles 2010). Glucocorticoid excess in fish tends to decrease the thrombocyte counts (Mary Ann Throll *et al* 2012). In the present study also it was clearly observed that the thrombocytes increased sharply on 1st day after infection indicating its immediate role in defence

against *A.hydrophila* infection, and its drop in count on 3rd day can be correlated with the increasing levels of blood glucose.

Total and differential leucocyte counts are important indices of non-specific defence activities in fish (Pedro *et al* 2005), as leucocytes are centrally involved in phagocytic and immune responses to parasitic, bacterial, viral and similar challenges (Houstan 1990). Similarly neutrophilia and monocytosis can be attributed to acute inflammatory response due to infection, as monocytes undergo transformation into macrophages and may be involved in phagocytosis and killing of pathogens upon first recognition and subsequent infections. Neutrophil is the first cell to respond to infection within 24 hours, increases during bacterial infections to phagocytose them, but they die after having phagocytosed a few pathogens as they cannot renew their lysosomes used in digesting microbes, whereas monocytes phagocytose the pathogens not only more efficiently as it is long lived and able to replace lysosomes but also presents the antigens to lymphocytes (Sivagurunathan *et al* 2012). In the present work also decline in neutrophil count especially in *Marsilea* and glucan diet fed groups when compared with control feed group, which may be due to early identification and active participation of more neutrophils in combating the pathogen leading to a significant decline in its population. Though the population of neutrophil might have declined but its early recognition of the pathogen may be of immense importance which was observed in the form of improved levels of lymphocytes, or otherwise the early initiation of non specific immune response by the neutrophils might have triggered the specific immune response early, this may be the reason for the increasing trend of neutrophils on 5th day. In fish, an increase of neutrophils due to stress response is frequently associated with a decreased overall leucocyte number (Slicher 1961). In the present work also similar trend was observed in control feed fed fishes.

Cyprinus carpio immunized with *Aeromonas hydrophila* bacterin, fed with *Aloe vera* supplemented diet for 8 weeks, exhibited significant increase in total luecocytes, serum protein and globulin levels only and no significant change was observed in erythrocyte counts, PCV (Alishahi *et al* 2010). Annalakshmi *et al* (2013) fed dietary *Phyllanthus amarus* to *Labeo rohita* for 14 days, infected with *Aeromonas hydrophila* and observed a increase in erythrocytes, haemoglobin, total leucocytes, lymphocytes and marginal decrease in neutrophil and monocytes.

The proliferation rate and number of lymphocytes produced is very important for the magnitude and duration of protection against disease. This supports the view that the magnitude of an immune activator may be a critical factor in maintaining long term protection against disease causing situations. Immunostimulants attach to specific receptors on the cell surface of the phagocytes and lymphocytes activating this cell to produce some enzymes that can destroy pathogens. Moreover, they can increase the production of some chemical messengers (Interferon, Interleukins and complement protein) that stimulate other arms of the immune system and increase the activity of T and B lymphocytes (Rao *et al* 1992). Monocytes/Macrophages possess specific receptor(s) for β-Glucans and produce mediators like leukotrienes, cytokines and prostaglandins in the presence of glucans (Jorgensen and Robertsen 1995). Macrophages are an integral part of the specific immune response as they recognize and process antigens, and trigger lymphocyte activation by the release of a wide range of immunomodulatory molecules (Secombes and Fletcher 1992). Enhanced levels of lymphocyte population with increase in time in the present investigation in both *Marsilea* and glucan diet reflects their role in disease protection.

The mechanism of immunostimulation of dietary herbs may be attributed to one or more of its components, especially catechins, flavonols, flavanones, phenolic acids,

glycosides and the aglycones of plant pigment (Pan *et al* 2003, Farhoosh *et al* 2007). These components have powerful natural antioxidants which protects cellular components against oxidative stress (Mohan *et al* 2006). Increase in lymphocytes may be due to the presence of flavonoids and terpenoids found in the leaf extract (Grayer *et al* 1996, Lemberkovics *et al* 1998). The qualitative analysis of *Marsilea* also revealed the presence of different above mentioned metabolites which may be the reason for its potential in improving the immune system of the fish.

