EVALUATION OF HERBAL EXTRACTS OF ARJUNA, *TERMINALIA ARJUNA* ON GROWTH, IMMUNE RESPONSES AND DISEASE RESISTANCE IN *LABEO ROHITA* (HAM, 1822)

Thesis submitted in partial fulfillment
of the requirements
for the degree of

Ph.D. (Fish Nutrition and Feed Technology)

by

Dharmendra Kumar Meena, M. F. Sc.
(FNFT-PA6-04)

ICAR-CENTRAL INSTITUTE OF FISHERIES EDUCATION
(University Established Under Section 3 of UGC Act, 1956)
Deemed- to- be University

Panch Marg, Off Yari Road, Versova,
Andheri (W), Mumbai – 400 061

AUGUST 2021

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Dharmendra Kumar Meena, 2021. Evaluation of herbal extracts of Arjuna, *Terminalia arjuna* on growth, immune responses and disease resistance in *Labeo rohita* (HAM, 1822). PhD Thesis, ICAR-Central Institute of Fisheries Education (University Established Under Section 3 of UGC Act 1956), Panch Marg, Off Yari Road, Versova, Andheri (W), Mumbai-400 061.

Dedicated To……

…….. My Family

Dated: December 2021

CERTIFICATE

Certified that the thesis entitled "**EVALUATION OF HERBAL EXTRACTS OF ARJUNA, *TERMINALIA ARJUNA* ON GROWTH, IMMUNE RESPONSES AND DISEASE RESISTANCE IN *LABEO ROHITA* (HAM, 1822)"** is a bonafide record of independent research work carried out by **Mr. Dharmendra Kumar Meena** during the period of study from September, 2016 to August, 2021 under our supervision and guidance for the degree of **Doctor of Philosophy (Fish Nutrition and Feed Technology)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ICAR-CIFRI, Kolkata-120

(A. K. SAHOO)
Senior Scientist,
REF Division
ICAR-CIFRI Kolkata-120

DECLARATION

I hereby declare that the thesis entitled "**EVALUATION OF HERBAL EXTRACTS OF ARJUNA, *TERMINALIA ARJUNA* ON GROWTH, IMMUNE RESPONSES AND DISEASE RESISTANCE IN *LABEO ROHITA* (HAM, 1822)"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

Date: 6 December 2021 **(Dharmendra Kumar Meena)**

Place: Mumbai PhD Student, FNFT

ICAR-CIFE, Mumbai

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Date: 6.12.2021
Place: Mumbai (Dharmendra Kumar Meena)

सार

वर्तमान अध्ययन में टर्मिनेलिया अर्जुना (अर्जुन) के विलायक अर्कों का लेबिया रोहिता में वृद्धि, रोग प्रतिरोधक क्षमता और प्रतिरक्षा प्रतिक्रियाओं पर प्रभावों की जांच की। इथेनाल छाल अर्क ने 17 जीवाणु उपभेदों, एक कवक, एफेनोमाइसीज इनवेडेन्स और एक परजीवी अर्गुलस बेंगालेंसिस, इसके बाद मेथेनालिक छाल के अर्क > एसीटॉन छाल अर्क > मेथेनॉल फल अर्क और इथेनॉल पत्ती अर्क ने प्रभाव दिखाया। गैलिक एसिड (m/z 170.028 g mL^{-1}) और एलैजिक एसिड (m/z 302.006 g mL^{-1}) को विलायक अर्क में एक प्रभावी एंटीऑक्सीडेंट के रूप में पाया गया। मायरेसिटीन और क्यूरसिटीन पत्ती और छाल के अर्क में पाये जाने वाले फ्लेवेनॉयड थे। दोनों, इनडोर आहार परीक्षण और आउटडोर आहार परीक्षणों ने अर्जुन छाल पाउडर (10 ग्राम/किलोग्राम) ने वृद्धि रोग प्रतिरोध और प्रतिरक्षा प्रतिक्रिया में आशाजनक परिणाम दिए। इंट्रापेरिटोरिनियल इनोक्यूलेशन प्रयोग ने सर्वोत्तम परिणाम 120 माइक्रो ग्राम/मिलीलिटर प्रति मछली में दिये। इनडोर आहार परीक्षण (90 दिन) तथा आउटडोर तालाब आहार परीक्षण (60 दिन) के दौरान अधिकतम वजन बढ़ने का प्रतिशत क्रमश: 148.41±0.854 और 134.51±3.31 था जबकि इंट्रापेरिटोनियल इनोक्यूलेशन प्रयोग (30 दिन) के तहत 30 दिनों के समय अंतराल की तुलना में पहले 15 दिनों में अधिकतम वजन प्रतिशत दर्ज किया गया था। इनडोर और इंट्रापेरिटोनियल इनोक्यूलेशन परीक्षणों में जीनों की अभिव्यक्ति का क्रम इस प्रकार देखा गया था- Mx>ISG15>STAT1। हालांकि संक्रमण के बाद, इनडोर आहार परीक्षण और इंट्रापेरिटोनियल इनोक्यूलेशन परीक्षणों में Mx तथा STAT1 का बढ़ा हुआ स्तर देखा गया। पांचन एंजाइम, विभिन्न सीरम पैरामीटर, ऑक्सीडेटिव तनाव एंजाइम और रक्त-जैव रासायनिक सूचकांक टीएवीपी और इसके अर्क के श्रेणीबद्ध स्तर के अनुरूप भिन्न थे, और विकास अध्ययनों के समान प्रवृत्ति का पालन करते है। इनडोर आहार परीक्षण तथा आउट डोर तालाब आहार परीक्षण में टीएवीपी की इस्टतम खुराक क्रमश: 7.9 ग्राम/किलोग्राम और 8.5 ग्राम/किलोग्राम पायी गयी। महत्वपूर्ण अंगों में हिस्टो-आर्किटेक्चरल परिवर्तन आहार परीक्षण के परिणामों से मेल खाते है। गट माइक्रोबायोम अध्ययनों में भी इस बात के पुख्ता सबूत मिले की टीएवीपी का गट माइक्रोबायोम पर सकारात्मक प्रभाव पड़ा जो कि नॉर्मोबायोसिस की परिकल्पना को प्रदर्शित करता है। फलस्वरूप, वर्तमान अध्ययन से पता चलता है कि टीएवीपी को एक प्रभावी आहारपूरक के रूप में उपयोग किया जा सकता है। इसके अलावा, इथेनॉलिक और मिथेनॉलिक छाल के अर्कों को लेबियो रोहिता के लिए सुरक्षित पाया गया और इसे मछली रोगजनकों के खिलाफ संभावित जैव कीटनाशकों को विकसित करने के लिए इस्तेमाल किया जा सकता है।

ABSTRACT

The current study investigated the effects of a solvent extract of *Terminalia arjuna* on growth, disease resistance, and immune responses in *Labeo rohita*. The ethanolic bark extract demonstrated broad spectrum antimicrobial activity against 17 different bacterial strains, as well as a fungal strain, *Aphanomyces invadans*, and a parasite, *Argulus bengalensis*, followed by methanolic bark extract> acetone bark extract > methanol fruit extract and ethanol leaf extract. Gallic acid (m/z 170.0208 g mol^{-1}) and Ellagic acid (m/z 302.0063 g mol^{-1}) were discovered as potential antioxidants in solvent extracts. Myrecetin and quercitin were common flavonoids found in leaf and bark extracts. In both indoor and outdoor pond feeding trials, dietary *T. arjuna* bark powder (TABP) at 10 g kg^{-1} feed yielded promising results in terms of growth, disease resistance, and immune responses. The intraperitoneal inoculation experiment (120 µg mL^{-1} per fish) yielded the best results. The maximum weight gain percentage during the indoor feed trial (90 days) and the pond feed trial (60 days) were 148.41±0.854 and 134.51±3.31, respectively. While in intraperitoneal inoculation experiment (30 days) maximum weight gain percentage was recorded in first 15 days as compared to 30 days' time interval. Mx (MX Dynamin like GTPase 1) had the highest level of real time expression in the indoor feed trial, followed by ISG15 (Interferon-stimulated gene 15) and STAT1 (Signal Transducer And Activator of Transcription 1), and the same trend was seen in the intraperitoneal inoculation experiment. However, after infection, an increased level of Mx and STAT1 was observed in the indoor feed trial and intraperitoneal inoculation experiment. Digestive enzymes, serum parameters of various functions, oxidative stress enzymes, and hemato-biochemical indices differed according to the graded level of TABP and its extracts, and followed the same trend as in growth studies. The optimal dose of TABP in both the indoor and outdoor pond feed trials was found to be 7.9 g kg^{-1} and 8.5 g kg^{-1}, respectively. The histoarchitectural changes in vital organs matched the feed trial results. Gut microbiome studies have also found strong evidence that TABP has a positive impact on the gut microbiota, lending support to the normobiosis hypothesis. As a result, the current study suggests that TABP can be used effectively as a feed supplement. Furthermore, ethanolic bark and methanolic bark extracts have been found to be safe for hosts and could be used to develop bio-pesticides against potential fish pathogens.

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AB: Arjuna Bark powder; **AF:** Arjuna Fruit powder; **AL:** Arjuna leaf powder

L1: Hexane extract of arjuna leaf; **L2:** Ethyl acetate extract of arjuna leaf; **L3:** Chloroform extract of arjuna leaf; **L4:** Acetone extract of arjuna leaf; **L5:** Ethanol extract of arjuna leaf; **L6:** Methanol extract of arjuna leaf; **L7:** Distilled water extract of arjuna leaf.

Br1: Hexane extract of arjuna bark; **Br2:** Ethyl acetate extract of arjuna bark; **Br3:** Chloroform extract of arjuna bark; **Br4:** Acetone extract of arjuna bark; **Br5:** Ethanol extract of arjuna bark; **Br6:** Methanol extract of arjuna bark; **Br7:** Distilled water extract of arjuna bark.

F1: Hexane extract of arjuna fruit; **F2:** Ethyl acetate extract of arjuna fruit; **F3:** Chloroform extract of arjuna fruit; **F4:** Acetone extract of arjuna fruit; **F5:** Ethanol extract of arjuna fruit; **F6:** Methanol extract of arjuna fruit; **F7:** Distilled water extract of arjuna fruit.

RPS: Relative percentage survival; **TI:** Therapeutic index; **LC50:** Mean lethal concentration; **EC-50:** Mean effective concentration; **TAAB:** *Terminalia arjuna* acetone bark extract; **TAAF:** *Terminalia arjuna* acetone fruit extract; **TAEB:** *Terminalia arjuna* ethanol bark extract; **TAMB:** *Terminalia arjuna* methanol bark extract; **AE:** Anti-parasitic efficacy; **DO:** Dissolved oxygen; **Suffix IM:** Immersion treatment; **Suffix BA:** Baath treatment; **Suffix IN:** *In-vitro* condition; **10, 20, 30, 40 and 50:** stands for concentration in ppm

APHA: American public health association; **DPPH:** 2, 2-diphenyl-1-picrylhydrazyl; **FCR:** Feed conversion ratio; **SGR:** Specific growth rate; **PER:** Protein efficiency ratio; **DO:** Dissolved oxygen; **CP:** Crude protein; **CL:** Crude lipid; **TABP:**

Terminalia arjuna bark powder; **CT:** Control feed at 0.0 % TABP; **T1:** Feed at 0.1 % TABP**;** **T2:** Feed at 1 % TABP; **T3:** Feed at 1.5 % TABP; **WG (%):** Weight gain percentage; **AWG (g):** Average weight gain; FCR- Feed conversion ratio; **FER:** Feed efficiency ratio; **SGR(% day-1):** Specific growth rate; PER- Protein efficiency ratio; **PI (g/fish):** Protein intake; **ANPU:** Apparent net protein utilization; **FI (g/fish):** Feed intake; **FTI (g/fish):** Fat intake; **FR (%):** Fat retention; **EI:** Energy Intake; **ER (%):** Energy retention; **SR (%):** Survival percentage; **GaSI (%):** Gonadosomatic index; **CSI (%):** Craniosomatic index; **HSI (%):** Hepatosomatic index; **IPF (%):** Intraperitoneal fat; **DORB:** De-oiled rice bran; **BHT:** Butylated hydroxytoluene; **GNOC:** Ground nut oil cake; **SOC Meal:** Soybean meal; **MOC:** Mustard oil cake; **FAO:** Food and agricultural organization

BDS1: DMSO hexane bark extract; **BDS2:** DMSO Ethyl acetate bark extract; **BDS3:** DMSO Chloroform bark extract; **BDS4:** DMSO Acetone bark extract; **BDS5**: DMSO Ethanol bark extract; **BDS6:** DMSO Methanol bark extract; **BDS7:** DMSO Distilled water bark extract; **FDS1:** DMSO hexane fruit extract; **FDS2:** DMSO Ethyl acetate fruit extract; **FDS3:** DMSO: Chloroform Fruit extract; **FDS4:** DMSO acetone fruit extract; **FDS5:** DMSO Ethanol fruit extract; **FDS6:** DMSO Methanol Fruit extract; **FDS7:** DMSO Distilled water fruit extract; **LDS1:** DMSO Hexane leaf extract; **LDS2:** DMSO Ethyl acetate leaf extract; **LDS3:** DMSO Chloroform leaf extract; **LDS4:** DMSO Acetone leaf extract; **LDS5:** DMSO Ethanol leaf extract; **LDS6:** DMSO Methanol leaf extract; **LDS7:** DMSO Distilled water leaf extract; **DMSO:** Dimethyl sulphoxide; **WHO:** World health organization; **Hex:** Hexane; **Etac:** Ethyl acetate; **Chlo:** Chloroform; **Acet:** Acetone: **Etoh:** Ethanol: **Meoh:** Methanol; **Dw:** Distilled water.

FRAP: Ferric Reducing Antioxidant Power; **ABTS:** 2, 2'-azino-bis,3-ethylbenzothiazoline-6-sulphonic acid; **DPPH-2:** 2-Diphenyl-1-picrylhydrazyl**; NO Scav:** Nitrous Oxide Scavenging; **TPC:** Total Phenolic Content; **TFC:** Total Flavonoids Content; **ROS:** Reactive Oxygen Species; RNS- Reactive Nitrogen Species; THF- Tetra Hydro Furan; Rf- Relative Front; RQ- Relative quantity, TLC-Thin layer chromatography, FT-IR- Fourier transform infrared spectroscopy; UV-VIS-Ultraviolet-visible spectroscopy; HRLCMS-High resolution liquid chromatography mass spectroscopy.

RBC: Red blood cell; **WBC:** White blood cell; **Hg:** Hemoglobin; **HCT:** Hematocrit; **MCV:** Mean Corpuscular Volume; **MCH:** Mean Corpuscular Hemoglobin; **MCHC:** Mean Corpuscular Hemoglobin Concentration; **PLT:** Platelets; **NBT:** Nitroblue tetrazolium; **Bact:** Bactericidal activity; **Lys:** Lysozyme activity; **PRO:** Protein; **ALB:** Albumin; **GLOB:** Globulin.

LIP: Lipase; **AMY**: Amylase, **PROT**: Protease; **SOD:** Superoxide dismutase; **CAT:** Catalase; **GST:** Glutathione-s-transferase; **AchE:** Acetyl choline esterase; **LDH:** Lactate dehydrogenase; **CKNac:** Creatine kinase; **Trig:** Triglycerides; **Creat:** Creatinine; **BIT:** Bilirubin total; **CHOL:** Cholesterol; **MPR:** Microprotein; **GLU:** Glucose; **GGT:** Gamma glutamyl transferase; **SGPTD:** Serum glutamic pyruvic transaminase; **SGOTD:** Serum glutamic oxaloacetic transaminase; **ALPU:** Alkaline phosphatase; **STAT1:** Signal transducer and activator of transcription; **ISG15:** Interferon stimulated gene.

RNA-M: RNA of muscle; **RNA-L:** RNA of liver; **RNA-K:** RNA of kidney; **RNA-I:** RNA of intestine**; RNA-B:** RNA of brain; **DNA-M:** DNA of muscle; **DNA-L:** DNA of liver; **DNA-K:** DNA of kidney; **DNA-I:** DNA of intestine; **DNA-B:** DNA of brain; **RDM:** DNA/RNA of muscle; **RDL:** DNA/RNA of liver; **RDK:** DNA/RNA of kidney; **RDI-I:** DNA/RNA of intestine; **RDB:** DNA/RNA of brain.

Ah: *Aeromonas hydrophila*; **Et:** *Edwardsiella tarda*

Appendix II

DRAGENOORFF'S REAGENT

• **Stock solution:** mix bismuth subnitrate (oxynitrate; 1.7 g) with water (80 ml) and glacial acetic acid (20 ml). Add potassium iodide solution (50 % w/v, 100 ml). Shake or stir until dissolved. Solution keeps indefinitely when stored in a dark bottle.

• **Working solution:** mix the stock solution with glacial acetic acid (200 ml) and make up to volume to 1 litre with distilled water. Keeps for 2-5 months when stored in a dark bottle.

MAYER'S REAGENT

Dissolve mercuric chloride (1.3 g) and 5.0g KI (potassium iodide) in distilled water (100 ml).

1. INTRODUCTION

Globally, aquaculture is considered as widely growing food production system, supplying nutrition and livelihood security to a large population. When compared to other animal proteins, fish protein is considered to be more easily digestible and less expensive (FAO, 2020). Indian major carps account for 60-70% of total fish production, with *Labeo rohita* being the major contributor to the total production. Due to its compatibility with other species and consumer preferences, the fish *L. rohita* plays an important role in six species composite aquaculture in South-East Asian countries including India. Current fish production is insufficient to meet the rising demand, with global per capita fish consumption hovering around 20.3 kg.

The strategies for increasing fish production as a prime demand include deploying new culture technologies in reservoirs and wetlands alongside existing culture practices, which are dependent on several factors such as scientific farming, quality feed, seed, and effective disease management measures. Intensification of aquaculture with high stocking density that makes fish more susceptible to various diseases, resulting in increased mortality rates and decreased fish production competence, which leads to a huge monetary loss to the fishers. The most common disease causative agents are, fungi, bacteria and viruses. The significant bacterial species are, *Aeromonas hydrophila*, *A. salmonicida*, *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa*, *Flavobacterium columnare, Edwardsiella tarda, Vibrio alginolyticus and V. parahaemolyticus, Streptococcus* (Das et al., 2020).

Among the pathogenic bacteria, *A. hydrophila* causes significant mortality in carps including *L. rohita* (Palanikani et al., 2018). As a result, disease outbreaks in aquaculture are impeding both economic and social development. In practice, it is difficult to estimate the monetary loss caused by disease outbreak problems however, Klesius et al. (2008) reported that 10-15% of the total value of fish production worldwide is lost due to different diseases. In India, for example, Andhra Pradesh shares a major chunk of 40,000 tons of major carps, worth 600 million rupees per year, but the loss due to disease outbreak is estimated to be

Rs. 40 million. Among the fungal diseases, Saprolegniosis is regarded as leading factor of economic degradation in aquaculture (Meyer, 1991) and hold second rank after bacterial disease caused in terms of economic importance.

The conventional approach to disease treatment is antibiotics, and the irrational use of chemicals and substances for growth promoters and immunity boosters has raised concerns about the expansion of disease resilient strains of bacteria and the presence of noxious chemicals in fish flesh. Antibiotic residues in fish flesh are a major source of concern when it comes to human consumption. Furthermore, antibiotics not only affect pathogenic bacteria but also beneficial and mineralizing microbiota, resulting in the loss of beneficial flora, which are responsible for harbouring and recycling useful nutrients from sediments. Vaccination, in addition to antibiotics, is proved to be an efficient prophylactic process for monitoring fish diseases, but it is comparatively expensive and pathogen-specific, and managing it to a huge number of fishes is tough (Ma et al., 2019). Because of these constraints, researchers set out to find an alternative that is less expensive, more easily applicable with health benefits.

Herbal products including ethno-medicinal plants are used for overcoming of communal contagious diseases since prehistoric periods (Rios and Recio, 2005), and medicinal plants have emerged as a potential alternative to obnoxious chemicals and antibiotics used in aquaculture (Stratev et al., 2018). The search for biologically active extracts based on traditionally used plants is still ongoing (Fierascu et al., 2020). This approach has also resulted in the discovery of sources rich in antibiotic and antibacterial compounds (Gorniak et al., 2019). Secondary metabolites such as volatile oils, phenolics (polysaccharides, tannins, saponins, alkaloids, and polypeptides) have been reported to be operative antibiotic alternatives (Othman et al., 2019). Bibliographic searches revealed that some researchers investigated the effects of herbal materials as a growth promoter, immunity booster, stress reliever, and health supplement in *L. rohita* (Shakya, 2017; Ali et al., 2020).