In goldfish (*Carassius auratus*) infected with *A.hydrophila* were fed with mixed herbal extract (*Azadirachta indica, Ocimum sanctum and Curcuma longa* in equal proportion) supplemented diet, the total leucocytes increased significantly, the erythrocytes, haemoglobin decreased in low dose of herbal extract where as restored near to control uninfected fish in higher dose. The haematocrit decreased significantly, the MCV, MCH and MCHC values altered significantly than control. The total protein, glucose and cholesterol levels declined initially and restored near to control fish in higher dose of herbal extract feed (Ahilan 2010). Thus herbal supplemented feeds restored the altered haematological parameters and triggered the innate immune system of goldfish against *A.hydrophila*. Ramasamy Harikrishnan *et al* (2010) observed a significant reduction in erythrocytes, haemoglobin, hematocrit, serum protein, cholesterol, glucose and increase in leucocytes in *Cirrhina mrigala* infected with *Aphanomyces invadens*, fed with control diet, where as no significant changes were observed in *Azadirachta indica* extract incorporated diet (provided effective protection).

There is a close relationship between the level of protein synthesis in liver tissue and plasma protein pools, total protein levels in plasma may be elevated due to the increased levels of protein synthesis in liver tissue of fish treated with herbal extract (Asadi *et al* 2012). Increase in the serum protein, albumin and globulin levels is thought

to be associated with a stronger innate response in fishes (Wiegertjes *et al* 1996). The increase in serum protein content might be in part due to an increase in the leucocytes, which is a major source of serum protein production such as lysozyme, complement factors and bactericidal peptides (Misra *et al* 2006). Proteins include albumin and globulin; some globulins are produced in the liver, while others are made by the immune system (Sandnes *et al* 1988). Globulin is made up of subunit of α-1, α-2, β and γ globulins, which are considered as the source of almost all the immunologically active proteins in the blood (Jha *et al* 2007). Increased levels of serum protein and globulin may be responsible for the bactericidal activity of the serum as the complement fraction constitutes a large part of the gobulin. Serum albumin not only maintains osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues but also acts as plasma carrier protein to transport steroid hormones, hemin, fatty acids and also compounds like drugs (Asadi *et al* 2012, Wikipedia-serum_albumin).

In the present investigation also significant increase in serum protein, albumin and globulin was recorded in both *Marsilea* and glucan diet fishes, especially in *Marsilea* diet fed fishes which may probably due to plant induced effect on liver. Thus *Marsilea* has the potential to improve the innate defence response in fish. Gupta *et al* (2008) observed an increase in serum protein and globulin levels in *Labeo rohita* juveniles after feeding with 1% Levan. They also observed significant decrease in the albumin/globulin ratio at 1.25% levan. The albumin/globulin ratio is a measurable humoral component of non-specific defence. The reduction might be due to increased globulin levels, which may play a significant role in the immune-protective mechanisms of fish. Misra *et al* (2006) found an increase in total protein and globulin content in

Labeo rohita after feeding with Beta glucan, similar results were observed by Anderson and Ramsey (1994) also.

It has been shown that glucose level increases in the infected or stressed animals to ward off the infection or stress (Citarasu *et al* 2006). Similar trend observed in the present work in fishes fed with especially *Marsilea* diet indicates its potential in inducing the body's immune system to fight against infection. The lipids are the usual economic form of biomolecules used by fish to stock energy and can be stored in many different organs (Guijarro *et al* 2003). Improved levels of cholesterol in the present investigation implies improved reserved energy.

However, decrease in serum protein levels were observed by some investigators. Sudagar Mohamad and Hajibeglou Abasali (2010) observed a remarkable decrease in serum protein, albumin, globulin, glucose, haemoglobin, erythrocyte counts, leucocyte counts and serum lyssozymal activity in fishes infected with *Aeromonas hydrophila* in both control as well as herbal diet(*Inula helenium, Brassica nigra, Tussilago farfara, Chelidoniume majus and Echinacea purpurea*) fed common carp when compared with their unchallenged counterparts.

Hajibeglou Abasali and Sudagar Mohamad (2010) fed common carp (*Catla catla*) with dietary *Ocimum basilicum, Cinnamomum zeylanicum, Juglans regis and Mentha piperita* (mixed in equal proportion) fed for 45 days and infected with *Aeromonas hydrophila*, observed a significant decrease in serum protein, globulin, albumin, erythrocytes, total leucocytes and haemoglobin levels but no significant change in glucose levels, further they have also observed that the serum lysozyme concentration and serum bactericidal activity has increased significantly in post challenged fishes.

Marked rise in serum total protein, albumin and globulin levels observed in *Marsilea* diet fishes endorses its Immunostimulant potential when compared with the well documented immnostimulant "Glucan". Further, sharp increase in blood glucose and serum cholesterol levels after pathogenic challenge observed in *Marsilea* and Glucan diet fed fishes reflects their active participation in preparing the animal during stress conditions.

Jayakumari and Sahoo (2006) has observed that when *Clarias batrachus* (Asian catfish) was fed with different Immunostimulants like lactoferrin. β1-3 glucan, levamisole and Vitamin-C for 30 days the Antibody titre levels have increased significantly. Soltani *et al* (2010) has administered dietary *Zataria multiflora* (essential oil) to *Cyprinus carpio* for 8 days and observed that the total leucocytes, total serum protein, globulin, albumin levels has increased after 15 days of administration, further the antibody titer levels has increased significantly in fishes treated with formalin killed *Aeromonas hydrophila.*

Rahim *et al* (2014) classified the SDS-PAGE serum proteins in grass carp (*Ctenopharygodon idella*) into six protein fractions as pre-albumin, albumin, alpha – 1, alpha-2, beta and gamma globulins from low to heavy molecular weight. According to Ford (2001) transferrin is a beta globulin which not only binds and transports iron, but restricts bacterial infections by keeping the free iron concentration low. Interaction of transferrin with bacteria is being currently looked upon as a principal factor of natural selection in evolution of teleosts. In the present study it was clearly observed denser globulin bands in challenged fishes fed with Immunostimulants either glucan or *Marsilea* which was not that conspicuous in control challenged fishes, indicating feed induced enhancement in specific immunity.

Lysozyme is a humoral component of the non-specific defense mechanism that has the ability to prevent the growth of infectious microorganisms by splitting β-1,-4 glycosidic bonds between N-acetyl muramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls (Alexander and Ingram 1992, Gopalakannan and Arul 2006, Grinde 1989 and Choi *et al* 2008). Lysozyme is localized in the lysosomes of neutrophils and macrophages and is released into the blood from these cells (Murray and Fletcher 1976). Although the kidney appears to be the major site of lysozyme activity in teleosts, the blood is recommended as a more practical, less variable tissue for monitoring lysozyme activity in fish (Lie *et al* 1989, Mock and Peters 1990). Several reports are available in which Immunostimulants can enhance the lysozyme activity (Ardo *et al* 2008, Hanif *et al* 2005, Chen *et al* 2003 and Puangkaew *et al* 2004). A large number of immunostimulants have been reported to increase serum lysozyme levels in fish that may be due to either an increase in the number of phagocytes secreting lysozyme or due to an increase in the amount of lysozyme synthesized per cell (Engstad, Robertsen and Frivold1992, Kumari and Sahoo 2005).