Herbs can act as immunostimulants, activating innate defense machineries in fish and increasing the adaptive immune system. Recently, there has been a surge in using the herbs as immunostimulants in aquaculture (Ngo, 2015). The

advantages of herbal materials over their synthetic or formulated counterparts, according to Stephen et al. (2007), are as follows: 1) a total of 90% consumption by the culture animal; 2) offers many phyto-chemicals in a multifaceted manner that yields a synergistic outcome; 3) not reported to cause harm to the biological system; 4) easy enzymatic digestion and breaks down; and 5) a small number of residues formed (EUS). Furthermore, numerous studies have shown that herbal additives promote fish growth and protect against disease (Rao et al., 2006; Sahu et al., 2007a & b; Muniruzzaman and Chowdhury, 2008; Sahu et al., 2008).

Green growth, green labels, aquaponics, and green fish are some of the new terms that have been coined to describe either healthy fish or associated products. Nowadays, the concept of producing healthy fish, free of noxious traces of chemicals, using herbal material called "Green fish" in the line of organic crop products, has entered the picture and is rapidly gaining popularity around the world. This type of product is gaining popularity because of its health benefits, and it harnesses the medicinal benefits of ethno-medicinal properties in the form of dietary products. Aquaponics is another method for producing healthy fish by effectively utilizing fish wastes by the plant (Dutta, 2015). The use of herbs and ethnomedicinal plants is essential for producing this type of end product.

Terminalia arjuna, also known as arjuna, is a plant native to India. Arjuna tree bark is used medicinally in humans for a variety of health benefits. Secondary metabolites known as bioactive compounds mediate the health benefits. Although a detailed study on solvent extract and fractions has not been conducted exhaustively, the bioactive compounds have been categorized in relation to plant parts. Nugroho et al. (2016) has studied the effects of leaf extract of *Terminalia catappa* on water quality, blood profile and survival of *Betta* species. The study revealed that immersing *T. catappa* leaf extract beyond 375 ppm is advantageous in enhancing the survival, WBC, RBC and Hg of *Betta* species. Amalraj and Gopi (2016) discovered that *T. arjuna* has antioxidant, hypotensive, antiatherogenic, anti-inflammatory, anticarcinogenic, antimutagenic, and gastroprotective properties in humans. In recent past, Aly et al. (2016) investigated the role of some probiotics like Vet-YeastTM and Organic GreenTM) and immunostimulants such as garlic, Echinacea in enhancing the response towards vaccine and immunity in

overwintered (juveniles) tilapia. Importance of herbal plants and their different forms in aquaculture (tilapia) for enhancing growth, immune system, resistance to diseases and utilization of feed is well mentioned (Kuebutornye and Abarike, 2020). Diab et al. (2008) also suggested the positive effects of the black seed, garlic and commercial Biogen on artificial infection with *Pseudomonas fluorescens* in Nile tilapia, *Oreochromis niloticus*.

Nutrigenomics is an emerging area to study the dietary gene interaction of such herbal extracts in fish. Moreover, a comparative study and detailed information for evaluating the health beneficial properties of these plant extracts on fish are deficient. As a result, the current study was designed to generate detailed information about the effects of herbal extracts on fish, with the following goals in mind.

Objectives

I. *In-vitro* screening of *T. arjuna* extracts for its anti-microbial and anti-oxidant properties
II. To evaluate the effect of herbal extracts of *T. arjuna* on the growth and survival of *Labeo rohita*
III. To evaluate the immune-modulating properties of *T. arjuna* in *Labeo rohita*

2. REVIEW OF LITERATURE

2.1. Inland fish production

2.1.1. Fish production and outbreaks of disease

Fish has been considered as an easily available, cheaper means of animal protein globally, representing 17% of total protein (animal) used and 7% of entire proteins (Fish site, 2020). Aquaculture forms a major share in total fish production and has received a great stride as a growing food production sector across the globe. From 2016 onwards, aquaculture has become an avenue, that provides fish for human consumption in most of the least developing and middle developing countries (FAO, 2020).

A dramatic rise in production was recorded by 527% from 1990 to 2018, which stressed upon the significance of aquaculture in years to come (FAO, 2020). In Asian subcontinents including India, the annual population growth rate is more than that of the annual growth rate of fish production. Therefore, it is imperative to intensify the culture practices. Intensification has led to many environmental issues and subsequently, offers a platform for disease outbreaks in culture systems.

In, India 10-15% economic loss was observed due to disease outbreaks mostly parasitic infestation (Mishra et al., 2018). Kibenge et al. (2012), estimated the virulency of different microbial organism with maximum by bacteria (55%) and minimum by fungi (3%) in aquaculture operation. Others such as viruses (23%) and parasites (19%) are also equally virulent. Among the bacterial pathogens, *A. hydrophila, Pseudomonas* spp*., Edwardsiella tarda, Vibrios* Spp., and *Flavobacterium columnare* are frequently associated with diseases in fish, which in turn to heavy mortalities both in cultured and wild fish (Shotts and Bullock, 1975; Menasveta, 1985).

Kumar et al. (1986) isolated both gram-negative and gram-positive bacterial strains from the diseased fish sample and recorded, *A. hydrophila*, *E.*

tarda, *Arthrobacter* spp. and *Clostridium* spp. As predominant species, a large number of gram-negative strains were isolated such as *Aeromonas, Proteus, Citrobacter, Pseudomonas, Flavobacterium*, and *Chromobacterium*, which were potentially pathogenic to *Cyprinus carpio* and *Salmo gardneri* fish fingerlings (Farkas, 1984). There have been many studies on *A. hydrophila* infection in carps (Supriyadi, 1986; Mukherjee et al., 1991).

2.2. Microbial pathogens

2.2.1. Bacterial pathogens

2.2.1.1. *Aeromonas hydrophila*

Aeromonas, is one of the deadly gram-negative bacterium which is widely distributed in aquatic environments and is responsible for a variety of human and animal diseases (Janda and Abbott, 2010). *A. hydrophila* is a naturally occurring aquatic organism that is responsible for a variety of diseases that have resulted in significant losses in numerous fish species including both freshwater (Rahman et al., 2004) and marine (Lilley et al., 1997b).

Aeromonas species is a deadly pathogen for various aquatic species (Carriero et al., 2016; Ni et al., 2016; Yang et al., 2016) and causes serious mortality at large-scale in culture operation of ornamental fishes (Abdi et al., 2014; Harikrishnan et al., 2015). A serious disease outbreak was reported in red hybrid tilapia (Musthafa et al., 2016) *rohu* (Das et al., 2015) and goldfish (Anusha et al., 2014; Sahoo et al., 2016).

Motile *Aeromonas* species are also causative agents in a variety of fish septicemias (Joseph and Carnahan, 1994) and leads to hemorrhagic septicemia in cultured and wild freshwater fish, *Oncorhynchus mykiss*, *Salmo trutta*, *Oncorhynchus kisutch*, *Plecoglossus altivelis*, *Ictalurus tilapia*, goldfish, eel, and carp (Aoki, 1999; Austin and Austin, 1999; Nielsen et al., 2001). It was also associated with epizootic ulcerative syndrome in South-East Asia, including Thailand and India (Llobrera and Gacutan, 1987; Nayak et al., 1999).

2.2.1.2. *Edwardsiella tarda*

The *E. tarda*, leading to disease Edwardsiellosis which is short, gram-negative bacterium (diameter =1 pm and length=2-3 pm) and rod-shaped motile bacterium that targets marine as well as freshwater fish (Thune et al., 1993, Maiti et al., 2009). Initially, *E. tarda* was identified as *Paracolobactrum anguillimortiferum*, which was linked with disease of *Anguilla japonica* termed as Japanese eel red (Hoshina, 1962). Later, this was observed in different fish culture *i.e.* mullet (Kusuda et al., 1976), red sea bream (Yasunaga et al., 1982), Asian catfish (Alcaide et al., 2006), channel catfish (Meyer and Bullock, 1973), Japanese flounder (Nakatsugawa, 1983), Chinook salmon (Amandi et al., 1982) and turbot (Nougayrede et al., 1994).

Edwardsiellosis is considered as the most serious threat in Indian major carps (Sahoo and Mukherjee, 2002). Edwardsiellosis is a subacute to chronic septicemic disease that infects fish such as channel catfish, carp, chinook salmon, eels, mullet, flounder, tilapia and striped bass that are commercially important and causes extensive lesions in the skin, muscle, and internal organs (CAB International 2006; Park et al., 2012). The bacterium is causing economic harm to the aquaculture industry in the majority of the world (Xu and Zhang, 2014). Sahoo et al. (1998) isolated *E. tarda* from Koi, *Anabas testudineus*, and studied the infectivity pattern and pathology (Mohanty and Sahoo, 2007). It was also isolated from *O. punctatus* after examining healthy fish from ponds and fish markets in Uttar Pradesh (Kumar et al., 2007).

2.2.2. Fungal pathogens

Among all the fungal pathogens, *Aphynomyces invadans* is the most dreaded one that causing mass mortalities in the major carps and other wild freshwater and estuarine fish species (Lilley and Roberts, 1997a). The causative agent of EUS was unknown but recently based on some speculations, it could be due to a combination of bacteria and viruses. It was reported that this is due to the fungus *A*. *invadans* (Callinan et al., 1995; Baldock et al., 2005). The *A. invadans* has been recognized as the responsible factor Epizootic Ulcerative Syndrome

(EUS). For the first time, EUS was reported from Japan in 1971 (Egusa and Masuda, 1971) and later spread to different continents of the world such as, South African, North America, Australia, and Asia (Iberahim et al., 2018). It has been reported to infect more than 160 fish species including *L. rohita*, and the range of the host is still expanding. Based on the etiological demography and pattern of disease occurrence, it is expected to infect many more species in years to come (Pradhan et al., 2020).

2.2.3. Parasites as fish pathogens

2.2.3.1 Argulosis in fish

Argulus spp., also known as fish lice, is a parasite that causes a significant economic loss of approximately 418.61 US dollars per hectare and reduces 30% net profit/ha/year in carp culture, including mortality and disease (Sahoo et al., 2013). From both wild and culture system, about 129 species of *Argulus* have been described throughout the world (William, 2008), with 14 species from India. Fish, including *L. rohita*, have been reported to be infected by *Argulus* spp. during culture in confined conditions due to their intrinsically sensitive and stressful environment (Sheila et al., 2002, Sahoo et al., 2013).

Except for Antartica, the genus *Argulus* is found all over the world. In India, the genus *Argulus* is represented by 17 species and one subspecies, 14 of which are freshwater dwellers. It has been reported that *L. rohita* had recorded maximum rate of infection 65.71% and intensity (25.86%), while, it was less in *Cyprinus carpio* and lowest in *C. mrigala* (Alom et al., 2019).

2.3. Control measures towards fish pathogen

Chemotherapy is commonly used to treat harmful fish pathogens in many freshwater fishes. Many antibiotics, including enrofloxacin, furazolidone, amoxicillin, erythromycin, and oxytetracycline, have been efficiently used to minimize the risk of diseases in aquaculture species (Agnew and Barnes, 2007). However, chemotherapeutic overdoses lead to mortality and other side effects in

fish (Punitha et al., 2008). As a prophylactic measure in intensive aquaculture systems, utilization of synthetic chemotherapeutants has been criticized due to their adverse effects such as immunosuppression and storage of residue in tissues (Harikrishnan et al., 2009a & b). Indiscriminate applications lead to the expansion of pathogens sturdy to drugs (Smith et al., 1994) and is considered to be unhealthy for both environment and consumers (Abutbul et al., 2004; Cabello, 2006).

Vaccines are proposed as an active alternate to control infection in aquaculture caused by bacterial and virus and to minimize or eliminate the dependency on synthetic chemicals. Vaccination is a highly effective measure for controlling fish and shellfish diseases but it is not cost-effective. Herbal material including extract, ethno-medicinal plants has taken centre stage as an alternative to conventional strategies for disease protection. Herbal material and medicinal plants are known for excellent phytochemical profiling and have proved their efficacy as an alternative prophylactic and control measure in aquaculture (Jana et al., 2018; Stratev et al., 2018; Gabriel et al., 2019; Yilmiz et al., 2019).

2.3.1. Herbal extracts and disease resistance in fish and shellfish

Both feed companies, as well as researchers, have recently shown progress in the utilization of plant materials in animal nutrition. Ethno-medicinal plants and their extracts have been used to regulate disease in aquaculture across the globe, with researchers reporting success in India, Thailand, Mexico, and Japan (Direkbusarakom et al., 1996a & b; Logambal and Michael, 1997). Improvement in innate immune responses (bacteriolytic activity and leukocyte abundance) was enhanced by incorporating some Chinese herbs in the diet of aquaculture species (Chansue et al., 2000).

Many researchers have reported that *O. Sanctum, A. aspera,* Oroglo layer dry, a xanthophyll prepared from Marigolds, *Solanum trilobatum, Azadirachta indica*, *Catharanthus roseus, Rosmarinus officinale*, and *Eclipta alba* extracts improve innate immunity in fishes (Logambal et al., 2000; Venkatalakshmi and

Michael, 2001; Nguyen et al., 2002; Rao et al., 2004, 2006; Rao and Chakrabarti, 2005; Christybapita et al., 2007; Divyagnaneswari et al., 2007; Xie et al., 2008).

Extract of *Polygonum multiflorum* has been shown both scavenging of free radicals (Chen et al., 1999) and antioxidative properties (Chiu et al., 2002). Extract of ginger (*Zingiber officinale*) reported a significant elevation in extracellular properties of phagocytic cells of the blood in Rainbow trout (Dugenci et al., 2003). Trout upon feeding with the extract of mistletoe and nettle showed enhanced synthesis of extracellular superoxide anion (Dugenci et al., 2003).

Abutbul et al. (2004) studied the dietary impact of *R. officinalis* to cure infection by *Streptococcus* in *Oreochromis* sp. Leaf extract of *O. sanctum* contains some phenolics of water-soluble nature and other compounds such as caryophyllene, methyl eugenol and eugenol, they may be used as immunostimulant (Chopra et al., 1956). Extracts of the plant have been applied to treat different diseases such as lymphocystis disease virus (Harikrishnan et al., 2010c), EUS (Campbell et al., 1998), and some other parasites mediated diseases such as, gyrodactylosis, scuticocliates, myxobolasis, argulosis, trichodinosis, and others have been reported in tropical freshwater farmed fishes (Dey, 1997; Harikrishnan et al., 2010a & b).

2.3.2. Antimicrobial activity of plant extracts on fish pathogens

The “Secondary" plant compounds (metabolites) are a diverse group of compounds extracted from plant tissues that include saponins, tannins, glucosides, alkaloids, organic acids, essential oils, and others (Fraenkel, 1959). The use of various herbs in the treatment of bacterial diseases has been proven as safe in organic agriculture, veterinary medicine, and human medicine (Direkbusarakom, 2004). Herbal medicines with antibacterial activity have the potential to be beneficial in aquaculture (Ocapo et al., 1993; Raman, 1996; Ravikumar et al., 2010; Abdul Kader Mydeen and Haniffa, 2011; Asimi and Sahu, 2013).

It has been reported that five Chinese herb extracts have antimicrobial activity against 13 bacterial fish pathogens. These extracts were found to be more toxic to *E. ictaluri* and *A. salmonicida* (Shangliang et al., 1990). Antibacterial properties of some Brazilian, Iranian, and Malayan edible herbs have been used to treat fish pathogenic bacteria (Castro et al., 2008; Najiah et al., 2011; Pirbalouti et al., 2011). Some antimicrobial properties of marine plants, mangroves, and sea grasses has been used against fish pathogens (Dhayanithi et al., 2010; Ravikumar et al., 2011a & b; Arivuselvan et al., 2011). The disc method was used to test 15 plant extracts against three microorganisms, *A. hydrophila, Vibrio harveyi* and *V. parahaemolyticus*. Exposure of *P. monodon* nauplii to very low concentration of 0.025 $mgmL^{-1}$ in *Lawsonia inermis* Linn. extracts, showed about 80% survival rate (Asha et al., 2008).

Emulsified product, 'Aquaneem' prepared from neem, was used in fish against four pathogenic bacteria, namely *Pseudomonas fluorescens, A. hydrophila*, *P. tyxobacteria* spp., and *Escherichia coli*. From the study, it was observed that the growth of these organisms was significantly inhibited at 10 mg L^{-1} of aquaneem to minimise the risk of bacterial disease out breaks in pond culture (Das et al., 1999). Similarly, other researchers have used aquaneem as an effective fish bactericide and as a substitute for chemicals and antibiotics (Sahu et al., 1996; Chitmanat et al., 2005).

AqE from *A. indica* leaves could be used to control infection of *A. hydrophila* in *C. carpio*. The antimicrobial activity of AqE from three herbal plants, *Solanum torvurn* (Sundakai fruit coat), *A. indica* (leaf) and *C. Tonga* (rhizome), was tested *in vitro* against the growth of *A. hydrophila* collected from fresh-water fish, *Channa striatus* infected by the pathogen (Abdul Kader Mydeen and Haniffa, 2011).

By using the good diffusion method, ethanol, chloroform, and acetone extracts of three plants, *Withania somnifera*, *Asparagus racemosus,* and *Mucuna pruriens*, were studied *in-vitro* against four different fish pathogens, *A. hydrophila, P. fluorescens, Vibrio cholera,* and *Klebsiella pneumoniae* (Jameela et al., 2011). At 500 ppm, Indian Almond, *Terminalia catappa*, showed improvement in the survival, improved hematological indices and protection against *A. hydrophila* in

Betta sp. (Nugroho et al., 2017). The efficacy of *T. arjuna* bark and leaf extracts has proved its potential against the bacterial isolates of human health significance (Kumar et al., 2017).

2.3.3. Phytochemicals in fish disease control

Medicinal plants are treasures for exploring novel phytochemicals with therapeutic importance and drug development potential (Kumar et al., 2006). Phytochemical's content certain active compounds that work as antimicrobial towards controlling the pathogenesis activities. Different medicinal plants like *Bidens pilosa* L. (Asteraceae), *Bixa orellana* L. (Bixaceae), *Cochlearia officinalis* L. (Rubiaceae)*, Cecropia peltata* L. (Moraceae), *Justicia secunda* Vahl. (Acanthacea) and *Jacaranda mimosifolia* D. Don (Bignoniaceae) have been studied for their antimicrobial activity (Rojas et al., 2006). In most cases, phenolic compounds were active chemicals in these plants. Plant extracts with different properties are part of folk medicine like antimicrobial activity (Ngwendson et al., 2003), alkaloids (Klausmeyer et al., 2004), essential oils (Alma et al., 2003), triterpenes (Katerere et al., 2003), flavonoids (Sohn et al., 2004), sesquiterpene lactones (Lin et al., 2003), diterpenes.

Antibacterial activity of the arjuna plant's bark and leaves as a whole is well evidenced by previous reports (Kumar et al., 2017; Singh et al., 2018). *T. arjuna's* protective effects towards the fish pathogens have been reported (Meena et al., 2020a). The study provided evidence for the safe and effective use of prospective solvent extracts of *T. arjuna* against *A. bengalensis* in *L. rohita* juveniles, as well as first-hand information on the acute toxicity of solvent extract in *L. rohita.*

2.3.3.1. Flavonoids

Flavonoids are responsible for providing antioxidant properties. Flavonoids are water-soluble phytochemicals and polyphenolic compounds with 15 carbon atoms (Harnafi and Amrani, 2007). They are divided into five major subcategories: anthocyanidins, flavones, flavanones, flavanols and flavonoids (Kuhnan, 1976; Nijveldt et al., 2001). Flavones have a planer symmetry due to the double bond in the middle aromatic rings. One of subgroup, Quercetin, is abundant in apples,

onions, broccoli, and berries. Naringenin which is a citrus fruit, is considered as a potential source of flavanones. Flavonoids derived from medicinal plants have showed an excellent array of antioxidant potential and DNA nicking inhibition activity against free radical-related disease, arteriosclerosis (Vaya et al., 2003).

Flavonoids participate in the foraging reactive oxygen species (ROS) and providing antimicrobial activity against pathogens of human health and livestock and aquaculture (Gorniak et al., 2019)**.** Quercetin is considered as one the potential antioxidant abundantly found in plant kingdom (Nijveldt et al., 2001). The antimicrobial properties of quercetin against bacterial fish pathogens have been reported (Jaisinghani, 2017). The study pointed that quercetin inhibited the growth of *S. aureus* and *Pseudomonas aeruginosa* at 20 µg mL^{-1} and 400 µg mL^{-1}, respectively.

2.3.3.2. Saponins

Saponins are a type of natural soap. Saponins are surface glycosides that are naturally produced by plants, certain bacteria and lower marine creatures (Yoshiki et al., 1998). Saponins are classified into two types: triterpenoid saponins and steroid saponins. Triterpenoid saponins are found in a variety of legumes, including soyabean, beans, peas, lurcene, and others. Many studies on the biological significance of saponin have been published. Antimicrobial properties of saponin have a lytic effect on erythrocyte membrane (Francis et al., 2002).