The level of serum lysozyme was enhanced in *Labeo rohita* after feeding the fish with *Achyranthus aspera* seed (Rao *et al* 2006). Elevated lysozyme was also observed in japanese Eel (*Anguilla japonica*) after feeding with Korean mistletoe extract (*Viscum album*) (Choi *et al* 2008). Similarly plasma lysozyme activity was increased in crucian carp by feeding four Chinese herbs (*Rheum officinale, Andrographis paniculata, Isotis indigotica and Lonicera japonica*) (Chen *et al* 2003). Sung and Sang (2012) have recorded that dietary inclusion of Onion powder (0.5%) for 40 days has improved the lysozyme activity and survival in Olive Flounder (*Paralichthys olivaceus*) infected with *Edwardsiella tarda*. Similarly dietary incorporation of ethanolic extract of propalis for 4 weeks to *Oreochromis niloticus* has enhanced serum lysozyme and serum bactericidal activity than administration of crude propalis and also better survival against *Aeromonas*

hydrophila infection (Azza and Abd-El-Rhman 2009). Swagatika *et al* (2007) has observed that dietary *Magnifera indica* (Mango) kernel to *Labeo rohita* for 60 days has improved the specific growth rate, serum protein, albumin, globulin, superoxide anion production, lysozyme activity, serum bactericidal activity and also improved the survival rate when challenged with *Aeromonas hydrophila*.

Christybapita *et al* (2007) administered dietary aqueous leaf extracts of *Eclipta alba* to *Oreochromis mossambicus* for 3 weeks, challenged with *Aeromonas hydrophila* and observed that the serum bactericidal activity, reactive oxygen species production, myeloperoxidase content has increased significantly after one week of feeding itself. Sivaram *et al* (2004) has reported that the serum bactericidal activity was enhanced in juvenile greasy groupers (*Epinphlus tauvina*) fed antibacterial active principles of *Ocimum sanctum* and *Withania somnifera*. Similarly, groupers (*Epinphlus tauvina*) juveniles fed with diets containing different doses of extract mixture of some herbs showed a significant increase in their serum bactericidal activity (Punitha *et al* 2008). Najmeh *et al* (2011) has observed that inclusion of decaffeinated green tea (*Camellia sinensis*) extract in the feed of *Oncorhynchus mykiss* has increased the serum bactericidal activity, serum lysozyme activity, antitrypsin activity and peroxidase content. Nya and Austin (2009) has observed a significant increase in specific growth rate, total erythrocytes, total leucocytes, lymphocytes, monocytes, serum total protein, globulin, phagocytic activity, superoxide anion production (respiratory burst activity) serum lysozyme activity, serum bactericidal activity, serum alternate complement pathway activity in Rainbow trout (*Oncorhynchus mykiss*) fed with dietary *Zingiber officinale* for 14 days and infected with *Aeromonas hydrophila*.

The release of myeloperoxidase enzyme mostly by the azurophilic granules of neutrophils during oxidative respiratory burst activity was measured by serum

peroxidase content. Degranulation is essential for the release of myeloperoxidase and activation of the halide production pathway and release of diverse antimicrobial enzymes (Quade and Roth 1997). The myeloperoxidase in the presence of chloride ion, which is ubiquitous, hydrogen peroxide is converted to hypochlorous acid (50 times more potent in killing pathogen), a potent oxidant and antimicrobial agent (Thomas and Kalyanaraman 1997). Ying-rui Wu *et al* (2012) fed diet supplemented with different concentrations of Chinese traditional herbal medicine *Sophora flovescens* to Tilapia (*Oreochromis niloticus*) for 30 days and observed enhanced serum lysozyme, antiprotease, complement, reactive oxygen, nitrogen species production and myeloperoxidase activity, when infected with *streptococcal agalactiae* increase in survival was observed. *Catla catla* fed with ethanolic extract of *Cynodon dactylon* for 60 days has improved non-specific humoral (Lysozyme activity, anntiprotease activity, haemolytic complement) and cellular production of (reactive oxygen & nitrogen species and Myeloperoxidase activity) immunity and also increased the survival against *Aeromonas hydrophila* infection (Kaleeswaran *et al* 2011). Harikrishnan *et al* (2012) also observed similar results in Olive flounder *Paralichthys olivaceus* when fed with diet supplemented with Chaga mushroom *Inonotus obliquus* and infected with *Uronema marinum*. Gultepe *et al* (2014) fed Tilapia (Oreochromis niloticus) with Thyme (*Thymus vulgaris*), Rosemary (*Rosmarinus officinalis*) and fenugreek (T*rigonella foenum graecum*) for 45 days and observed that all have significantly improved phagocytic activity, haematocrit, WBC, RBC, neutrophil and monocyte counts, plasma myeloperoxidase and lysozyme activity and increased survival against *Streptococcus iniae*. Kumar Srivastava and Chakrabarti (2012) fed *Labeo rohita* fry with dietary *Achyranthes aspera* seed for 30 days and immunized with chicken RBC and analysed immune parameters on 7, 14 and 21 days after immunization. They have observed that the feed conversion ratio, serum protein, albumin, globulin, antibody titre, serum

glutamic oxaloacetic transaminase, serum glutamate, myeloperoxidase, pyruvate transaminase and alkaline phosphatase have increased in all treated groups. Four weeks of dietary incorporation of Tetra (*Cotinus coggygria*) to koi carp (*Cyprinus carpio*) challenged with *Vibrio anguillarium* has significantly increased RBC, MCHC, nitroblue tetrazolium activity, lysozyme and myeloperoxidase activity, and also improved their survival (Bilen *et al* 2013). Behra *et al* (2011) has injected curcumin to *L.rohita* and after 15 days observed that the non-specific immune parameters like respiratory burst, myeloperoxidase, haemagglutinaion, haemolytic and bacterial agglutination has increased significantly.

The administration of *E. alba* leaf extract enhanced the leucocyte myeloperoxidase activity after 1 week of feeding. In another study reported, the leucocyte myeloperoxidase activity was found to be significantly higher in gilthead seabream fed with 10 g/Kg yeast supplemented diet after 2 weeks of feeding (*Ortuno* 2002).

From the present study in challenged groups, declining trend in total erythrocyte, haemoglobin and haematocrit was observed, but this decline was efficiently resisted and in higher doses the values were enhanced in fishes fed with both *Marsilea* and Glucan. Thus *Marsilea* is as potent as Glucan in improving total erythrocytes which can be correlated with increased haematocrit, thus ensuring maintenance of general health which is a prerequisite to face any threat. Enhanced levels of platelets, total leucocytes and lymphocytes were observed in both *Marsilea* and glucan diet groups, which can be correlated with the improved levels of serum proteins, albumins and globulins indicating the role of Immunostimulants in activating the secretory nature of either blood and immune cells and/or liver cells thereby improving the non specific immunity. Significant enhancement in the agglutination titer indicate immunostimulant

induced enhancement in the production of specific immunoglobulins which can be correlated with the increase in the lymphocyte population in the challenged fishes. Further, significantly improved lysozyme concentration, serum bactericidal activity and myeloperoxidase activity indicates the Immunostimulant induced improvement in the production of serum proteins, globulins, complement or other immune related proteins as reported by earlier works. Dense protein fraction bands observed in the SDS-PAGE indicates the presence of more globulin fraction of protein indicating enhanced humoral immunity in Immunostimulant diet groups, further, additional low molecular weight protein fraction observed in Immunostimulant diet groups may belong to prealbumin which may facilitate the transport of immune related substance. Thus from the present investigation it is evident that *Marsilea* also possesses immune potential like a well known Immunostimulant beta glucan thereby improving the immune response of the fish ensuring good health and physiological status of the fish.