Levels of serum cholesterol were lowered by saponins in some of the animal studies (Al-Habori and Raman, 1998; Matsuura, 2001). The uncontrolled utilization or overdose of saponins has been reported to intensify such as haemorrhagic septicemia (red sore disease), infectious dropsy, rubella, ulceration, exophthalmia, abdominal distension, tail rot, fin rot, and scale protrusion, which have resulted in significant losses in the aquaculture industry.

Saponin causes motile aeromonad septicemia (MAS), bacterial haemorrhagic septicemia (BHS), and epizootic ulcerative syndrome in a variety of marine (Lilley et al., 1997b) and freshwater types (Rahman et al., 2004). Stratev et

al. (2018) reported that phytochemicals including saponins are known to enhance specific immune responses, globulin and Innate defense responses *i.e* lysozyme activity, NBT and phagocytic activity, in fish species of aquaculture importance.

2.3.3.3. Alkaloids

Alkaloids compounds are containing nitrogen. They are obtained by plants, microbes and marine organisms by a complex biosynthetic pathway and more than 12,000 structures have been explicated from different plants (Wink, 1999 & 2003; Verpoorte and Memelink, 2002;). Alkaloids are also abundantly available in the plant kingdom and reported as one of the major constituents of a plant leaf. Alkaloids are the responsible bioactive principles for the credence of antimicrobial effects of most plants (Windisch et al., 2008).

Alkaloids are known to provide antibacterial, antibiotics enhancing and anti-virulence properties (Thawabteh et al., 2019). Further, Srichaiyo et al. (2020) has evaluated the dietary (0, 5, 10 and 20 g kg^{-1}) effects of gotu kola (*Centella asiatica,* as a potential source of alkaloids) on immunological parameters in Nile tilapia.

The study pointed that fish fed with 5 g kg^{-1} demonstrated significantly elevated lysozyme activity and mucus peroxidase activity as compared with control. Similarly, Reverter et al. (2017) reported that phytochemical including alkaloids are primarily responsible for immunomodulation in aquaculture fish species.

2.3.3.4. Tannins

Tannins are polyphenolic compounds derived from plants that are divided into two types: hydrolysable (which are polyesters of gallic acids and different individual sugars) and condensed tannins (Mc Sweeney et al., 2001). Ellagic and gallic acid are tannins that can be hydrolysed and are further divided into gallo tannins that produce gallic acid and glucose, while ellagic tannins produce ellagic acid and glucose (Haslam, 1989). Prusty et al. (2007) investigated the dietary (0, 2.5, 5, 10, 15 and 20 g kg^{-1} tannins on hemato-immunological parameters in *L.*

rohita. The results summarized those humoral responses such as NBT, total leucocyte count and lysozyme activity enhanced remarkably in tannin-fed treatment as compared with control.

Soltanian and Fereidouni (2016) highlighted the potential immunomodulatory effects of Henna (*Lawsonia inermis*) extract tannin as a major phytochemical on *C. carpio.* The study revealed that extract at 60 and 600 mg kg^{-1} body weight could considerably ($p<0.05$) elevate the range of parameters of innate immune system i.e. total leucocyte count, increase in a number of neutrophils, monocytes and phagocytes and enhanced humoral immune responses, NBT, lysozyme activity and bactericidal activity and showed significant protection towards *A. hydrophila.*

Penga et al. (2020) assessed the effects of incorporation of dietary condensed tannin on immune response and oxidative stress in *Lateolabrax japonicus.* The study suggested an improved SOD activity and enhanced immune response as compared to control. The condensed tannins are reported to possess potent antioxidant and immunomodulatory effects hence recognized as a prospective substitute to dietary synthetic antimicrobials. Further, condensed tannins was also reported to have antimicrobial activities and sustained immune augmentation capability in fish species (Huang et al., 2018).

2.3.3.5. Cardiac glycosides

Cardiac glycosides are assumed to be a group of natural steroids found in both animals and plants. Cardiac glycosides are reported to possess both antioxidant and immunomodulatory properties (Skubnik et al., 2021). Nahak and Sahu (2014) reported the immune-stimulating effects of dietary aqueous leaf extract of *Ocimum basilicum* in *Clarius batrachus*. The results showed enhanced specific and non-specific immune responses including protein, globulin etc., at 2.5% and 5% extract concentration.

Sutthi et al. (2020) evaluated the dietary (0, 0.25, 0.50 and 0.75 g kg^{-1}) effects of ethanolic extract (mixture of different bioactive constituents including

cardiac glycosides) of *Apium graveolens* on lysozyme activity and disease resistance against bacterial pathogens in *Labeo chrysophekadion* (Bleeker, 1849). The study revealed that 0.75 g kg^{-1} could enhance the disease resistance and lysozyme activity in treatment over the control.

2.4. Purification and extraction of compounds as antimicrobial agent

Isolation of pharmacologically active compounds in pure form from the identified plants is a time-consuming and labour-intensive method. As a result, methods that eliminate unnecessary separation procedures must be applied. Thus, chemical screening is carried out for a preliminary screening of the targeted new or useful constituents with potential activities. Based on the chemical structure and property of a solvent, the extraction capabilities of solvents vary. Other factors involved in solvent selection include density, boiling point, viscosity, surface tension, corrosiveness, toxicity, flammability, compatibility, stability of product, availability and cost (Cowan, 1999).

Many studies have used a variety of solvents in the extraction of plant materials. For the extraction procedure, both single extraction and sequential extraction methods were used. In sequential extraction methods, the solvent was used from lower polarity to higher polarity. For the isolation of antimicrobial principles mediated by antioxidants and polyphenols are extracted and further isolated in high polar solvents (Chemat et al., 2020).

2.4.1. Solvents used for extraction of the compounds

1. Water: Antimicrobial activity is a process that uses water to extract plant products, and water is known as a universal solvent. Traditional water is used as primary base, however, extracts from plant-derived organic solvents have been shown to have more stable antimicrobial action. Furthermore, water-soluble flavonoids (mainly anthocyanins) have shown

no antimicrobial properties, whereas water-soluble phenolics are considered as potential antioxidants (Das et al., 2010).

2. Acetone: Acetone dissolves and is primarily used in antimicrobial studies where extraction of a large number of phenolic compounds is required. According to study, extraction of phenolics including tannins was improvised in aqueous acetone as compared with aqueous methyl alcohol (Eloff, 1998; Das et al., 2010). Saponins are extracted using acetone and methanol (Ncube et al., 2008).
3. Ethanol: Ethanol extracts are very effective at reducing the activity of aqueous extracts. Water forms a good medium for the growth of microorganisms in comparison to ethanol (Lapornik et al., 2005). Aqueous ethanol (70%) fetches better results in terms of extraction of bioactive compounds as compared to absolute ethanol (Bimakr et al., 2011).
4. Chloroform: Chloroform is a less polar solvent which mainly used to extract terpenoid. Terpenoid lactones were extracted from dry barks using chloroform, methanol and hexane, with high activity in the fraction of chloroform. On occasion, terpenoids and tannins were seen in the aqueous phase, but both were extracted using less polar solvents (Cowan, 1999).
5. Ether: Ether is commonly used to selectively extract coumarins and fatty acids (Cowan, 1999).

2.5. Compound characterization

2.5.1. Column chromatography

Column Chromatography is a type of chromatography that uses columns. The purification of crude herbal extracts was accomplished using chromatographic techniques. Chromatography can be either preparative or analytical in nature. Though thin-layer chromatography (TLC) is the most simple and cheap process of detecting plant compounds, hyphenated high-performance liquid chromatographic (HPLC) method is in demand (Marston et al., 1997; Hostettmann et al., 1997). HPLC in a combination of UV photodiode array detector (LC/UV) has been widely

preferred for plant extracts characterization in crude form (Hostettmann et al., 1984).

A newer hyphenated method is HPLC combined with mass spectrometry (LC/MS). Mass spectrometry is a highly sensitive method for molecular analysis that provides data on the molecular weight and structure of the samples. For the HPLC, thermo spray, continuous flow fast atom bombardment, and electro spray were used. These methods are used for the ionization of non-polar compounds (Aglycones, MW around 200) to highly polar protic bioactive principles (Glycosides, MW. 2000). The compound having mass ranged from 200-800 amu, LC/TSP-MS is preferred for complete ionization of medium polar compounds, polyphenols or terpenoids. Further, CF-FAB or ES can be applied for larger-size high polar compounds saponins that possess a molecular weight of more than 800 (Wolfender and Hostettmann, 1995).

Recently, HPLC in combination with NMR spectrometer (LC/NMR) was rarely used due to sensitivity issues. But, advancements in solvent suppression method and pulse field gradients, in conjunction with advances in techniques and the creation of high field magnets, now allow for a characterization of the varied array of compounds.

The LC/NMR has a lot of potential for identifying natural product structures. The NMR spectroscopy is the most powerful technique to obtained detailed information about the assembly of organic compounds existing in the solution (Albert, 1995). It is simple to connect to an HPLC machine and techniques like solvent suppression give an opportunity to use non-denudated solvents like methanol or acetonitrile under different conditions (reversed-phase) in which D20 is used in place of water.

2.5.2. Fourier transformation infra-red spectroscopy (FT-IR)

Fourier transformation infra-red spectroscopy is a technique that enables the non-destructive analysis of biological samples and provides the nature of functional groups of the bioactive compounds/ samples based on chemical

constituents and elucidation of the structural compounds. The molecular bonds with the electric dipole moments can be changed by atomic dislocation due to normal shakings are infra-red active (IR).

In FT-IR, these vibration modes are measured by IT spectroscopy for the quantification and further elucidation of molecular configuration and subtleties without disturbing the samples (Bellisola, 2012). For the biotic sample, the most spectral region would be finger print (600–1,450 cm^{-1}) and the amide I and II (1,500–1,700 cm^{-1}) regions. Larger wave number (2,550–3,500 cm^{-1}) is directly proportional to the stretching of the vibrations *i.e.* S-H, C-H, O-H and N-H (Diem et al., 2004).

On other hand, the lower wave number reflects the bending and carbon skeleton fingerprints vibrations. It is crucial to standardise IR spectra to the explanation for puzzling aspects like sample thickness (Griffiths and Haseth, 2007). The most common normalization methods include Amide I/II peak and vector normalization.

After baseline rectification, amide I/II standardisation is commonly used, whereas vector standardisation is commonly used after spectra distinction. For chemical imaging or unsupervised clustering, leaving spectra non-normalized reveals tissue arrangements mainly based on absorbance intensity, while standardisation highlights alterations in biochemical structure.

Certain form of spectral standardisation is used for diagnosis. IR-microscopy is used for rapid detection and providing the number of spectra which can be chosen by utilizing different scanning methods (Bhargava, 2012; Kole, 2012). Chaudhary et al. (2015) reported a strong band at 3427 cm^{-1} could be due to widening of N-H.

2.5.3. UV-VIS spectroscopy

The UV-Vis provides the peak when the atom gets activated and that peak intensity was measured in UV-VIS. The two maximum can be received in the

solvent extract of high polar solvents but it rely on the type of compound and solvent used (Kalaichelvi and Dhivya, 2017). The spectra of flavonoids compounds have two maxima in the wave range of 230-285 and 300-385. The exact location and relative intensities of these maxima provide an important clue about the nature or group of flavonoid compounds present in the particular sample (Saxena and Saxena, 2012).

2.6. Plant extracts and minimum inhibitory concentration (MIC)

Previous researchers have illustrated the minimum inhibitory concentration (MIC) as the lowermost concentration on which inoculum viability is reduced (Carson et al., 1995). The lowest concentration that is needed to inhibit the completion of the test sample after 48 h incubation (Canillac and Mourey, 2001) or the least dose that impedes noticeable progression of the sample micro-organisms (Delaquis et al., 2002). Different authors used various methods to assess MIC in plant extracts, including turbidimetric methods (Lambert, 2001; Mejlholm and Dalgaard, 2002), broth dilution methods (Marino et al., 2001), gradient plates methods (Ting and Deibel, 1992), and inhibition or kill curves (Ting and Deibel, 1992; Mejlholm and Dalgaard, 2002; Nakamura et al., 2004).

Igbinosa et al. (2009) investigated the minimum inhibitory concentration (MIC) for different crude ethanolic, methanolic, and water extracts of *Jatropha curcas* and discovered that the MIC value of ethanol extract for diverse samples varied from 0.5 - 6.25 mg mL^{-1}, whereas the value for methyl alcohol extract varied from 0.5 to 10 mg mL^{-1}. The MIC for streptomycin control was also described to be varied from 0.065-0.5 $mgmL^{-1}$. The minimum bactericidal activity (MBC) of the extract for various bacteria strains was observed to be from 2.0 to12.50 mg mL^{-1} for the ethyl alcohol extract and between 2.0 to 20 mg mL^{-1} for the methanolic extract.

Aneja et al. (2012) tested *T. arjuna* for antimicrobial activity against bacterial pathogens that cause ear infections, such as *Acinetobacter sp., E. coli*, *S. aureus*, *Proteus mirabilis,* and *P. aeruginosa*. The study found that *S. aureus,*

P. mirabilis, and *Acitenobacter* sp. had MICs of 3.12 mg mL^{-1}, 6.25 mg mL^{-1}, and 25 mg mL^{-1}, respectively.

2.7. Toxicity studies of plant extracts using brine shrimp lethality assay (BSLA)

The plants' products are considered to be safe in most of the cases, however, sometimes their overdose or presence of certain antinutritional factors impose detrimental effects on the health of the test animals (Samtiya et al., 2020). Such plant material, if not tested scientifically for its toxic effects, may exert side effects on the health of the animal. So, it is imperative to screen such material by adopting animal models such as brine shrimp (*Artemia salina*) lethality assay.

The *A. salina* model can efficiently be used for the cytotoxicity of plant materials or any other material that is going to be used as a feed supplement could be assessed. The *A. salina* bioassay was evaluated for its ability to predict the toxicity of extracts of herbal materials by comparing the LC_{50} values of *Artemia salina* with the LD_{50} values of serious toxicity in mice and rat (Sharma et al., 2013; Naidu et al., 2014).

Fatema et al. (2017) studied the toxicity of the methanolic extracts using brine shrimp lethality assay. The results showed that LC_{50} values (0.491 & 12.5 mg L^{-1}) of two fractions of methanolic extracts, evaluated in BSLA, are greater than that LC_{50} (0.781 mg L^{-1}) of its standard vincristine and aqueous fractions.

The brine shrimp lethality assay (BSLA) was used in the current investigation, to assess the *in-vitro* toxicity of *T. arjuna* solvent extracts as well as preliminary selection of solvent extracts. Solvent extracts showed that acetone, chloroform, ethyl acetate, ethyl alcohol, hexane, methyl alcohol and water hexane, had LC_{50} values as, 93.36, 101.75, 118.50, 528.78, 278.32, and 477.67 ppm, respectively. Based on this value they are designated as a medium, highly medium, low medium toxic (Meena et al., 2020b). Thus, the study recommends

that ethanol can be used further for dietary supplements in fish feed without compromising the overall performances and health of the animals.

2.8. Effects of herbal biomedicines on fish immune system

The application of natural immune stimulants may be used to control prevalent fish disease (Siwicki et al., 1989), but due to deficit standard procedure, the research community has still rejected this approach (Ponni, 2002). It is well known fact that herbal items are inexpensive means for therapeutic application and have higher efficiency in comparison to other agents with chemotherapeutic use. They also provide sufficient and accurate solutions to all of the problems that are faced by the aquaculture system. Many plant products with properties such as growth-promotion, immunostimulant, antimicrobial and anti-stress, have a significant impact on the larviculture of shrimp or fish (Citarasu et al., 1998, 2003a & b).

Natural plant products have been used in finfish and shrimp larviculture as aphrodisiacs and antimicrobials (Citarasu et al., 2002; Sivaram et al., 2004). Herbal immunostimulants are a less expensive and more effective alternative to different drugs, antibiotics, and chemicals that are currently used for the treatment of fish and shellfish diseases (Jeney and Jeney, 2002; Hou and Chen, 2005; Yeh et al., 2009a). Immunostimulants like plant extracts, significantly increase phagocytic properties in a variety of fish (Logambal et al., 2000; Venkatalakshmi and Michael, 2001; Chakrabarti and Rao, 2006; Gopalakannan and Arul, 2006).

2.9. Mode of administration of herbal immunostimulants

Immunostimulants can be administered through the oral method, injection (intraperitoneal inoculation), or bathing, where oral administration is the most practical approach (Jeney and Anderson, 1993a & b; Mulero et al., 1998a & b; Sakai, 1999; Yin et al., 2006). Though intraperitoneal injection is the most rapid and effective method for administration, many researchers believe that dietary inclusion is the best option for aquaculture because it is low in cost and effort,

stressless and can be used for a large number of fish (Siwicki et al., 1989; Blazer, 1992; Anderson, 1992; Esteban et al., 2001). Immunostimulants are typically administered intraperitoneally in aquaculture, and this appears as a more effective route of administration compared to oral administration (Ainsworth, 1994; Yoshida et al., 1995; Duncan and Klesius, 1996a & b).

However, the limitation of intraperitoneal inoculation is its time-demanding nature and stressful situation for the fish. Injection administration allowed the immunostimulant to absorb as quickly as possible and show the action, whereas oral administration of immunostimulant makes the fish body to absorbed gradually (Yoshida et al., 1995). Both particulate and soluble compounds of phyto-medicine are applied to treat fish and both can trigger the immune response in a protective manner. Treating goldfish with water-based leaf extract of *A. indica* showed a noticeable enhancement in serum glucose, cholesterol and total protein levels (Harikrishnan et al., 2009b).

Oral administration is a feasible substitute for applying a large amount of medicines to fish of various size (Selvaraj et al., 2005). Oral administration of *C. roseus* extract in *L. rohita* (Nguyen et al., 2002) and glycyrrhizin in *S. quinqueradiata* (Edahiro et al., 1990) boosted disease resistance and immune system against *A. hydrophila* and *E. seriola* on the experimental infection (Matsuo and Miyazano, 1993), whereas glucans enhanced resistance against *V. anguilla* (Raa et al., 1992).

2.10. Dietary feeding and intraperitoneal inoculation of herbal extracts

Dietary supplements and alternative therapies are becoming more popular around the world as people strive to prevent disease and improve their health (Eisenberg et al., 1998; Durante et al., 2001). The regulatory requirements for the sale of dietary supplements are less stringent than those for pharmaceuticals (Marwick, 1995), and efficacy data supporting use is the exception rather than the rule for many of these supplements (Angell and Kassirer, 1998). Dietary

supplements have a broad range of benefits in terms of health. Several reports on the administration of immunostimulants either through feed or orally are available (Siwicki et al., 1989; Ainsworth et al., 1994; Yoshida et al., 1995).

The immunomodulation in *L. rohita* Hamilton (Cyprinidae) juveniles was studied for haemato-immunological fluctuations by both dietary and inoculation study followed by challenge with *A. hydrophila*, and reported promotion in growth and enhanced immunity (Kumar et al., 2007). Balasubramanian et al. (2008) reported feeding of plant extract of *Cynodon dactylon* (Linn.) against infection caused by WSSV in *Penaeus monodon* (Fabricius) showed sufficient preventive activity.

Koo et al. (2001) carried out a research to determine the ideal dose of dietary chlorella powder as potential fed supplement in coherent to better growth of olive flounder, *Paralichthys olivaceus* juveniles. When *Sargassurn fusiforme* polysaccharide extract (SFPSE) was supplemented in the diet of juvenile shrimp, *Fenneropenaeus chinensis*, it was evaluated as a feed additive to enhance growth performances and disease resistance (Huang et al., 2006). The addition of *Achyranthes aspera* seed to the diet increased both resistance and immunity against infection caused by *A. hydrophila* in *L. rohita* (Rao et al., 2006).

Four ethanolic extracts of different herbal medicinal plants (*Ocimurr basilicum, Cinnamomum zeylanicum, Juglans regia*, and *Menthe piperita*) were mixed with artificial feeds and fed to *Cyprinus carpio* to study the immunological effects (Abasali and Mohamod, 2010). The results showed an enhanced synergistic effect on disease resistance of the mixed solvent extract as compared to individual dietary incorporation.

The use of leaf aqueous extract from *Eclipta alba* as a feed supplement improved the majority of the innate defense factors tested in Nile tilapia (Chirstybapita et al., 2007). A chloroform extract of *Nyctanthes arbortristis* seed was also given orally as a feed supplement to *Oreochromis mossambicus* (Peters) at various doses to improve the non-specific immune system parameters (Kirubakaran et al., 2010). Wu et al. (2010) calculated the effects (4 and 8 μgg $^{-1}$)

of hot aqueous extracts of *Toona sinensis* on survival and biochemical parameters in *Oreochromis mossambicus.*

Similarly, other researchers also used herbal extracts in the form of injection for the enhancement of disease resistance and immunomodulation to various aquaculture species (Sudhakaran et al., 2006; Divyagnaneswari et al., 2007; Alexander et al., 2010; Kirubakaran et al., 2016; Kumar et al., 2019).

2.11. Fish hemato-biochemical changes associated with application of herbal products

One of the diagnostic tools for identifying diseases is hematologic evaluation. Clinical hematology can assess normal deviation due to inherent or extrinsic variables, as well as diseases upsetting blood cells and counts. Changes in blood parameters reflect physiological changes in the organism as a result of environmental influences, pathogenic infestation, and various toxic effects. Many factors influence and change these values, including handling (Houston et al., 1971), metabolism (Booke, 1964), age (Tugarina and Ryzhova, 1970), dlot (Smith, 1968), stress (Bouck and Ball, 1966), and pollutants (Sprague, 1971).

The majority of fish have nucleated erythrocytes that impart a critical part in oxygen transport, influenced by the gas exchange mechanism and the amount of haemoglobin in the cell. Fish leucocytes, like those of other vertebrates, are part of defense mechanisms and offer protection against microbial infections (Anjusha et al., 2019). When common carp were fed different concentrations of ethanolic extracts of herbal medicinal plants, their WBC, RBC, and haemoglobin levels increased (Abasali and Mohamod, 2010).

Plasma protein contributes to the maintenance of acid-base balance and osmotic pressure in body fluids. Plasma proteins, as a source of nutrition for tissue proteins, establish a dynamic equilibrium with tissue proteins. When there is a protein deficiency, tissue proteins are broken down to keep the plasma protein level stable (Shanmugam et al., 1977). It has been established that any type of

stress that does not result in gross changes and mortality causes certain changes in the blood characteristics of fish (Christensen et al., 1972).

Abasali and Mohamod (2010) investigated the effect of four ethanolic extracts of different herbal medicinal plants (*O. basilicum, C. zeylanicum, J. regia* and *M. piperita*) on biochemical parameters such as albumin, globulin and total serum protein, when fed to *Cyprinus carpio*. He also reported a decrease in glucose levels with an increase in herbal extract in the diet. Both Rao et al. (2006) and Sahu et al. (2007a) reported increase in the values of albumin, globulin and serum total protein in *L. rohita* juveniles fed *A. aspera* seed and *M. indica* kernel. After feeding various herbal extracts to rainbow trout, the fish's plasma total protein level increased (Dugenci et al., 2003). After being fed herbal immunostimulant diets, the glucose levels of *L. rohita* and black tiger shrimp (*Penaeus monodon*) were reduced (Citarasu et al., 2006; Sahu et al., 2007b).

Role of various sources of green tea as a feed supplement on growth, composition of body and blood chemistry in olive flounder, *Paralichthys olivaceus*, was also determined (Cho et al., 2007). Fish fed *Chlorella* protein at 2.0 g kg^{-1} of feed had remarkable higher serum albumin values than control (Lucia-pavon, 2001). Hsieh et al. (2008) reported that rutin from *Toona sinensis* had beneficial effects in terms of enhancing haemato-biochemical parameters in white shrimp (*Litopenaeus vannamei*) at >10 µg g^{-1} dose following infection with *Vibrio alginolyticus*.

Ojha et al. (2016) evaluated the effects of diets of two herbs, *Mucuna pruriens* and *Pedalium murex* (1:1) with an inclusion level of 0.0 g kg^{-1}, 0.06 g kg^{-1}, 0.08 g kg^{-1} and 0.0 g kg^{-1} feed, fed to *L. rohita*. The study revealed that blood parameters such as packed cell volume (PCV), haemoglobin content (Hg), erythrocytes (RBC) drastically increased in treated groups. A proliferation of WBCs was also observed in treatments as compared with control and also, the enzyme of protein metabolism increased significantly in treated groups.

Suely et al. (2016) evaluated the possible toxic effect of varied doses of ethanol extract of *T. arjuna* in *Heteropneustes fossilis* by immersion method. The

study demonstrated that the extract showed the toxic effect on hematology of fish and the LC_{50} was recorded as follows, 12.7, 8.95, 5.63 and 4.71 at 24, 48, 72 and 96 h, respectively. Kumar et al. (2019) assessed the effects of intraperitoneal injection (10, 20 and 30 ppm) of ethyl alcohol leaf extracts of *Avicennia marina* on blood profiling of *L. rohita.* The result showed an enhanced total serum protein, WBCs, RBCs and Hg content after one month of treatment that indicates the beneficial impact of the extract on hematology in rohu.

2.12. Enzymatic changes associated with herbal product application to fish

2.12.1. Fish digestive enzymatic changes associated with feeding

The success of aquaculture operation is largely depending upon the feed ability to enhance growth, utilization of feed and to prevent disease outbreaks. It has proved that nutrient assimilation and utilization is greatly depending on digestive enzyme activity in aquatic animals (Day et al., 2011). The herbal stimulants have shown their efficacy to increase the activity of digestive enzymes that confirms that herbal products are efficient to alter the activities of the digestive enzymes.

Gabriel et al. (2017) assessed the impacts of dietary *Aloe vera* extract (0.5, 1, 2 and 4%) on digestive enzyme activity in GIFT tilapia. The study suggests that an inclusion level of 1.76, 1.82 and 2.10 %/ kg was found to be best suitable for enhancing the activity of amylase, protease and lipase in GIFT tilapia. The study of Ali and Kaviraj (2018) showed that the inclusion of 25% *I. aquatica* leaf meal fermented with bacteria could enhance the activity of α-amylase in *L. rohita.*

Goswami et al. (2020) studied the combined effects of macrophytes and almond oil cake on digestive enzyme activity in *L. rohita*. The experimental diets were as follows, containing 300 g -g^{-1} protein using plant constituents and fishmeal in a 1:1 blend: *T. catappa* (FTC), duckweed (FLM), water fern (FSM) and combined of these three (FTCLMSM). The results of the study showed that

amylase, trypsin and chymotrypsin activities enhanced noticeably in FLM. FLC and FLM diets significantly increase the protease activities and FTL alone could increase the activity of lipase in *L. rohita*.

Bibliographic studies showed that many researchers recorded better and enhanced digestive enzymes activities upon feeding *L. rohita* and other fish species with herbs, and other plant products. The effect of garlic on digestive enzyme activities in *L. rohita* was estimated by several workers (Jocelyn, 1972; Sheela and Augusti, 1992; Ahmed and Shrama, 1997; Augusti et al., 2001; Ojha et al., 2016).

Levels of digestive enzymatic activities such as, SGOT, ALP and SGPT were investigated through feeding *L. rohita* with *Achyrrmlho aspera* (Rao et al., 2006), rainbow trout, and *Oreochromis nilloticus.* The effect of all green tea sources on fish serum LDL cholesterol and GPT was investigated (Cho et al., 2007). Sahu et al. (2008) investigated the impact of *Curcuma longa* on the enzymatic profiling of *L. rohita*, challenged with *A. hydrophila*, including ALP, SGOT and SGPT. The result showed a better enzyme profile in treated fish as compared to control.

The Akbary et al. (2017) investigated the effects of four ethno-medicinal plants, *Mentha piperita, Matricaria chamomilla, Terminalia chebula* and *Zataria multiflora* on digestive enzyme activity in white leg shrimp. The study revealed a significant increment in activity of lipase, protease, and amylase in treated group as compared with control.

2.12.2. Physico-metabolic enzymatic responses associated with herbal products applications in fish

Herbal products possess a varied range of bioactive constituents that affects the metabolic responses along with growth performances in fish (Kumar et al., 2015). Because growth, metabolic, and hematological responses are important paraments for studying the physiological condition of fish under stressful circumstances that are mediated by biotic or abiotic factors, they have been

extensively studied. This can help to monitor the status of the physiological condition of fish by using the changes in growth, metabolism and hematological parameters. Enzymes of protein and carbohydrates metabolism, SGOT, SGPT and LDH, are also a good stress marker (Abhijith et al., 2016).

In response to stress, fish has to adjust their metabolism, and changes in enzymes like SGOT, SGPT, LDH, ACP, and ALP that imitate the fish's overall well-being (Malarvizhi et al., 2012). The SOD and CAT, which are found in a variety of fish tissues, are both powerful antioxidant enzymes.

To meet energy demands, teleost fishes convert amino acids into glucose *via* gluconeogenesis process during stress by inducing an increase in the transamination pathway. Kumar et al. (2015) reported a reduction in AST and ALT level in muscle as well as liver in *Cirrhinus mrigala* fed with Anthraquinone extract at 1% inclusion level, found to have stress-relieving properties. Gupta et al. (2013) revealed a decreasing trend in *C. carpio* ALT activity in muscle and liver as dietary levan was gradually increased. This finding indicates that dietary levan has discriminatory effects among the treatments as compared to control.

Ojha et al. (2014) found that dietary ethyl alcohol extract of *Mucuna prurient* with 0.06% inclusion level in the fingerlings of *L. rohita*, had the lowest AST activity, implying immune-enhancing and health-boosting properties. Gora et al. (2018) reported a reduced level of MDH and SGPT activity in liver on feeding *L. rohita* with 4% fucoidan level.

Huang et al. (2020) had studied the synergistic effects of dietary (0, 2.5, 5, 7.5, 10, 12.5 g kg^{-1}) mixture of Chinese herbal medicines on serum biochemical and metabolic responses in European eels, *Anguilla anguilla* for 42 days. The results showed an improved serum biochemical and metabolic responses in terms of total antioxidant capacity of eel was found to elevate at 9 g kg^{-1} doses.

Adineh et al. (2020) showed the beneficial impact of dietary micro-encapsulated garlic (0, 0.25, 0.5, 1, and 2%) on the antioxidant status of *rainbow trout*. The study recommends that microencapsulated garlic extract could be

reflected as a good food additive to advance the antioxidant capability of rainbow trout. Latif et al. (2021) reported that dietary black seed fed to *L. rohita* resulted in the decrease of lipid peroxidation and lower level of hepatic-nephric marker enzymes in treated groups as compared with control.

Yousefi et al. (2021) revealed that the dietary level of *Hibiscus sabdariffa* at 0.5% could reduce the metabolic stress in rainbow trout. Mahima and Begum (2012) evaluated the effects of hydroalcoholic extracts of *Achyranthes aspera* Root and *T. arjuna* bark on the activity of oxidative enzymes in aerotolerant capability of *Streptococcus mutans.* It was observed the activity of MnSOD, NADH oxidases, and glutathione peroxidase (GPx) was inhibited in *S. mutans*.

Dash et al. (2016) highlighted the beneficial effects of *T. arjuna* bark powder on various enzymes, ALP, LDH, CKNAc and gamma-glutamyltransferase (GGT) in plasma of buffalo, ingesting arsenic contaminated fodder and water. The study revealed remarkable higher level for all the enzymes in arsenic-treated groups as compared with control, while following the treatment with *T. arjuna* bark powder, values subsequently ($p<0.05$) reduced in arsenic exposed groups and was found to close to control group. This is an indication of the hepatoprotective and nephroprotective effects of *T. arjuna* bark powder.

2.12.3. Herbal products and response of oxidative enzymes in fish

The SOD and catalase are the two important oxidative enzymes that react against reactive oxygen species. Kumar et al. (2014) studied that microalga strains have both anti-oxidant and non-antioxidant enzymes due to that it functions against free radicals. Sharma et al. (2019) studied the effects of microalgal strain *Ascochloris* spp. on oxidative enzymes in African catfish. The study concluded that diet supplemented with microalga showed discriminatory differences in treated groups as compared to control.

The oxidative enzymes, SOD, catalase and glutathione-s-transferase showed higher value in control as compared to treated groups. Similarly, other researchers also showed the potential beneficial effects of herbal-based diets in

different fish species *i.e.* when dietary *Laminarin, Chlorella vulgaris* and *Chlorella* spp. were fed to, olive flounder, orange-spotted grouper and *C. carpio*, respectively (Stara et al., 2014; Yin et al., 2014; Rahimnejad et al., 2017).

Gora et al. (2018), in their investigations on metabolic effects of dietary (0-4%) inclusion level of fucoidan revealed the stress regulating properties in *L. rohita*. The results showed an elevated level of AST activity in muscle tissue in 4% inclusion while antioxidant enzyme SOD and CAT activity was reduced in liver and kidney at 4% level.

The enzyme, GST is mainly accountable for cleansing of toxic constituents through conjugating with glutathione reductase enzyme, GSH (Jancova et al., 2010). It has been reported that incorporation of microalga in fish fed did not influence the level of GST in *Dicentrarchus labrax* and *Clarias gariepinus* (Peixoto et al., 2016; Raji et al., 2018). Though, contrary to this, the inclusion of sources of non-conventional animal protein such as housefly and cricket meal in the feed of African cat fish and Nile tilapia caused an elevation in the level of GST in treated groups as compared with control (Ogunji et al., 2007; Taufek et al., 2016).

Hebbani et al. (2021) evaluated the protective effects of *T. arjuna* on oxidative damage of rats. The results showed that bark extract could regenerate the alcohol degenerated liver to its normal condition. However, such evidence of application of *T. arjuna* in fish is still lacking.

2.13. Antioxidant potential, free radical scavenging and DNA nicking inhibition activity of herbal extracts/medicinal plants

Medicinal plants are considered to be promising source of antioxidant potential and free radical scavenging activities. Due to this antioxidant potential, they protect living cells from ROS and RNS thereby offer DNA nicking inhibition activity also. Genus, *Terminalia* has been studied for its antioxidant potential. It has been reported that arjunic acid of *T. arjuna* has antioxidant properties (Sun et al., 2008). Similarly, triethylchebulate, an aglycone obtained from *T. chebula* was

proved to be an excellent antioxidant biomolecule (Chen et al., 2011). Rajmohamed et al. (2017) has explored the anti-cholinesterase and antioxidant properties of ethyl acetate extract of *T. chebula*, and further, in their studies, it has been reported that extract has potential activity in inhibiting neurodegenerative diseases. The study showed that crude extract has more butanol fractions of leaf and bark that correspond to more antioxidant activity as compared with their other solvent extracts.

Chatha et al. (2014) investigated the comparative antioxidant potential and potential of scavenging of free radicals in two extracts of leaf and bark, water: ethanol (20:80 v/v) and water: methanol (20:80 v/v). The results highlighted a significant ($p<0.05$) variation in the bioactive constituents thereby leading to possess a varied array of antioxidant potential and scavenging of free radicals. Fatema et al. (2017) evaluated the antioxidant potential of methanolic extract of *T. arjuna,* and results revealed antioxidant activity to be linked with the presence of quercetin and vincristine. Kumar et al. (2017) has evaluated the antioxidant potential of ethanolic fractions of leaf and bark of *T. arjuna.*

The study showed that crude extract has more butanol fractions of leaf and bark has more antioxidant property as compared to their other solvent extracts. Ghadigaonkar et al. (2021) assessed the free radical scavenging potential and antioxidant property of leaf extracts of *T. arjuna* prepared by Soxhlet apparatus using different solvents. The results showed that *T. arjuna* extracts possess, 37.69-42.23 µg GAE/mg total phenolic content, 44.68-263.41 µg QE/mg total flavonoid content and also exhibited DPPH and NO scavenging activity as 10.47-11.13 µg mL^{-1} and 9.49-11.32 µg mL^{-1}, respectively.

In the present study, Meena et al. (2021) evaluated antioxidant potential and DNA damage scission inhibition potential of the 21 solvent extracts comprising bark, fruit and leaf of *T. arjuna*. The results showed that ethanol bark extracts exhibited maximum total phenolic content,144.67-1794 µg mL^{-1} GAE and total flavonoid content, 2.5-34 µM Fe (II)/g, respectively. A trend of combined solvent extracts showed maximum antioxidant potential in ethanol bark extract followed by Ethanol leaf>Methanol bark≥ distilled water fruit extract. Similarly, DNA Scission

inhibition activity variables such as, relative front (RF), relative quantity (RQ), band (%) and percentage of bands and lanes, established a significant ($p<0.01$) correlation, exhibiting $R^2= 0.94$ with operative concentration for relative DNA scission inhibition was found to be 0.48 mg.

2.14. Immunological changes in fish due to herbal feeding

Lysozyme is an important compound in fish immune systems and bactericidal because it hydrolyzes peptidoglycan of the bacteria cell wall, resulting in bacteriolysis. Lysozyme function in serum can be increased by Immunostimulants that either increasing the quantity of lysozyme synthesized per cell or by increasing the number of phagocytes that secretes lysozyme (Engstad et al., 1992). Neutrophil activity is another indicator of a non-specific response (Galina et al., 2009). Introduction of a Chinese herb mixture increases phagocytosis in phagocytic cells, respiratory burst activity, and lysozyme activity in carp (Yin et al., 2009).

The bactericidal activity, serum lysozyme and NBT activity in *C. carpio* has increased significantly upon fed with herbal immunostimulant diets (*O. basilicum, C. zeytanicum, J. regia, and M. piperita* (Abasali and Mohamod, 2010). Chen et al. (2003) reported that dietary treatment of crucian carp with four Chinese herbs (*lsatis indigotica, Rheum officinale, Rographis paniculatal*, *and Lonicera japonica*) elevated plasma lysozyme activity. Considerable increment ($p<0.05$) was reported in the serum lysozyme activity when fed with 100 and 200 mg kg^{-1} marine algae, *Dunaliella salina* for nine weeks in *Oncorhynchus mykiss* (Amar et al., 2004).

Feeding *Pseudosciaena crocea* (large yellow croaker) and Jian carp (Jian and Wu, 2004), with traditional Chinese medicine (TCM) that is articulated from the roots of *Angelica* (*R. angelicas sinensis*) and *Astragalus* (*Radix astragalin* seuheydsari) showed elevated lysozyme activity. The serum lysozyme level in *L. rohita* was also increased when fish was fed with *A. aspera* seeds (Rao et al., 2006). After feeding aqueous extract of *Eclipta alba* to tilapia, *Oreochromis*

mossambicus, lysozyme activity increased significantly ($p<0.05$) (Divyagnaneswari et al., 2007).

After feeding Indian major carp, rohu (Das et al., 2009) with *euglena vulgaris* elevated lysozyme activity. Japanese eels (*Anguilla japonica*) when provided a dietary supplementation of a Korean mistletoe extract (KM-110; *Viscum album* Coloratum) showed elevated levels of lysozyme (Choi et al., 2008). Appropriate concentration of chloroform-based extract from seeds of *Nyctanthes arbortristis,* were fed to tilapia for two weeks, which resulted in subsequent ($p<0.05$) increase in serum lysozyme level (Kirubakaran et al., 2010). It was also discovered that *Chlorella vulgaris* increases phagocytic activity of leucocytes significantly in broilers and the intestinal lymphatic tissue, as well as Harder's gland (Kotrbacek et al., 1994).

Bactericidal activity of serum was found to increase in juvenile greasy groupers (*Epinphelus tauvina*) when fed with dietary *Osmium sanctum* and *Withania somnifera* (Sivaram et al., 2004). Similarly, feeding of juvenile grouper (*E. tauvina*) with some of the herbs increased serum bactericidal activity (Punitha et al., 2008). Administration of dried crude *M. indica* elevated the serum bactericidal level in *rohu* (Sahu et al., 2007a). Superoxide anions are produced when there is a respiratory burst in phagocytes, and this is treated as important for hindering the development of pathogen (Rairakhwada et al., 2007). The NBT activity can be assessed by NBT assay that quantifies the number of intracellular superoxide radicals generated by leukocytes (Sahu et al., 2007a; Ardo et al., 2008).

Superoxide anion were increased in blood leucocytes when rohu was fed with a diet containing *Curcuma longa, E. viridis*, and *Achyranthus* (Rao et al., 2006; Sahu et al., 2008; Das et al., 2009). Similarly, feeding Nile tilapia with extracts of *Astragalus membranaceus* and *Lonicera japonica* individually or in two herbal mixture or in combination showed a significant increase in respiratory burst and blood phagocytic activity (Ardo et al., 2008). Jang et al. (1995) pointed that *in vitro* treatment of rainbow trout with glycyrrhizin obtained from *Glycyrrhiza glabra*

showed an increase in NBT values of macrophage and lymphocyte proliferative reactions.

2.15. Fish histopathological changes associated with feeding of herbal materials

The histological observations are very much important to correlate biochemical parameters of fish to ensure their healthiness (Telli-Karakoça and Barlas, 2019). Changes in the histology of vital organs at the tissue level with respect to feeding mechanisms have also been reported (Veterinaria, 2010; de Melo Germano et al., 2014). Fish gills are essential for gas exchange, ion transport, waste excretion, and metabolism of several xenobiotics (Evans et al., 2005). Chemical or active bio-ingredients of herbal origin cause anatomical changes in vital organs such as the gills, intestine, kidney and liver in response to the nature of the materials effects. The fish intestine is an important organ that provides the idea about the nutrient's digestion and assimilation. The important parameters in the fish intestine are morphometric and morphological changes in goblet cell, microvilli and enterocytes height and width and absorptive area of the digestive cells (Shashikala and Sahoo, 2018).