Summary

SUMMARY

Inland fish culture is a promising enterprise in India owing to its rich water resources. However aquaculture industry faces a serious threat due to several factors such as environmental deterioration, climatic changes, water quality, predators, pathogens etc. Disease outbreaks are common as opportunistic pathogens infect fishes and cause greater economic loss. Apart from this external stressors such as crowding, handling and poor water quality also influence fish health. In order to promote health in cultured fishes supplementary feeds with growth enhancers, antibiotic and vitamins are incorporated. These become cost-effective and lead to development of resistant pathogen and residual poisoning. Hence plants with medicinal qualities have been tried as feed additive to boost the health. These are classified as an Immunostimulant which elevates the nonspecific and specific immune response. Many medicinal plants used to enhance animal health especially in man, since they possess the ability to influence the immune system. Hence in the present investigation an aquatic fern *Marsilea quadrifolia* which has medicinal properties was incorporated in the formulated feed and supplemented to *Labeo rohita* a most preferred food fish.

In the present investigation it was observed that the aquatic fern *Marsilea quadrifolia* was rich in wide range of secondary metabolites like tanins, phenolic compounds, flavonoids, alkaloids, phytosterols and saponins besides sugars, proteins and amino acids. GCMS analysis has revealed the presence of 13 bioactive compounds most of which have antimicrobial, anti-inflammatory, anticancer and antioxidant properties. The antimicrobial studies has revealed the benzene extract and then ethanolic extract of *M.quadrifolia* has the potential to resist the growth of different pathogenic microbes.

The feeding trial with different concentrations of ethanolic extract of *M.quadrifolia* and beta glucan (0.25%, 0.5% and 1%) exhibited a significant increase in specific growth rate, haematological, serological and immunological parameters indicating a significant impact on improving the health status of the test fish. The densitometric analysis of the electrophoretogram of serum proteins revealed the presence of many new moderate molecular weight proteins (probably of globulin type) in the experimental feed fed fishes also supports feed induced enhancement in the immune response.

As a continuation of the study, the fishes of all the groups were challenged with *Aeromonas hydrophila* and the haematological, serological and immunological parameters were analysed on 1st, 3rd and 5th day post challenge. It was inferred that incorporation of *Marsilea* in diet has improved the erythrocytic parameters in the test fish indicating its potential in improving the general health of the animal which is of foremost important to face any challenge as anemic animals are prone to infection. As fishes rely mainly on nonspecific immune response, diet induced enhancement in the thrombocytes (which also functions as immune cells in fish) and leucocytes particularly lymphocytes in challenged fishes is a measure to protect themselves from infection probably by earlier initiation of humoral immune response. Increased levels of serum protein, globulin and lysozyme in experimental groups indicates the Immunostimulant ability of *Marsilea* in inducing the secretory cells of immune system by binding with their receptors like the known immunostimulant beta glucan. This is well reflected by the 2 to 3 fold increase in agglutinating antibody titer, serum bactericidal activity and also in myeloperoxidase activity. It was further observed that in challenged fishes the immune responses were significant mainly upto 3rd day. The densitometric analysis of serum protein electrophoretogram of challenged fishes has revealed the presence of

significantly high amount of different protein fractions (especially globular protein fractions) in *Marsilea* and Glucan feed fed fishes, which can be correlated to the enhanced immune response. Highly significant decrease was observed in many parametes in challenged fishes of control feed groups indicating the inability to withstand or succumbing to the infection, whereas the immune improving effect was significant and at the same time the decline was gradual and also minimal in both *Marsilea* and glucan feed fed fishes indicating the enhanced immunity. Thus it was inferred that *Marsilea quadrifolia* also possesses immunostimulant potential like the known immunostimulant β-glucan. As 0.5% of ethanolic extract of *Marsilea quadrifolia* has produced consistent improvement on various parameters evaluated, same can be incorporated in the diet of farmed fish to enhance the immunity.

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