Ragavan and Krishnakumari (2006) evaluated the effects of dietary (250 mg kg^{-1} and 500 mg kg^{-1}) *T. arjuna* bark extracts on histopathology of vital organs in alloxan-activated diabetic mice. The results summarized that the higher dose (500 mg kg^{-1}) was fond to be more hepato-nephro protective as compared to control. However, such evidence is not available in the case of fish. Palanikani et al. (2020) evaluated the effects of *Andrographis paniculata* extract on the health aspects of *L. rohita*. The investigation highlighted that 50 µL dose of *A. paniculata* also showed the protective effects on the histology of vital organs such as the intestine, kidney, liver, and gills towards *A. hydrophila* infection. Souza et al. (2020) evaluated the effects of microalga-based diet fed to Nile tilapia in relation to histoarchitextural changes in the intestine. The results showed an enhanced surface are for digestion and absorption in digestive cells of the intestine, increased height of enterocytes, increased number of microvilli, increased the level

of goblet cells and overall, the compactness of the intestinal morphology was reported in treated groups as compared with control.

Yadav et al. (2020) assessed the impacts of dietary algae-based feed on histological changes in the intestine of common carp. The investigation demonstrated that the foregut that contains tunica mucosa with numerous mucosal folds and villi shortened and gradually widened from foregut to hind gut, and intestinal bulb of common carp has grown in size in response to feeding assimilation in treatments as compared with control. It has also been observed that a high dose of herbal products/medicines/ herbal extracts can also lead to a negative impact on the histological changes in vital organs of the animals (Imo et al., 2014).

Raskovic et al. (2011) suggested comparative assessment methods and the impact of different plant and animal-based feed ingredients in fish species. And, also, pointed that the structural changes are linked with the landscape of feed and the existence of anti-nutritional elements, a bioactive compound present in the extract and the effects of the bioactive compounds on the tissues of vital organs.

2.16. Effect of plant material on survival and growth in fish

Herbal extracts/ powder of medicinal plants has been used to improve fish growth rate and feed utilization indices, which are thought to be due to the availability of polysaccharides in the feed (Zahran et al., 2014). They can improve nutrient absorption, digestion and assimilation capacity in animals (Heidarieh et al., 2013; Gabriel, et al., 2015; Kumar et al., 2016). Combination of different herbal extracts in feed showed increase in survival percentage (92.42), non- specific immunity and disease resistance against *A. hydrophila* of *C. carpio* (Abasali and Mohamod, 2010). It was discovered that feeding of herbal immunostimulant supplemented diets to black tiger shrimp, *Penaeus monodon*, increased their percentage survival (Citarasu et al., 2006). The growth indices, survival and immune-protection in black tiger shrimp *P. monodon* showed a remarkable higher survival (76%) and SGR (4.26 0.11%) with improved FCR (1.5) when shrimps

were fed dietary methyl alcohol extract at 2.5 mL kg^{-1} as compared with other groups (Uddin et al., 2017).

Chlorella powder supplementation in diets improves growth performances, nutrient retention, serum cholesterol levels, and total body fat contents of juvenile Japanese flounders (Kim et al., 2007). Pachanawan et al. (2008) discovered that tilapia (*Oreochromis niloticus*) fed with *Psidium guajava* had a higher survival capacity after being infected with *A. hydrophila*. The *T. catappa* extract was observed to improve survival in *Betta* sp. at 500 ppm dose. Shakya (2017) examined the effect of herbs and herbal products used as feed supplements on the growth of fish and mentioned that herbal products have emerged as feasible growth enhancers and immunostimulants. Studies of natural herbs like anise, basil, black seed, caraway, fennel, fenugreek, garlic, licorice, marjoram and onion, showed that they enhance promote growth, protein digestibility, energy retention and feed conversion in aquatic animals (Lee and Gao, 2012).

Recently, Gabriel (2019) reported the effect of herbs mostly studied in tilapia for their enhanced growth and survival. Use of *Aloe vera* (Gabriel et al., 2015) and *Carica papaya* (Kareem et al., 2016) in crude form revealed a considerable ($p<0.05$) increment in the intake of feed (FI), FCR, SGR and weight gain percentage in Nile tilapia. The growth performances and feed retention indices of Nile tilapia fed with dietary polysaccharides of *Astragalus* and blackberry syrup, demonstrated a subsequent ($p<0.05$) improvement in growth performances and survival (Zahran, et al., 2014; Mo et al., 2016; Yilmaz et al., 2019a).

However, at higher levels of herbal extract dietary inclusion led to poor growth or no growth, or poor health of fish which is associated with the presence of high concentrations of anti-nutritional factors such as saponin, tannin etc, presence of toxic constituents, and allergic reactions (Yilmaz and Ergun, 2012 & 2018; Madalla et al., 2013; Irkin et al., 2014). The Akbary et al. (2017) investigated effects of four medicinal plants, *M. chamomilla, Z. multiflora, M. piperita* and *T. chebula* on growth performances and survival in white leg shrimp. The results showed that survival and growth performances were noticeably higher in treatments as compared with control. Similarly, Goswami et al. (2020) evaluated

the combined impacts of macrophytes and almond oil cake on survival and growth performances in *L. rohita*. The study revealed that the inclusion of mixture at 300 g kg^{-1} could enhance the growth without compromising the health status of the fish.

2.17. Herbal extract/medicinal plants and gut microbiome

Medicinal plants are known to produce beneficial effects in animals due to their capability to alter the gut microbial community. Gut microbes reduced the load of the harmful organism and enhance the production of beneficial compounds termed as postbiotics (Zolkiewicz et al., 2020). The metagenomics studies showed that gut microbes also help in the proper metabolism of the nutrients in fish (Dimitroglou et al., 2009; Eichmiller et al., 2016; Tarnecki et al., 2017).

A varied array of microbes reported from the gut of carps heavily dominated by *Cetobacterium* (Eichmiller et al., 2016). Lyons et al. (2017) studied the role of microalga on rainbow trout. The results demonstrated that rainbow trout fed with microalga could alter the diversity and species richness witnessed with elevated Chao and operational taxonomic units (OTUs) numbers in treated groups as compared with control, however, community structure remained the same. This indicates that polysaccharides present in the microalga supplemented in diet might have efficiently utilized gut microbiota. Further, higher bacterial genera in the supplemented diet as compared with control, also revealed the potential beneficial effects of herbal material on the fish gut.

Similarly, Souza et al. (2020) revealed in their study that microalga-based fish feed modulated the microbiome of gastro-intestinal tract of Nile tilapia cultured in net cages. It has also been reported that microbial community structure also depends on the nature of feeding habits. As the microbial diversity tends to increase in herbivores fish as compared to carnivores their counter parts (Ley, 2008). Smriga et al. (2010) highlighted that microbiome in herbivore, whitecheek surgeonfish, *Acanthurus nigricans*, fed with a diet based on algae and detritus, was higher as compared to carnivore red snapper, *Lutjanus bohar*.

The bibliographic study reported that dietary herbal products slightly alter the gut microbiome but the core community structure remains the same (Wong et al., 2013; Zarkasi et al., 2014). Wu et al. (2020) also reported the beneficial effects of woody forages on the diversity and bio-activity of aerobic cultivable bacteria that resides in the gut of Nile tilapia, and pointed its probiotic effects in terms of increasing the number of beneficial bacteria present in the gut of the Nile tilapia.

Initially, the investigation used to carry out with culture-based and low-resolution micro-biotechnological approaches which can deliver only the fractional composition of microbiome structure and its potential reactions to dietary management (Nayak, 2010). Now day high throughput techniques are readily available which provides a complete picture of the gut microbiome of the fish species. Ingerslev et al. (2014a & b) applied Illumina HiSeq platform to unravel the variations among the community composition of microbiome of rainbow trout fry in the intestine, fed by marine or plant-based dietary components. In the present study, the Illumina HiSeq 2500 platform was used to sequence the prepared library, and V3-V4 metagenomics pipeline was used to in-silico data analysis to see the deviations in the gut microbiome of the fish, *L. rohita* in relation to differ feeding treatment and trials.

2.18. Herbal extracts/medicinal plants and gene expression in fish

Medicinal plants, herbs, herbal extracts and their associated products are well known for posing various health beneficial properties, and since ancient, they have been used to treat various geriatric and chronic diseases in human beings. Medicinal plants and their product have also been used in aquaculture as a growth enhancer, immunomodulator and nutraceuticals. The head kidney of fish is the vital organ for immunocompetence in fish (Feng et al., 2016). Tan et al. (2020) studied the impacts of dietary *ginko bibola* leaf (GBE) extracts (0.0-10.0 g kg^{-1} of feed) on growth performances and expression of immunogenic genes in hybrid group (*Epinephelus lanceolatus*♂× *Epinephelus fuscoguttatus*♀).

The study revealed that dietary dose of GBE was found to up-regulated, the anti-inflammatory cytokines (TGF-β1 and IL-10), antioxidative genes (CAT, GR

and GPx) and Immunogenic genes (TLR3 and MHC2 43). On the other hand, expression of genes related to apoptosis (p53, caspase-3, caspase-9 and caspase-8) was down regulated. This study suggests the efficacy of herbal extract to trigger the specific defense system of fish. Similarly, other researchers also assessed the impact of dietary herbal extracts on expression of immunogenic genes in red hybrid grouper *i.e. Radix Bupleuri* extract (Zou et al., 2019) and *Senecio scandens* buch-ham extract (Sun et al., 2020). Other genes such as IFN-γ has also been reported to be up regulated upon feeding with *Usnea barbata* leaf extract in *Oncorhynchus mykiss* (Bilen et al., 2019). Garg et al. (2020) evaluated the dietary impacts of *Hottunia cordata* leaf extract and meal (0.0-10.0 g kg^{-1} of feed) on two immune genes, TNF-α and IFN-γ in *L. rohita*. The study revealed that maximum expression of genes was observed in liver and kidney at 10 g kg^{-1} treatments.

Further, other workers also reported the up-regulation of IFN-γ gene *i.e.* dietary fucoidan that is a rich seaweed extract in *L. rohita* (Prabu et al., 2016), processed Rehmannia glutinosa polysaccharide in *Luciobarbus capito* (Wu et al., 2019), isolated sulphated polysaccharides isolated Codium fragile in *Paralichthys olivaceus* (Yang et al., 2019), mannan oligosaccharides (MOS) in *Seriola dumerili* (Fernandez-Montero et al., 2018). Yousefi et al. (2021) studied the immune-modulators effects of dietary *H. sabdariffa* on gene expression of interleukin-8 (il8), interleukin-1 beta (il1b) and heat shock protein 70 (hsp70) and hepatic expression of tumor necrosis factor alpha (tnfa).

The results showed *H. sabdariffa* could reduce the stress gene expression thereby showed its mitigating potential against stress-causing factors in fish. Das et al. (2019) highlighted the presence of Mx gene that was reported to be presented in all stages of fish. The Mx gets activated upon external stimuli to the fish. The present study is of its first kind to report the impacts of *T. arjuna* bark extract on the expression of STAT1 and ISG15 gene in *L. rohita,* however, their presence in fish has been documented (Sobhkhez et al., 2014).

3. MATERIAL AND METHODS

3.1. Experimental location and setup

The research was conducted at the ICAR-Central Inland Fisheries Research Institute in Barrackpore, West Bengal. The feeding trial was divided into two parts; a trial with powder of different parts of *Terminalia arjuna* on growth, its survival, resistance to diseases and immune responses in *Labeo rohita* (Hamilton, 1822). Trials were done with graded levels of the respective part of *T. arjuna* and it was set up in triplicate for each dose. All the glassware used throughout the experiment were purchased from Borosil and chemical also AR grade which is manufactured by Hi-media, Sisco laboratories, Sigma (USA) and Eurofins.

3.2. Collection of the material

The parts of arjuna plant namely, bark, leaf and fruit were collected from the trees located in ICAR-CIFRI, Campus, Kolkata, India (Authentication no.14) and confirmed for the botanical origin with the help of morphological characters and botanists.

3.2.1. Pre-treatment of herbal material and preparation of herbal extracts

After collection, the selected parts of arjuna were washed in tap water to get rid of the extraneous material and dirt. After washing, the plant materials were subjected to sundry till the removal of the moisture. Subsequently, the material was blended in the mixer and sieved through 50-micron sieve and the coarse materials again were put into the mixer till the course material appears not useful. About 100 g fine powder of each part was put into the individual four different solvents in 1:5 (material: solvent) *i.e.* acetone, methanol, ethanol and distilled water for individual fraction and serial fraction, it was kept in hexane to remove the greasy materials and then kept in the four solvent serially starting from one polar aprotic (acetone), and three polar protic (methanol, ethanol and distilled water) for

36 h at 36 ℃ in a shaking incubator. After 36 h, the extracts were centrifuged at 8000 rpm for 5 min in a ReMi R-24 (ReMi, India) to settle down the coarse particles, in a separate collection vial, supernatant was gathered. The supernatant was then filtered by a Whatman No.1 (40) and the residue was kept overnight in the existing solvent. The same process was repeated once more, with the residue being discarded for individual fractions and allowed to dry to evaporate the mother solvent before being kept in the next polar solvent for serial fractions. The supernatant was obtained using the same method. For proper distillation, the supernatant was vacuum dried in a rotary evaporator till, it attains final volume as to be 1/10 of its initial volume (Fig. 1). At room temperature the extracts were air dried and stored at 4 ℃ until further use.

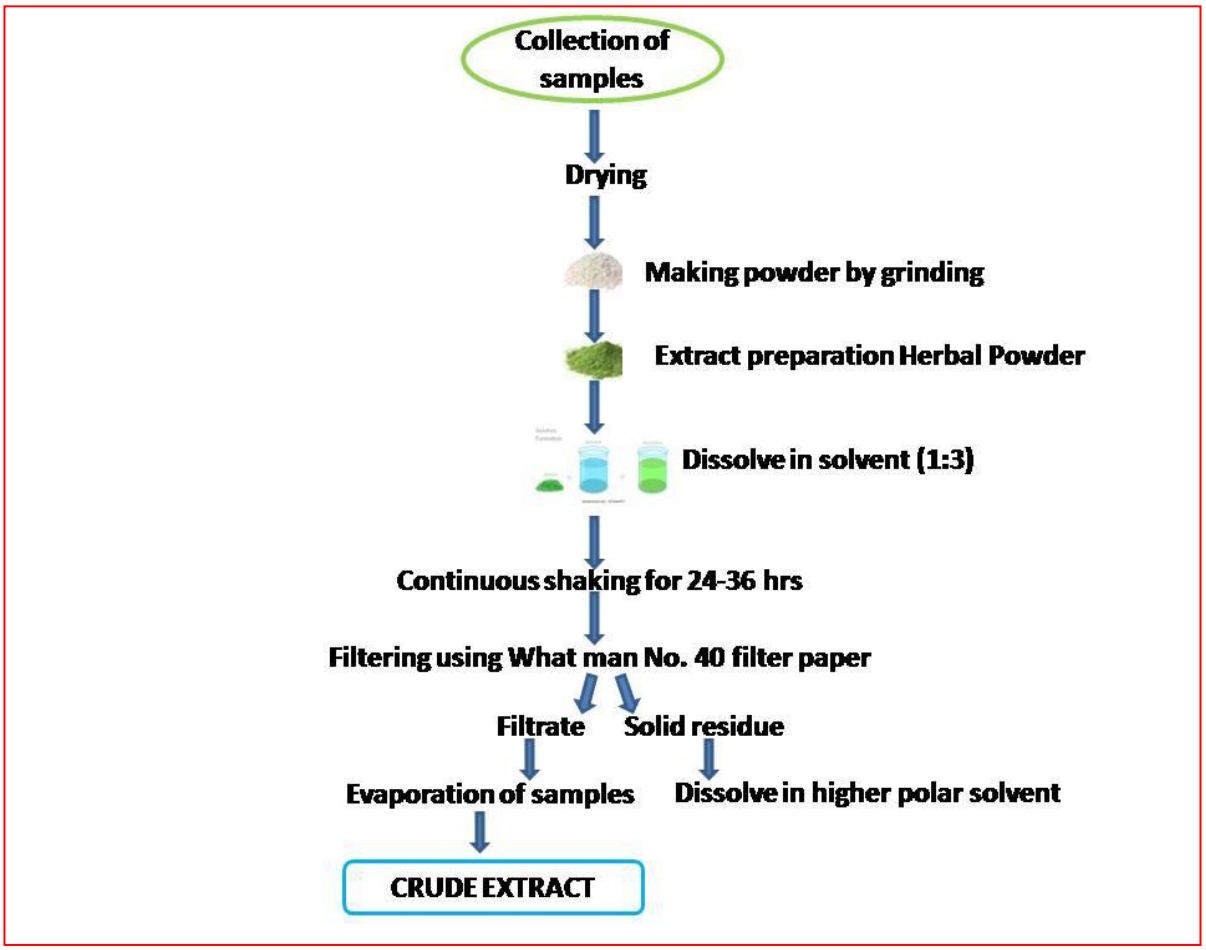


Fig. 1. Schematic representation of preparation of crude extract of *T. arjuna*

3.3. Antibiogram profiling

3.3.1. Estimation of minimum inhibitory concentration

A method of broth dilution with minor modification determined the minimum inhibitory concentration (MIC) (Wiegand et al., 2008). Briefly, extracts were diluted to achieve 12, 16, 20, 24, 28, 32 mg mL^{-1} of concentration. Each tube was pipetted with 3 mL Muller Hinton Agar (MHA), 0.5 mL culture of bacteria and 0.5 mL corresponding extracts and 37 ℃ incubated for 24 h. The mix was permitted to incubate at 37 ℃ for 24 h.

The test tubes with no turbidity visualization indicated no growth and *vice versa*. The tubes containing only growth medium were taken as the positive control. To assess bacterial inhibition, the optical density (OD) of the positive influence, as well as treated tube with no turbidity, was calculated at 560 nm. The MIC for *A. invadans* was assessed similarly.

3.3.2. Initial antibacterial efficacy study

The disc diffusion method (CLSI, 2011) performed an antibiogram investigation with slight modification. In brief, the 24 h fresh culture of desired bacteria was inoculated into the Muller Hinton Broth (MHB), Himedia and incubated at 37 ℃ in shaking condition. Thereafter, fresh overnight bacterial culture (10^7 CFU/ mL) @ 20 µL was aseptically transferred to the MHA plate.

Subsequently, the sterile blank disc (Himedia) saturated with four concentrations (0.25, 0.5, 0.75 and 1.0 mg mL^{-1}) was used on the MHA plate for initial screening. Simultaneously, the references antibiotics discs as a positive control such as Oxytetracycline (OTC), Streptomycin (SM) and Flucanazole (Flu) along with a disc of negative control of respective solvents were embedded in the plate. The plate was incubated 18 h at 30 ℃, with readings taken at 12, 16, and finally 18 h for a zone of inhibition, with the average of three replicates were considered in the final analysis.

3.3.3. Final efficacy study with broad-spectrum antibiotic

After initial screening, the higher concentration was fixed at 0.5 mg mL^{-1} as a ceiling dose and OTC was used as a broad-spectrum antibiotic as a reference one.

3.4. Antifungal activity

The fresh fungus, *Aphanomyces invadans* culture was taken to assess the antifungal activity of the selected extracts by disc diffusion method with slight modification. In brief, the four concentrations (0.25, 0.5, 0.75 and 1.0 mg mL^{-1}) of each extract were prepared. A blank disc having 20 µL of the extract was set on the plate and, the fresh culture of fungus was also put at different places representing the whole area of potato dextrose broth.

Plates were kept at 37 ℃ for 24 h in a shaking incubator to determine the ample growth of the fungus. Firstly, all the extracts were screened to check the efficacy as an antifungal agent, and thereafter the comparative efficacy of the screened extracts was applied as mentioned above.

3.5. Antiparasitic activity

3.5.1. *In-vitro* screening of selected solvent extracts and efficacy study of effective solvent extracts

The 21 extracts of *T. arjuna* have been used for efficacy against *A. bengalensis*, with the top five chosen for further research. Five effective solvent extracts were used for *in-vitro* testing at 10, 20, 30, 40, and 50 ppm, concentrations. In brief, 50 *Argulus* were dropped into 50 mL of distilled water in a sample container with a capacity of 100 mL using a dropper. To this, 10 mL of mother stock extract was added, and the mortality of the *Argulus* was noted at different intervals of time, and the mortality of the *Argulus* was recorded by seeing the movement of the specimen and under the microscope.

3.5.2. Estimation of LC_{50}

The total number of *A. benegalens is* stocked and numbers of dead *Argulus* after 1, 2, 3, 4 and 5 h was noted and the efficacy of the extract in terms of LC_{50} was calculated following the method of Finney (1952).

3.5.3. Estimation of anti-parasitic efficacy (AE) percentage

It is an indication of the potential of herbal extract and can be calculated as follows (Wang et al., 2009)

$$AE\ (\%) = \quad (B-T)/B*100$$

Where, B= Mean *A. bengalensis* survived in control; T= Mean *A. bengalensis* survived in treatment

3.5.4. Therapeutic Index

After calculating the LC_{50} at particular time and dose, the relationship of dose-response is used to determine the therapeutic index which can be calculated by the following formula.

$$TI = LC_{50}/EC_{50}$$

LC_{50}=Mean lethal concentration; EC_{50}=Mean effective concentration

3.6. Assessment of toxicity of herbal extract using *Artemia salina* model

3.6.1. Artificial seawater preparation and hatching of the *A. salina*

The critical element in the hatching of brine shrimp cysts is the availability of artificial sea water. For preparing 5% artificial seawater, a total of 200 g salt was added to 4 L distilled water. To extract foreign material, the mixture was mixed and sieved through a fine mesh muslin cloth net. To achieve the desired dimensions and densities, 1 g brine shrimp cyst was poured to 5 L capacity cylinder-glass pot maintaining 4 L water level.

The inclusion in two installations in these steps of the total amount of salt needed makes *Artemia salina* nauplii size homogeneous, which is crucial to the experiment's success. The jar mouth was properly tied with fine mosquito net the glass pot was kept under 100 V bulb providing yellow light and proper aeration were given. Active hatching began after 36 h, and it continued for up to 48 h.

3.6.2. Experimental setup and normalization of the experimental conditions

Firstly, mother solvents such as acetone, chloroform, ethyl acetate, ethanol, hexane, methanol, distilled water as well as DMSO (reference solvent) have been used to determine the value of LC_{50}. To summarize, 30 Artemia salina nauplii, along with 100-500 µL of mother solvents have been poured to a 50 mL (Tarson) sample container and incubated under 100 V yellow light for 24 h. No motion nauplii were classified as dead by mortality, as they were examined by visualization with a 50-X glass magnifying glass.

3.6.3. Estimation of LC_{50}, absolute LC_{50}, relative LC_{50} %, % absolute LC_{50} and fixing the toxicity level

The LC_{50} was measured with an excel sheet and the prism v.8.1.3 of graph pad; with the below formula, both the combined and absolute toxicity were used in calculating the solvent extract's actual toxicity to determine the relative percentage LC_{50} and absolute percentage LC_{50}.

Relative LC_{50} (%) = Combined LC_{50}/LC_{50} of a solvent or solvent extract *100

Absolute LC_{50}(%) = Mother solvent LC_{50}–Relative LC_{50}

The toxicity was determined on the basis of the LC_{50}, either in comparison with the toxicity index of Meyer or Clarkson. The toxicity index of Meyer categorizes extracts as, toxic with LC_{50} < 1000 µg mL^{-1} and extracts non-toxic with LC_{50}>1000 µg mL^{-1} (Meyer et al., 1982).

Criteria for toxicity by Clarkson for evaluating the plant extract toxicity of groups is extracted from the following categories.

LC_{50} > 1000 µg mL^{-1} – non-toxic; LC_{50}500 - 1000 µg mL^{-1} – are low toxic; LC_{50}100 - 500 µg mL^{-1} –medium toxic, and LC_{50}0 -100 µg mL^{-1} – high toxic (Clarkson et al., 2004).

3.7. Assessment of the antioxidant potential

3.7.1. Preliminary phytochemical screening

For preliminary phytochemical screening, 1% stock concentration was prepared for each solvent extracts. For this, one gram solvent extract was dissolved in 100 mL solvent of choice. The phytochemicals such as tannin, alkaloids, steroids, flavonoids etc. were evaluated qualitatively following standards methods described by Sofowora (1982) and Trease and Evans (1989).

3.7.2. Screening of alkaloids

a. Dragendroff's reagent test: Five millilitres of extract were mixed with 2 mm of HCl and 1 mL of Dragendroff's reagent (Appendix I). The presence of alkaloids was immediately indicated by an orange or red precipitate.

b. Mayer's reagent: Two millilitres of extract were mixed with 0.2 mL of dilute HCl and about 0.1 mL of Mayer's reagent (Appendix I). The presence of alkaloids was indicated by white precipitation.

3.7.3. Screening of flavonoids

Added some drops of dilute sodium hydroxide to 1 mL of extract. The extract's intense yellow colour, which turned colourless after addition of some drops of acid in dilute form, suggested the existence of flavonoids.

3.7.4. Screening of tannin

Ferric chloride test: 5 mL extract was put into 1 mL of a 5 % ferric chloride solution. The developed blackish green colour gives an idea about the presence of the tannin in the sample.

3.7.5. Screening of steroids

One mL extract was dissolved in one mL of chloroform and the test tube was added side by side with the same amount of concentrated sulfuric acid. The sulfuric acid layer was green, while the upper layer of chloroform was red. This meant the presence of steroids.

3.7.6. Screening of saponins

In a graduated cylinder, the extract was diluted and shaken for 15 min in 20 mL distilled water. Saponins were identified in 1 cm thick layer of foam that was formed.

3.8. *In-vitro* antioxidant activity

3.8.1. Total phenolic content

To identify total phenolic content, Folin-Ciocalteu reagent method was slight modified (Singleton and Rossi, 1965). About, 10 µL of sample from the stocks of 25-1250 µg mL^{-1} was mixed with 1.5 mL reagent (Folin-Ciocalteu) and 5.5 mL of triple distilled water in test tubes (triplicate) marked as concentration. Blank and control were taken to calibrate the spectrophotometer at zero. In blank, every constituent was there except sample, instead of sample the volume was adjusted with distilled water and standard were set up similarly, in standard marked test tube except sample, the standard was taken at 30 min and incubated at room temperature, 1.0 mL of 1 M In the reaction mixture, sodium carbonate has been added. Until cooling, the reaction mixture was kept for 20 min at 40 ℃ in a water bath.

The mixture was put in a water bath and the absorbance was measured at 760 nm using EpochTM2 Microplate Spectrophotometer, Biotek, USA. Gallic acid (1 mg mL^{-1}) was used as a standard to assess the TPC of solvent extracts (25-1250 µg mL^{-1}). The TPC was expressed as Gallic Acid Equivalent µg mL^{-1} (GAE).

3.8.2. Quantification of total flavonoids contents

The TFC was calculated applying the aluminium chloride ($AlCl_3$) method with slight adjustments (Harborne, 1998). About 10 µL of sample concentration was drawn out of stock of extracts (25-1250 µg mL^{-1}) and kept in triplicate in a test tube marked with concentration, having 200 µL DW and 150 µL $NaNO_2$ and incubated for 10 min in dark at room temperature. After 10 min, 200 µL of 10% $AlCl_3$ was added and kept at RT for another 10 min, in dark place. To this reaction mixture, 2 mL (4% NaOH) was added to make the volume 5 mL and kept for 20 min RT in a dark place. The appearance of pink colour in the sample after 20 min indicates the presence of flavonoids. The OD at 510 nm was taken in a spectrophotometer and about 1 mg mL^{-1} Quercetin was used as standard to estimate the total flavonoid content in the samples and the total content was represented as µg mL^{-1} Quercetin equivalents (QE).

3.8.3. DPPH free radical scavenging assay

Free radical scavenging activity in solvent extracts was assessed by DPPH assay by a slightly modified method (Bozin et al., 2006). About 10 µL of each stock concentration (25-1250 µg mL^{-1} of extracts) was taken into triplicate of test tube that is marked as concentration, to this 1 mL of newly prepared reagent DPPH (1 mgmL^{-1} in methanol). The volume was made up to 5 mL by adding DW and the test tubes were kept for 30 min at RT in a dark environment, and the absorption was taken at 536 nm. Using the equation below, the inhibition percentage was determined.

$$\text{Inhibition (\%)} = (A_0 - A_1/A_0) \times 100$$

A_0: absorbance of CT; A_1: absorbance of test material

3.8.4. ABTS assay

The ABTS was measured with standard procedure with slight modifications (Re et al., 1996; Ilyasov et al., 2021). Briefly, 10 µL of each stock concentration of extracts (25-1250 µg mL^{-1}) was taken into triplicate of test tube that is marked as concentration, to which 3 mL (ABTS prepared with 1:1 of Potassium persulfate 2.44 mM) was added and stored in the dark for about 12-16 h before use at room temperature; and the volume was made up to 5 mL by adding methanol. The test tube was held RT for 30 min before measuring absorption at 734 nm. Trolox has been used as a control and the inhibition percentage has been computed with the below mentioned formula.

$$\text{Inhibition (\%)} = (A_0 - A_1/A_0) \times 100$$

A_0: absorbance of CT; A_1: absorbance of test material

3.8.5. Scavenging activity of Nitrous Oxide

Scavenging activity of nitrous oxide was measured using the Griess Illosvoy reaction (Garrat, 1964). Sodium nitroprusside (10 mM) in phosphate-buffered saline was added in different proportions of extract and placed at 300 ℃ for 2 h. A sample volume of 10 µL from each concentration was taken into triplicate of test tube marked as concentration, in phosphate-buffered saline, sodium nitroprusside (10 mM) and mixed with samples.

To this mixture, 10-20 µL newly prepared Griess reagent (composition: 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid and 1% sulphanilamide in 2.5% phosphoric acid) was mixed with the sample and after 1 h absorbance was deliberated at 546 nm. The Gallic acid was taken as standard. The NO scavenging percentage was measured by below mentioned formula.

$$\text{Inhibition (\%)} = (A_0 - A_1/A_0) \times 100$$

A_0: absorbance of CT; A_1: absorbance of test material

3.8.6. Ferrous Reducing Anti-oxidant Potential (FRAP)

Ferrous Reducing Anti-oxidant Potential (FRAP) was measured following the standard method (Benzie and Strain, 1996) with slight modification. About 10 µL of each stock concentration of extracts (25-1250 µg mL^{-1}) was added into triplicate of test tube marked as concentration. About 1.5 mL FRAP reagent (10:1:1 of 300 mM Sodium Acetate buffer: 20 mM $FeCl_3$: freshly prepared TPTZ in 40 mM HCl) was adjusted to final volume to 5 mL by distilled.

Similarly, a standard (ascorbic acid) was set up in triplicate with various concentrations (25-1250 µg mL^{-1}) and a blank containing no sample was used as standard. This reaction mixture was allowed to stand for 30 min in dark room at room temperature. The absorbance at 593 nm was measured and FRAP was estimated and the expressed as µM (Fe II)/g of extracts.

3.9. DNA nicking inhibition

The DNA nicking inhibition potential of solvent extracts of *T. arjuna* on DNA nicking due to induced oxidative stress was investigated using pBR322 plasmid DNA (Thermo Scientific). The samples were first poured in 1% tetrahydrofuran (THF) at 50 mg mL^{-1} using a specific dose percentage concept. A reaction mixture of 20 µL final volume containing 2.5 µL of plasmid DNA pBR322 (0.25 µg μL^{-1}), 16 µL of 50 mg mL^{-1} sample and 1.5 µL of 1% H_2O_2 was made.

Tetrahydrofuran (1%) and plasmid DNA, H_2O_2 (1%) and tetrahydrofuran (1%) treated plasmid DNAs and only THF prepared solvent extract mixture was used for 24 h at 37 ℃ as control groups. In the samples 4 µL loading dye were added, and the mixture was loaded onto a 1% agarose gel for 45 min at 120 V. Under UV light, the Gel was pictured. Similarly, the inhibition of DNA nicking inhibition of sample was compared with standard antioxidants BHT, BHA, ascorbic acid, and gallic acid.

3.10. Mineral profiling of dry powders of bark, leaf and fruit and solvent extracts of *T. arjuna*

Mineral profiling was carried out at the ICAR-NIASM, Baramati, Maharashtra as per AOAC (2006). In a brief, a 1 g sample of each solvent extract was weighed and acidic digestion was performed in a microwave digestion system (Model START-D, SN- 135177, Milestone, USA). HNO_3 and H_2O_2 were mixed in a 5:1 ratio and kept in digestion vessels (Kumar et al., 2017).

The samples were cooled at room temperature following proper and complete digestion before filtering with 0.45 mm pore size of Whatman paper, which was developed in batches of up to 50 mL for inductively coupled plasma mass spectrometry (ICP-MS) trace element analysis (Agilent 7700 series, Agilent Technologies, USA).

3.10.1. Calculation of extract yield (%)

The yield (%) of solvent extracts was calculated for serial fractions and individual fractions as per the dry weight basis of the extracts with the following formula:

$$\text{Yield}\,(\%) = \frac{\text{Weight of extract}}{\text{Weight of herbal material taken for extraction}}\ \text{X100}$$

3.11. Compound characterization from the solvent extracts of *T. arjuna*

3.11.1. Standardization of column chromatography

3.11.1.1. Preparation of Column

Weighed 70 g of silica gel (60-120 mesh) and mix with 100 mL hexane and poured into the column.

3.11.1.2. Extract Preparation

A total of 0.5 g of sample was taken and added requisite volume of acetone/methanol to it and mixed properly with a glass rod so that it becomes like a fine powder. Thereafter, a requisite volume of chloroform was added to remove any trace fatty acids present in the sample.

3.11.1.3. Elution of fractions

After preparation of extract 100 mL of chloroform was passed through the column and elution fractions were collected. Likewise, the different solvent extract was obtained during the process of getting purified compounds.

3.11.2. Thin layer chromatography (TLC)

Chromatography is a strong separation tool used in all fields of science, and it is often the only way to separate components from complex mixtures. The term chromatography was coined in 1906 by Russian botanist Mikhail Tswett. Differential migration is the basis of chromatography. The solutes pass through a stationary phase in a mobile phase. Solutes with more mobile affinity will spend more time in them than those with a stationary phase. The solutes separate as they pass through the stationary phase. This procedure is known as the development of chromatography.

3.11.3. Standardization of the solvent system for thin layer chromatography

The 80:20 eluted fractions were subjected to thin layer chromatography with a different solvent system like methanol: chloroform 80:20, methanol: chloroform: water 80:20:3 with 1 mL formic acid, methanol: chloroform: water 80:20:5 with 1 mL formic acid, methanol: chloroform 80:20 with 1 mL formic acid. For the identification of antioxidant, flavonoid and phenol compound, the plates were sprayed with DPPH, anisaldehyde sulphuric acid and vanillin sulphuric acid.

3.11.4. Thin layer chromatography analysis for antioxidant constituents

Plates of TLC were loaded with about 20 µL of *T. arjuna* extract (Merck, 20 cm X 20 cm). To separate antioxidant components of the extracts, the plates were developed in 80:20 methanol: chloroform (v/v) with 1 mL formic acid. The plates were air dried after they had been developed. The antioxidant constituents were then analysed using DPPH spraying. This was accomplished by spraying the surface of developed TLC plates with 0.05% DPPH solution in methanol and incubating them for 10 min at RT.

3.11.5. Thin layer chromatography analysis for flavonoid constituents

TLC plates were loaded with about 20 µL of *T. arjuna* extract (Merck, 20 cmX20 cm). To separate flavonoid compounds in the extracts, the plates were formulated in methanol: chloroform (80:20) (v/v) with 1 mL formic acid. The developed plate was dried in the open air. The plate was then sprayed with anisaldehyde sulfuric acid and incubated at 110 ℃ for 20 min.

3.11.6. Thin layer chromatography analysis for phenolic constituents

TLC plates were loaded with about 20 µL of *T. arjuna* extract (Merck, 20 cm X 20 cm). Methanol: chloroform (80:20) (v/v) with 1.0 mL formic acid were used to produce the plates. The developed plate was dried in the open air. The plate was then sprayed with anisaldehyde sulfuric acid and left as it is for 20 min at 110 ℃. The principle behind TLC analysis is represented (Fig. 2).

3.11.7. Determination of retention factor (RF) value

$$\text{Rf value } [-] = \frac{\text{Distance covered by the compound to be analysed [cm]}}{\text{Distance travel by the solvent[cm]}}$$

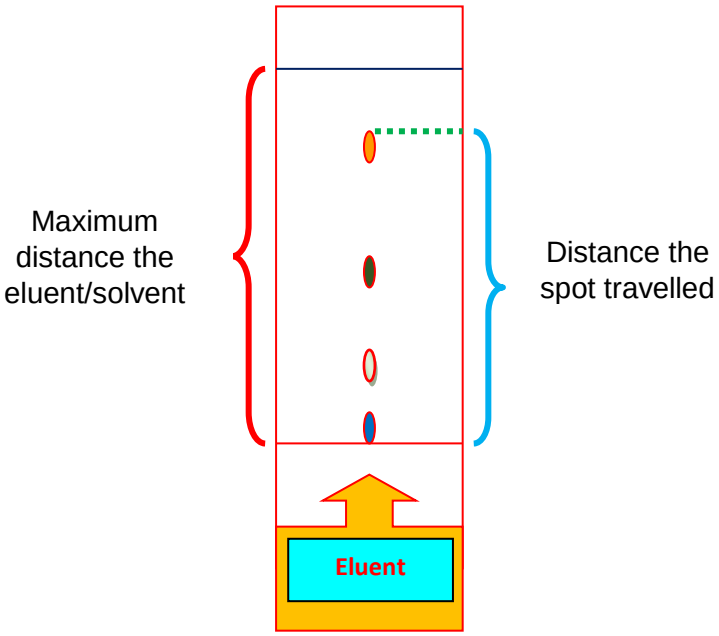


Fig. 2. Principle of TLC-analysis

3.11.8. Characterization of compounds by gas chromatography mass spectrometry (GCMS)

The 80:20 elution fractions were increased in volume by repeated column chromatography. Then, it was evaporated in a rotary evaporator at 50 ℃. Finally, the left-over small volume was allowed to air dry. The dried product was analysed by GC-MS for its structure.

3.11.9. Liquid chromatography combined to electrospray-Orbitrapmass spectrometry

In a 2.1 mm Hypersili gold micron column C18 1.9 (Thermo Fisher Scientific) was injected and separated 3 µL of samples. Mobile phase A containing 100% miiliq-water and phase B containing 100% methanol. Both the phases containing 0.1% formic acid, flowed at 0.3 mL min^{-1}. The gradient was a 2 min isocratic step in phase A at 95%, followed by a linear gradient in phase A from 5 to 95% before the next 18 min, and 5 min on hold. In the next minute, the gradient

was brought to its initial phase and held for the next 4 min before returning to 100% A for 4 min.

A Q-Exactive plus-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) with an electrospray source operating ion mode positive and negative has been used for mass spectrometric detection. The mass spectrometer was supplied with a capacity of 4.2 Kv capillary voltage and temperature at 340 ℃. Both sheath gas and auxiliary gas flow rates were set with nitrogen gas at 37 and 13 arbitrary units, and the auxiliary gas heater at 400 ℃, respectively.

At the MS and MS/MS levels, the detection was obtained from 100 to 1200 m/z at a resolution of 35000 and 17500, respectively. A repeated count was set to 10 s for dynamic exclusion and the microscope count was united. Thermo Compound Discoverer software (Version 2.1 SP1 Thermo Fisher Scientific) was used to analyze the data, with the workflow Max ID-Detect unknowns with ID using online database searches and the mzCloud search engine.

3.11.10. Extraction of chromatogram and mass spectra of representative bioactive compounds

After data acquisition, the raw files (raw) obtained were processed in Thermo Compound discoverer software (Version 2.1 SP1). For compound identification, Max ID workflow was used to detect and identify all compounds in a single sample (with ddMS2). This workflow performs unknown compound detection and predicts elemental compositions for all compounds. The compounds were identified using mzCloud (ddMS2) search engine. It also uses mzCloud to perform a search for similarity for all ddMS2 compounds.

The intensity threshold for unknown compound detection was set to 500000, Mass Tolerance of 5 ppm, S/N Threshold: 3, Ions: [M+H]+1; [M-H]-1; [M+K]+1; [[M+NH4]+1; M+Na]+1. Following parameters were kept for mzCloud database search: Compound Classes: Endogenous Metabolites, Natural Products/Medicine, Steroids/Vitamins/Hormones, FT Fragment Mass Tolerance:

10 ppm, Precursor Mass Tolerance: 10 ppm and IT Fragment Mass Tolerance: 0.4 Da.

3.11.11. FT-IR analysis

A Thermo Fischer 6700 FTIR system was used to measure the FTIR spectra. Sample (0.1-1.0%) is mixed well with 200-250 mg fine powder of potassium bromide (KBr) and pulverized. Degassing followed by drying was used to eliminate moisture and air from the samples. FTIR in KBr pellet method was carried out at a specific resolution (4) and wave number (0.01 cm^{-1}). The data were collected at 32 scans for atmospheric CO_2 correlation. The data were corrected for the background spectrum.

3.11.12. UV-VIS

In the bark, leaf, and fruit extracts, UV-VIS analysis was used to detect phytoconstituents as well as compounds containing diverse bonding pattern and lone pair of electrons, chromophores, and aromatic rings. The samples were dissolved in methanol and diluted further 1:10 times using the same solvent. Methanol was kept as a reference for blank correction. Absorbance was recorded from 200-1000 nm wavelength range using Perkin Elmer UV Spectrophotometer.

3.12. Feeding experiment with bark powder of *Terminalia arjuna*

3.12.1. Assessment of anti-nutritional factors

Tannin content was calculated using a modified version of Makkar et al. (1993) spectrophotometric technique. In a test tube, 100 mg of solvent extract was placed and the final volume was made to 2.5 mL with DW. Subsequently, 800 mL Folin Ciocalteu was mixed properly. 2.5 mL (20%) Na_2CO_3 solution was subsequently mixed and incubated for 40 min at RT. At 725 nm, a spectrophotometric reading was taken, and the concentration was calculated using the standard curve.

To compute the following equation for saponin using Harborne's (2005) method and phytates by Darambazar (2018). In brief, it is a spectrophotometric method that accounts for the phytate available in the solvent extracts. It is based on decolouration of a ferric-sulfosalicylic acid complex as the phytate in the sample replaces the sulfosalicylic acid.

A standard curve was drawn and concentrations plotted on Y and their absorbance on X for the standard curve.

Thus, phytate expressed in percent that is converted in g kg^{-1} by applying rationalization of percentage =10 g kg^{-1}.

$$\text{Saponin (g kg}^{-1}\text{)} = \text{Wt2} - \text{Wt1} \times 1000 / \text{Wt.}$$

Where, Wt. = weight of sample Wt1 = weight of empty container, W2 = weight of container + extract.

3.13. Proximate composition of ingredients, experimental diets and fish

The biochemical analysis of fish diet used in the experiment (proximate composition) was evaluated with the Association of Official Analytical Chemists' standard procedures (Cunniff, 1995). Proximate analysis is just before the beginning of the experiment of the diet was performed. After 90 days of experimental phase for feed trial, 15 and 30 days for injection experiment, and 60 days for pond experiment, carcass analysis was performed.

3.13.1. Moisture

To achieve a constant moisture content weight, the samples were thoroughly dried at 105 ℃ in a hot-air oven. Using the formula below, the moisture content was calculated.

$$\text{Moisture (\%)} = \frac{\text{(Wet weight of sample – Dried weight of sample)}}{\text{(Wet weight of sample)}} \times 100$$

3.13.2. Crude protein (CP)

The crude protein content was measured using Kjeldahl method (Mariotti et al., 2008). Total nitrogen is measured in this process, and the CP percent is estimated as follows:

$$CP\ (\%) = N_2\ (\%)\ X\ 6.25$$

3.13.3. Ether extract (EE)

A solvent extraction method in a soxhlet apparatus was used to determine the ether extract with a 40-60 ℃ organic solvent (petroleum ether) (Bhattacharya & Company, India). For EE calculation, the following formula was used.

Ether Extract (%) = (Weight of ether extract / Weight of the sample) x 100

3.13.4. Ash

To calculate the total ash content, samples were dried and incinerated for 6 h at 550 ℃ in a mufflel furnace using the equation below.

Ash % = (Weight of ash / Weight of sample) x 100

3.13.5. Nitrogen free extract

The nitrogen-free extract (NFE) was measured by subtracting the percentage of other nutrients such as ash, fiber and crude protein from 100 (Hasting, 1969).

NFE (%) =100 – Crude protein (%) – Ether extract (%) – Ash (%) – Fiber (%)

3.13.6. Digestible energy value (DE)

The digestible energy content of diets and tissues in the study is calculated using the formula below, which is based on normal physiology values (Halver, 1976).

Digestible energy (kcal/100g) =

Protein (%) x 4 + Lipid (%) x 9 + Carbohydrate (%) x 4

3.14. Diet preparation

Four iso-nitrogenous (30.36% crude protein) and iso-caloric (423.76 kcal GE/ 100 g) experimental diets have been formulated using the chemical composition of basic ingredients. The following are four experimental diets based on the graded bark powder level of *Terminalia arjuna* control diet without bark powder 0.0% (0 g kg^{-1}) inclusion (CT); 0.1% (1 g kg^{-1}) inclusion (T1); 1% (10 g kg^{-1}) inclusion (T_2) and 1.5% (15 g kg^{-1}) inclusion (T3).

For homogeneous blending, the required quantitative feed ingredients were weighed and mixed and then cooked at 15 psi in a pressure cooker for 15 min. After the mixed ingredients had cooled, the rest of the ingredients were added to the dough, including fish oil, BHT, sunflower oil, choline chloride, vitamins and minerals mixture, and proper mixing was ensured. To obtain uniform-sized pellets, the combined ingredients are pressed with a 1 mm die of the pelletizer (1.0 mm diameter). Pellets were collected in an aluminium tray and dried evenly in a rotating hot air oven at 60 ℃ for 14 h. Dry pellets were collected and stored in an airtight polythene bag at 4 ℃ until required.

3.15. In-door feed trial

Indoor feed trial was conducted for 90-days. A total of 700 fish juveniles of *L. rohita* were purchased from the local fish market (average weight, 20.3±0.34 g) and reared in a 5000 L cement cistern for two weeks acclimatization at ICAR-

CIFRI, Barrackpore, Kolkata, India. A 90-day study was designed out in triplicates using a fully completely randomized design (CRD). In triplicates, 540 fish (average weight: 20.7 ±0.34 g) were assigned to four different dietary treatments. Fish were reared in a flow-through system in a 500 L tank and were fed twice daily (9.30 am and 5.00 pm) up to satiation. Sampling was carried out every month to check the growth and health status of fish.

3.16. Outdoor pond feed trial

A 60-day outdoor pond feed trial was conducted concurrently with the in-door feeding trial to assess and validate the results of the in-door feeding trial, specifically for disease resistance and growth performances. A total of 720 fish (23±0.67 g) was stocked in 12 Hapa (3 m x 1.5 m x 2 m) in a pond at ICAR-CIFRI, Barrackpore. Monthly sampling was conducted for growth and health monitoring.

3.17. Intraperitoneal inoculation experiment

A 30-day Intraperitoneal inoculation experiment was performed to evaluate the effects of fractionated ethanolic bark extract of *T. arjuna*. The *L. rohita* with initial weight of 20.7±0.34 g was stoked in a 500 L capacity tank. The experiment was conducted in triplicate with 20 individuals in each tank. *T. arjuna* ethanol bark extract at concentrations above the MIC of the corresponding extract (7.02±0.20 µg mL^{-1}) and treatments at doses of 0, 80, 100 and 120 µg mL^{-1} (CT, T1, T2, and T3). The fixed volume of 50 µL was inoculated intraperitoneally to each fish.

3.18. Challenge study

In-door feed and outdoor pond feed trial was conducted for 90 and 60-days, respectively followed by challenge study with *A. hydrophila* and *E. tarda* for 10 days. Whereas, intraperitoneal inoculation experiment was conducted for 30-days, however after 15 days fish were challenged with *A. hydrophila* and *E. tarda* for 10 days. The pathogenic bacterial strains were grown for 24 h in a BOD incubator at 30 ℃ on tryptone soya broth (TSB). The cells were collected, washed thrice in

sterile PBS before putting into PBS at a concentration of 1.97 x 10^8 cells/mL. Each fish was challenged with 100 µL of bacterial suspension, which corresponded to 10^5 cells/mL. The relative survival percentages (%) in challenged fishes and changes in behavioural morphology were observed for up to 10 days. After the experiment, other parameters were analysed.

Table 1. Proximate composition of basal ingredients (% dry matter basis)

Ingredients	Crude protein	Ether extract	Ash	Crude fiber	NFE[3]
Fish meal	65.67±1.53	10.39±0.18	20.99±0.39	-	*2.95±0.03
GNOC[1]	37.74±0.23	8.43±0.07	6.46±0.05	7.98±0.27	39.39±0.25
Soybean Meal	45.15±0.08	1.48±0.04	11.48±0.15	8.01±0.27	33.88±0.17
Mustard oil Cake	35.58±0.15	8.79±0.21	6.67±0.17	8.15±0.08	40.81±0.32
De-oiled Rice Bran	18.34±0.13	0.64±0.03	6.62±0.06	16.67±0.31	57.73±0.21
Wheat flour	12.14±0.16	0.77±0.08	10.32±0.23	7.89±0.14	68.88±0.17
TABP[2]	3.54±0.78	1.07±0.35	28.1±1.26	18.89+1.87	48.40±1.24

[1]GNOC- Groundnut oilcake; [2]TABP- *T. arjuna* bark powder; [3]NFE- Nitrogen free extract. *Total carbohydrate content in fish meal.

Table 2. Feed formulation and chemical composition of experimental diets

Ingredients (g kg^{-1})	**CT**	**T1**	**T2**	**T3**
Fish meal	5.00	5.00	5.00	5.00
Groundnut oilcake	16.00	16.00	16.00	16.00
Soybean meal	28.00	28.00	28.00	28.00
Mustard oilcake	13.50	13.70	14.00	14.20
De-oiled rice bran	14.50	14.50	14.50	14.50
Wheat flour	14.95	14.25	13.45	12.75
Fish oil	3.00	3.00	3.00	3.00
Sunflower oil	3.00	3.00	3.00	3.00
*Vitamin mineral mix	2.00	2.00	2.00	2.00
Choline chloride	0.02	0.02	0.02	0.02
Phytase	0.01	0.01	0.01	0.01
Butylated hydroxytoluene	0.02	0.02	0.02	0.02
[1]TABP	0.00	0.50	1.00	1.50
Total	100.00	100.00	100.00	100.00
[1]Chemical composition of the diets (% dry matter basis)				
Dry matter	92.37±1.15	93.27+1.64	94.12+1.89	94.55+1.96
Crude protein	30.24±0.24	30.78±0.10	30.45±0.16	30.89±0.27
Ether extract	6.92±0.08	6.85±0.05	6.78±0.09	7.02±0.12
Ash	9.98±0.07	10.14±0.17	9.87±0.13	10.54±0.09
Crude fibre	8.34±0.02	8.96±0.15	9.44±0.07	10.09±0.05
Nitrogen free extract	44.52±0.23	43.27±0.16	43.46±0.27	41.46±0.14
[#]Gross energy (kcal/ 100 g)	433.32	434.51	433.56	433.32
**Digestible energy (kcal/100 g)	361.32	357.85	356.66	352.58
P: E ratio (mg protein/ kcal DE)	83.69	86.01	85.38	87.61

[1]Data expressed as mean (n=3); [1]TABP-*T. arjuna* bark powder.

*Composition of vitamin mineral mix (Premix Plus) (quantity/kg feed): vitamin B_2- 40 mg; vitamin E− 15 mg; vitamin A (retinol)- 33 mg; vitamin D_3(cholecalciferol) - 0.55 mg; vitamin K- 20 mg; vitamin B_6- 20 mg; vitamin B_{12}- 0.12 mcg; calcium pantothenate-50 mg; nicotinamide- 200 mg; choline chloride- 3000 mg; Mn- 540 mg; I- 20 mg; Fe- 150 mg; Zn- 100 mg; Cu- 40 mg; Co- 9 mg; L-lysine- 200 mg; DL-methionine- 200 mg; selenium- 2.5 mg. [#]The gross energy (GE) contents of crude protein, crude fat, and total carbohydrate were computed using gross calorie values of 23.6, 39.5, and 17.2 kJ/g, respectively (Brett,1973).

**Digestible energy (kcal/100g) = Protein (%) x 4 + Lipid (%) x 9 + Carbohydrate (%) x 4 (Halver, 1976).

3.19. Growth parameters and nutrient utilization indices

The fish were sampled to determine their body weight at 30-day intervals. For 24 h, the fish were starved before they were weighed. An electronic weighing balance was used to calculate the weight.

3.19.1. Weight gain percentage (WG%)

Weight gain percentages were calculated using the following formula:

$$\text{Weight gain}(\%) = \frac{\text{Final weight} - \text{initial weight}}{\text{initial weight}} \text{ X100}$$

3.19.2. Specific growth rate (SGR)

The following formula was used to measure the specific growth rate

$$\text{SGR (\%)} = \frac{\text{Log}_e \text{ (final weight)} - \text{Log}_e \text{ (initial wei}}{\text{Number of days}} \text{ X 100}$$

3.19.3. Feed conversion ratio (FCR)

Feed conversion ratio was calculated by below mentioned formula:

$$\text{FCR} = \frac{\text{Feed given(dry weight)}}{\text{Body weight gain(wet weight)}}$$

3.19.4. Protein efficiency ratio (PER)

To estimate the protein efficiency ratio, the following formula was used:

$$\text{PER} = \frac{\text{Net weight(wet weight)}}{\text{protein fed}}$$

3.19.5. Apparent Net Protein Utilization

$$\text{ANPU}(\%) = \frac{\text{Final Carcass protein} - \text{initial Carcass protein}}{\text{Protein fed}} \text{ X100}$$

$$\text{FR}(\%) = \frac{\text{Final Carcass lipid} - \text{initial Carcass lipid}}{\text{Fat fed}} \text{ X100}$$

3.19.6. Survival rate

All of the experimental tubes were dewatered at the end of the experiment, the number of fish in each tub was recorded to calculate the survival rate (percent) using the formula below:

$$\text{Survival}(\%) = \frac{\text{Total number of harvested}}{\text{Total number stocked}} \text{ X100}$$

3.19.7. Calculation of relative percentage survival (RPS)

The relative percentage survival provides an estimate of the potency of herbal extracts/ vaccines. The RPS was calculated as per the following equation (Austin and Austin, 2007).

RPS=(1-% mortality in treated fish)x 100)/ % mortality in control

3.20. Physio-chemical parameters of water

The parameters like water temperature, air temperature, dissolved oxygen, pH, specific conductivity, were analysed by using a water quality multiprobe analyzer. The total alkalinity, total hardness, TDS, Nitrate-N, ammonia and free CO_2 were estimated by using a standard titrimetric method (APHA 1998). The sampling for

water quality parameters was conducted fortnightly for indoor feed trail and outdoor pond feed trial and weekly for intraperitoneal inoculation experiment.

3.21. Body indices

Body indices were determined as follows:

3.21.1. Hepatosomatic Index (HSI)

In different treatment groups, the hepatosomatic index was calculated using the liver weight of fish:

$$\text{Hepatosomatic index(HSI)} = \frac{\text{Liver weight (g)}}{\text{Weight of fish(g)}} \text{X100}$$

3.21.2. Gastrosomatic Index (GaSI)

The gastrointestinal tract in different treatment groups was recorded, and the gastroisomatic index was calculated as follows:

$$\text{Gastrosomatic index(GaSI)} = \frac{\text{Gastrointestinal tract weight (g)}}{\text{Weight of fish(g)}} \text{X100}$$

3.22. DNA, RNA estimation

The nucleic acid fraction of DNA and RNA in vital tissues was assessed using a slightly modified Pentose analysis method (Schneider, 1945). On a dry basis, the concentrations were expressed as µg/100 mg tissue, and the equations below were drawn (Hassan et al., 2016).

µg DNA / mL = (OD at 600 nm)/0.019

µg RNA / mL ={(OD at 600 nm+0.08)-(µg DNA /mL X 0.013)}/0.116

3.23. Sampling, blood and serum collection

After the commencement of trial (feeding), fish were weighed to assess the growth indices. Final sampling was carried out to determine different response parameters. From each replicate, three fish had been randomly chosen and with 0.015 mL L^{-1} of clove oil anaesthetized. Following anesthesia, a 24-gauge needle and a 2 mL Preheparinized syringe were used to extract blood samples from the caudal vein of each sample group. The fish were then sacrificed to assess body composition, hepatosomatic index (HSI), gastrosomatic index (GaSI), and craniosomatic index (CSI). Fish have been dissected and tissues were collected in 70% alcohol from vital organs to determine the DNA-RNA ratio. Before sampling, the fish were starved for 24 h.

Fish from each subgroup have been randomly chosen for serum and blood from every experimental set and anaesthetized at 0.1 ppm MS 222 during sampling. Using a 24-gauge needle or 2 mL preheparinized syringe, after anesthesia, blood samples were obtained from each study group's caudal vein into a plastic eppendorf tube. It was then maintained at 4 ℃ for further day. Samples of blood were drawn from the caudal veins of anaesthetized fish and processed in eppendorf tubes without anticoagulant for serum analysis (heparin). On an ice pack in a slanting location, blood samples were allowed to clot for 40 min. After clotting, the serum supernatant was collected and processed at -20 ℃ after centrifugation for 5 min at 5000 rpm.

3.24. Blood parameters

The blood samples were analyzed for complete blood count using Sysmex XP-100 3-part differential fully automated haematological analyzer (Sysmex Corporation, Kobe, Japan). Following parameters were estimated in the instrument. CBC count like such as, RBC ($x10^6$ / µL), WBC ($x10^3$ / µL), HCT (Hematocrit) (%), Hg (Haemoglobin) (g dL^{-1}), MCV (Mean Corpuscular Volume) (fL), MCH (Mean Corpuscular Haemoglobin) (pg), PLT (Platelets) ($x10^3$ nos./ µL) and MCHC (Mean Corpuscular Haemoglobin Concentration) (g/dL).

3.25. Serum parameters analyzed

The serum enzyme was assessed employing fully automated biochemistry analyzer Transasia-Erba EM – 2000. The following paraments were assessed in the instrument: serum glutamic pyruvic transaminase(SGPTD) (U/L), serum glutamic oxaloacetic transaminase (SGOT) (U/L), amylase (AMY) (U/L), lipase (LIP) (U/L), albumin (ALB) (U/L), protein (PRO) (U/L), globulin (Glob) (U/L), A:G ,glucose (GLU) (mg dL^{-1}), gamma glutamyl transferase (GGT)(U/L), microProtein (MPR)(mgdL^{-1}), MxGB (mg dL^{-1}), trglycerides (Trig) (mg dL^{-1}), creatinine kinase(CKNac) (U/L), serum alkaline phosphatase(ALPU) (U/L), bilirubin total (BIT) (mgdL^{-1}), lactate dehydrogenase (LDH) (U/L), Cholestrol (CHOL) (mg dL^{-1}), and creatinine (mg dL^{-1}). For graphical representation the bacteria *Aeromonas hydrophila* and *Edwardsiella tarda* were designated as Ah and Et, respectively.

3.26. Digestive enzyme activities

3.26.1. Lipase

The method of Cherry and Crandell (1932) was used to measure lipase activity (EC 3.1.1.3). The amount of NaOH used to maintain a constant pH is used to calculate the amount of fatty acid released per unit of time. Phosphate buffer (pH 7.0), distilled water, homogeneous tissue, and olive oil emulsion make up the reaction mixture. The mixture was thoroughly mixed before being incubated at 40 °C for 24 h. Following that, 95% alcohol was added along with few (2) drops of phenolphthalein, then the mixture was titrated with 0.05 N NaOH until the colours became permanently pink. Before the buffer and oil emulsion were added, an inactivated enzyme source was used as a control. The milliequivalent of alkali is used to calculate lipase activity.

3.26.2. Amylase

The Dinitro salicylic acid (DNS) method was used to assess the sugar reduction caused by the effects of alpha-amylase and gluco-amylase on carbohydrates (Rick and Stegbauer, 1974). The recipe is made up of phosphate

buffer, 1% (w/v) starch solution and homogeneous tissue. These mixtures were incubated at specific temperature 37 °C for 30 min followed by addition of DNS and were further held in a boiling water bath for about 5 min. After cooling, the mixture was diluted to calculate the absorbance at 540 nm. Maltose was considered as the starting point and the activity of amylase was measured in moles of maltose released from starch at that temperature per minute.

3.26.3. Protease

The Drapeau's (1974) casein digestion technique was used to evaluate proteases. The enzyme-reaction mixture was incubated in Tris phosphate buffer (0.05 M) containing 1% casein (w/v) for 5 min (pH 7.8) at 37 °C. After that, the tissue was homogenised and mixed with the other components. The reaction was stopped by adding 10% trichloroacetic acid (TCA) to the mixture after 10 min and filtering the entire contents. The blank was created by adding tissue homogenate to the reaction just before it was stopped with TCA without incubation. The amount of enzyme required to release 0.001 (A280) acid-soluble fragments per min at 37 °C and pH 7.8.

3.27. Enzyme of oxidative injury/stress

3.27.1. Superoxide dismutase (SOD)

SOD activity was assessed using the Misra and Fridovich (1972) method. The enzyme that oxidises the epinephrine-adrenochrome transition is included in the assay. The reaction mixture consists of 50 µL, 1.5 mL phosphate buffer, and 0.5 mL epinephrine. After the solution had been thoroughly mixed, the optical density change was measured in a Transasia Elisa reader for 2.0 min. Action of one unit of SOD was equal to the amount of protein required to suppress epinephrine autooxidation by 50%.

3.27.2. Catalase

The Catalase activity (EC 1.11.1.7) was determined using the catalase method (Takahara et al., 1960). About 50 µL of homogenized tissue was mixed with

2.45 mL phosphate buffer (50 mM, pH 7.0), and the reaction was initiated with 1.0 mL of H_2O_2 solution. Decrease in the absorbance was measured at 240 nm for 2 min at 30-sec intervals. The enzyme blank is run with distilled water (1.0 mL) rather than hydrogen peroxides. The activity of the enzymes was measured in terms of moles of H_2O_2 decomposed per minute by mg protein.

3.27.3. Glutathione-s-Transferase (GST)

GST (Glutathione-s-transferase) catalyzes glutathione conjugation of a wide range of potentially toxic materials, including aliphatic, aromatic, heterocyclic, epoxides, and arene oxides. The method of Habig can be used to calculate GST (Habig et al., 1974).

3.28. Enzymes of neurotransmission, Acetylcholine esterase (AchE)

The Hestrin (1949) procedure, which was improved by Augstinasson (1957), was used to measure the activity of the enzyme AchE (E.C.3.1.1.7). The acetylcholine esterase assay used 1.0 mL acetylcholine (0.004 M, pH 4.0) substrate buffer mixture (1:9 dilution), 1.0 mL M/15 phosphate buffer (pH 7.2), and 0.2 mL homogenate, all of which were kept at 37 °C for 30 min. Alkaline hydroxylamine (2.0 mL) was added to complete the reaction. About 1.0 mL of HCl (2:1) was added and thoroughly mixed. The enzyme solution was poured into the control tubes. 1.0 mL $FeCl_3$ (10%) was added after careful mixing, and the OD was calculated at 540 nm to produce the colour. It is critical to mix the solution at each stage of this assay to avoid the trapping of air bubbles.

3.29. Serum hormones

3.29.1. Serum cortisol

Cortisol was quantified with the Immuno Assay Technique in fish serum (E0053Fi). The kit was imported from the Bio-Assay Technology Laboratory. The serum cortisol measured in the blood was $ngmL^{-1}$.

3.29.2. Serum Triiodothyronine (T3) and thyroxine (T4)

The enzyme immune assay technique and the bioassay technology laboratory (E0054Fi & E0055Fi) kit were used to identify the thyroid hormones (T3 and T4) in serum fish. The blood level of T3 and T4 was measured in ng mL^{-1}.

3.30. Gene expression

3.30.1. Total RNA Extraction and cDNA transcription

Total RNA was extracted from *L. rohita* brain tissue using an RNA isolation kit from Quigen Rnase mini kit, as directed by the manufacturer. Based on 1% agarose gel, two notable bands at the 28S and 18S were evaluated in the quality of the RNA following RNA extraction. Spectrophotometric verification of RNA purity and quantity was performed using a NanoDrop ND-1000 (Nano Drop Technologies Inc., USA). To ensure gDNA-free samples, Reverse transcriptase-deficient reactions (RT minus) were carried out. Using the Super Script III first-strand synthesis super mix for qRT-PCR, about one microgram of total RNA was reverse-transcribed for preparing cDNA (Invitrogen).

3.30.2. Mx, ISG15 and STAT1 genes amplification and Semi-quantitative PCR

Semi-qPCR PCR was done taking the cDNA sample, using specific primers and housekeeping gene β-actin.

3.30.3. qPCR analysis

Immune genes were deployed to evaluate the expression pattern in *Labeo rohita* by RT- PCR (qPCR). The Light Cycler 480 I master mix was used for qPCR (Roche, Germany). Amplification was carried out using a MX specific primer and the details of primer is given in Table (3). The amplicon size of Mx, ISG15, STAT1, and β-actin is,165, 200,146, and 265 bps, respectively.

Table 3. Details of the primer used for qPCR analysis

Primer	Forward	Reverse
Mx	5'-GTCCAGTACCACATGCTGGACC	5'- TTTGCCAGCACTCCTCAGGCGT-3'
ISG15	5'- GGCAAAAGATCGTGTCTCGT-3'	5'- CATCACGGCATTGAAAACA-3'
STAT1	5'- AGAAGGGCCAGGTCAAAACT-3'	5'- TCCACAGCCAGAATGGTACA-3'
β-actin	5'-TTCGAGCAGGAGATGGGCACTG- 3'	5'-GCATCCTGTCAGCAATGCCA-3'

Primers were designed using Primer 3 software. The Primer efficacy output was checked based upon the standard curve line slope, and a melting curve. Approximately 1.0 µL of 10-fold diluted cDNA was mixed with 0.5 µL (5 pmol) of each primer of Mx forward primer and reverse primer (10 µL) and 5 µL of 2x I master mix on the Light Cycler 480 SYBR Green (Roche, Germany), 3 µL of nuclease-free water provided in the kit in a final volume of 10 µL. The qPCR amplification was performed in triplicates with the β-actin (housekeeping gene) as control under the following conditions: 95 ℃ for 10 min, followed by 40 cycles of denaturation at 95 ℃ for 10 sec, amplification at 55 ℃ for 10 sec and extension at 72 ℃ for 10 sec.

The 2−ΔΔCT technique was used to calculate the relative gene expression of the targeted genes in comparison to the reference gene (β-actin). The quantification cycle (cq) values for each gene were calculated using Light Cycle SW 1.1 software and a second derivative, maximal method for absolute quantification. When the efficiency was ~100%, $2^{-\Delta\Delta Cq}$ fold was calculated by method (Livak and Schmittgen, 2001) taking the β-actin relative gene expression. $2^{-\Delta\Delta Cq}$ was used to compute the fold expression for each sample in relation to the calibrator. Each group's average (in triplicate) folding expression was derived and presented as a median value. The 1.5% agarose gel was used to measure the desired length of the band for 8 µl of the qRT-PCR product.

3.31. Gut microbes

DNA isolation and 16s rRNA amplicon-based Illumina Library preparation The DNA was isolated from the given samples and the amount of DNA in the sample was

calculated. using Qubit Fluorimeter (V.3.0). V3 Forward primer: CCTACGGGNBGCASCAG and V4 Reverse primer: GACTACNVGGGTATCTAATCC were used to amplify the V3-V4 region of 16S rRNA. On a 2% agarose gel, the amplified product was tested, and gel purification was performed to eliminate non-specific amplifications. A 5.0 ng amplified product has been developed for library preparation with the NEB Next Ultra DNA library preparation kit. The Agilent 2200 Tape Station was used to quantify the library and estimate its quality. The Illumina HiSeq 2500 platform was used to sequence the prepared library. The library preparation steps are illustrated (Fig. 3).

3.32. Histoarchitectural changes

For the histopathological study, gill, liver, kidney and intestine were isolated from the normal and experimental fish. Tissues were then fixed in a neutral formalin buffered solution of 10% and processed through a series of graded alcohols before they were cleared with chloroform and embedded in paraffin wax. With the assistance of a Leica RM2125RTS microtome, sections were trimmed at 5-micron thickness, stained with eosin as well as haematoxylin (Humason, 1972), and mounted in D.P.X. The images of prepared sections were taken at 100X, 200X & 400X magnification under axiostar carl zeiss microscope.

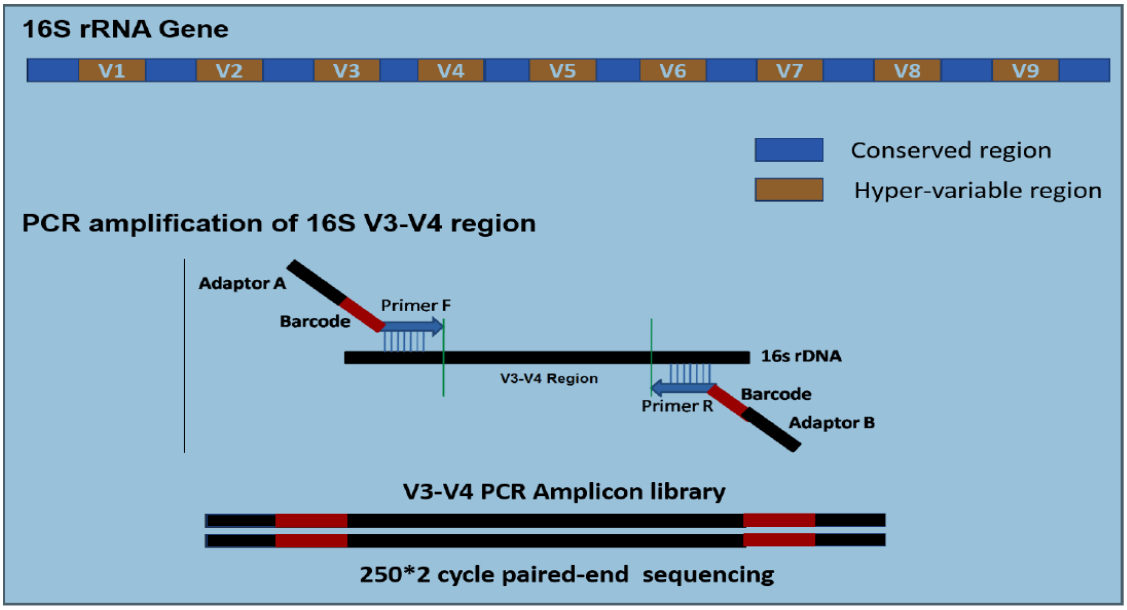


Fig. 3. Graphical representation of 16S rRNA V3-V4 library preparation and sequencing 3 Bioinformatics analysis pipeline.

3.33. Estimation of immunoglobin (IgM) by ELISA

The IgM level was asses using ELISA following Anil et al. (2002) with few modifications. Briefly, serum was diluted in 50 mM carbonate–bicarbonate buffer (1:1000) maintaining pH at 9.5 which was further coated with ELISA plate and kept for incubation at 4 ℃ for overnight. The plate was subjected to thrice washing with washing buffer prepared in 10 mM PBS of 0.05% Tween-20 for removing unbound antigen. Further, wells were blocked in 200 µ blocking solution of 5% skimmed milk for 1 h at RT. Plates were washed thrice with washing buffer to pour off the blocking solution. The coating of anti-MrNV serum from immunised mice was diluted properly and inoculated 100 µL/well for 1.3 h. Again plate was washed thrice with washing buffer following conjugation with anti-mouse IgM HRP in 1:2000 dilution in 3% BSA for 45 min at RT. Washing was done thrice for removing the unconjugated. A total of 100 µL Tetramethyl benzidine (TMB)/H_2O_2 in DW (1:20) was added and kept for incubation for 5-10 min. For stopping the reaction 100 µL of 2 N H_2SO_4. Using an ELISA reader, the OD was measured at 450 nm (Bio-Tek Instruments Inc., USA).

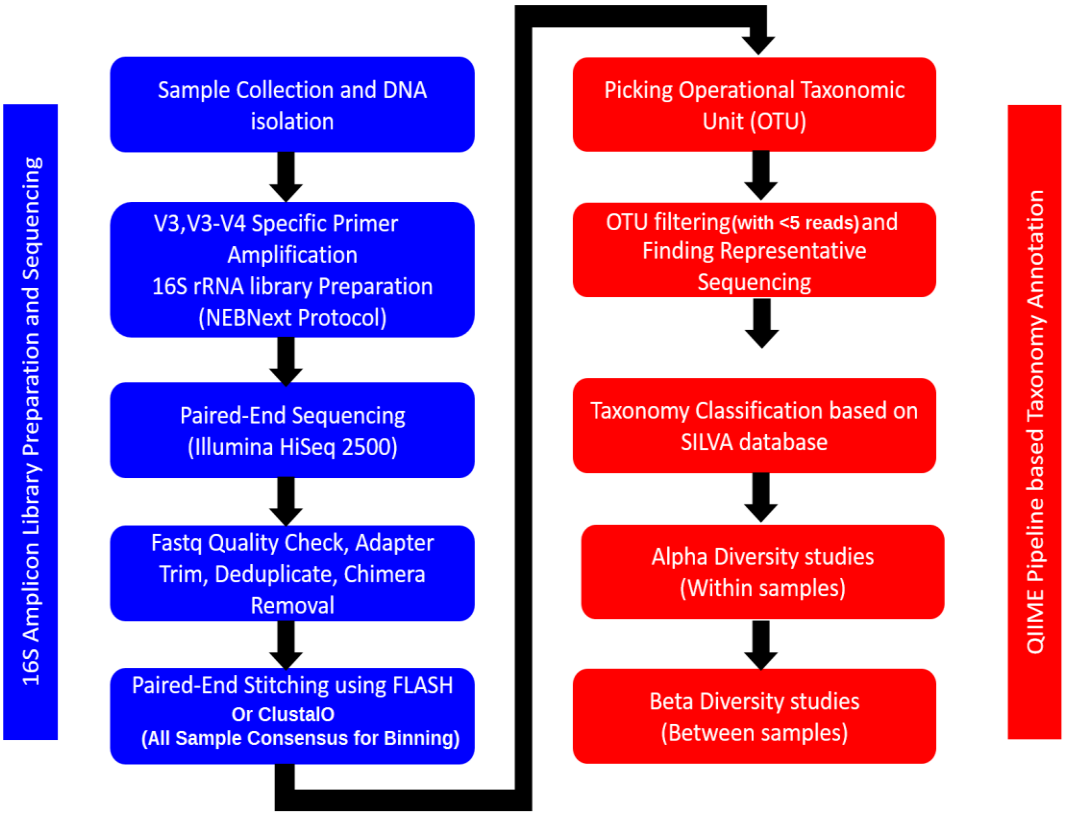


Fig. 4. The bioinformatics analysis pipeline for the V3-V4 metagenomics

Schematic representation of the bioinformatics pipeline is presented in Fig. 4. The following analysis (NGS P6297 Amplicon (V3-V4) Metagenome Seq. CIFRI is performed as mentioned below.

I. Fast Quality checking: Base quality, base composition, GC content

II. From paired-end data, filtering and identification of the V3-V4 region

a. Identification and read trimming of V3-V4 sequences

b. Building consensus sequence from paired-end reads

III. Operational Taxonomy Unit (OTUs), Taxonomy classification and Relative abundance

a. Identification of OTUs

b. Assignment of taxonomy to each OTU

c. Identification of number of reads and abundancy of OTU

IV. Alpha diversity with samples and rarefaction curves

a. Shannon

b. Chao1

c. Observed species

V. Beta diversity between samples and beta diversity plots (only for multiple samples)

a. Distance matrix calculation using different approaches

b. Principal component analysis

3.34. Data analysis and editing

The values were represented as mean± SE. The data were analysed in Microsoft Excel v.16, and significance was determined using SPSS 20 in accordance with Duncan's multiple range test (DMRT) (Duncan, 1955) to determine significance difference at 5% ($p < 0.05$). Paint 3D v.16 was used to edit the photos. The PAST4.01 and Minitab 18 software's were used to determine overall networking, association patterns, significance and ordination scaleograms between solvent extracts based on antibiogram studies, toxicity parameters, and antioxidant potential. LAB IMAGE software was used to quantify and characterise the gels (Bio-Rad). The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before and after challenge study and this is represented in the result section by double astreik (**).

4. RESULTS

4.1. Antibiogram profiling

The antibiogram profiling and MIC values of solvent extracts against bacterial isolates given below.

4.1.1. Antibacterial screening

The antibacterial screening results of arjuna extracts showed various patterns of their efficacy in the selected microorganism including gram (+) ve, gram (-) ve, OTC sensitive, OTC resistant and partially resistant. Overall maximum efficacy in maximum numbers of bacteria was showed by bark extracts followed by leaf and fruit. However, in some bacterial strains such as B10 and B11, better efficacy was reported in the order of leaf, fruit, and bark, respectively (Table 4). The maximum zone of inhibition showed by B1 (20.83±0.10 mm) and B2 (15.5±0.23 mm) for ethanolic extract of bark. The values were found equal for ethanolic extracts of fruit and leaf (Table 5a).

Anti-biogram profile of 17 bacterial strains against 21 solvent extracts, indicated strain specific activity of the particular solvent extract. For instance, most of the cases, ethanolic and methanolic extracts of bark and leaf showed wider efficacy, however, acetone extract of bark also recorded to exhibit the maximum anti-biogram against *Stayphylococcus aureaus* (SA-5) represented as B17. Out of the 17 bacterial strains, 11 were gram negative which have showed maximum anti-bio-gram as, ETML-3, AH-1, VA-06 being most susceptible.

Ethanolic extract of arjuna bark was found to be most effective solvent extract showing maximum antibacterial activity against most of the bacterial strains particularly, *Edwardsiella tarda* (ETML-3) represented as B1, and *Aeromonas hydrophila* (AH-01) represented as B6 (OTC resistant), witnessed by zone of inhibition (Plate 1).

4.1.1.1. MIC values of bacterial strains

The combined (over-all solvent extracts) MIC value (µg mL^{-1}) ranged from 7±0.20-18±0.16; 9±0.16-19±0.07; 9±0.16-18±0.16, was found to be in order of bark, fruit and leaf, respectively. The minimum MIC was observed for B1 (7±0.20 µg mL^{-1}) and B2 (7±0.20 µg mL^{-1}) for ethanolic extract of bark and maximum by B6 and B7 for fruit, leaf and bark extract of distilled water (Table 5b).

Table 4. Test microorganisms and their characteristics

Sl. No.	Name of bacteria	Strain	Sample code	gm (–) ve/ + ve	Reaction against OTC	Effective solvent extracts
1	*Aeromonas hydrophila*	AH-01	B6	(-) ve	R	Br5, Br4, F4, Br6
2	*Aeromonas hydrophila*	AH-04	B7	(-) ve	S	L6
3	*Aeromonas hydrophila*	AH-11	B8	(-) ve	S	Br5
4	*Aeromonas hydrophila*	AH-05	B12	(-) ve	R	L4, Br5
5	*Acinetobater pittii*	ACI-10	B10	(-) ve	R	Br5, Br4, F4, F6, F5
6	*AcinetobaterJunii*	ACI-02	B16	(-) ve	S	Br5, F6
7	*Edwardsiella tarda*	ETML-3	B1	(-) ve	S	Br4, F4, Br5, Br6
8	*Edwardsiella tarda*	ETML-14	B15	(-) ve	S	F6, L6, Br5
9	*Flavobacterium columnare*	FC-50	B5	(-) ve	S	F5, Br6, L6
10	*Pseudomonas aeruginosa*	PA-06	B2	(-) ve	S	Br5
11	*Pseudomonas aeruginosa*	PA-02	B13	(-) ve	R	Br4, F4, Br5, L5, Br6
12	*Pseudomonas putida*	PP-03	B3	(-) ve	R	Br5, Br4, F6, Br6
13	*Pseudomonas putida*	PP-04	B11	(-) ve	S	Br5
14	*Staphylococcus aureus*	SA-01	B4	(+) ve	S	Br5, F5, Br4
15	*Staphylococcus aureus*	SA-05	B17	(+) ve	S	F6, F5
16	*Vibrio anguillarum*	VA-20	B9	(-) ve	PR	Br5, Br6, F6
17	*Vibrio anguillarum*	VA-06	B14	(-) ve	S	Br4, Br5, Br6

S: Sensitive; **R:** Resistant; **PR:** Partial Resistant
L4: Acetone extract of arjuna leaf; **L5:** Ethanol extract of arjuna leaf; **L6:** Methanol extract of arjuna leaf; **Br4:** Acetone extract of arjuna bark; **Br5:** Ethanol extract of arjuna bark; **Br6:** Methanol extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **F5:** Ethanol extract of arjuna fruit; **F6:** Methanol extract of arjuna fruit.

Table 5a. Antibiogram (zone of inhibition in mm) of solvent extract against bacterial isolates

Code	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**
L4	14±0.10	9±0.31	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	8±0.35
L5	19.5±0.16	11.5±0.06	7.5±0.28	8.33±0.23	9.5±0.07	9.5±0.07	10±0.14	10.5±0.20	10.5±0.34
L6	14.83±0.08	14.33±0.09	7±0.40	8±0.35	11±0.13	10±0.28	11±0.13	9.5±0.22	0±0.0
Br4	16.2±0.02	0±0.0	0±0.0	11±0.26	10±0.28	9.5±0.37	9±0.31	0±0.0	0±0.0
Br5	20.83±0.10	15.5±0.23	0±0.0	12.17±0.31	10.5±0.20	11.83±0.21	10.83±0.24	8±0.18	7.5±0.28
Br6	15.5±0.08	15.33±0.12	9.5±0.22	12.33±0.21	10.5±0.20	12±0.0	11±0.13	8±0.18	7.5±0.28
F4	15.33±0.07	4.5±1.41	10.92±0.20	10.42±0.32	9.61±0.25	9.81±0.33	0±0.0	0±0.0	0±0.0
F5	19.5±0.06	12.5±0.17	15.7±0.13	15.5±0.13	14.6±0.14	15.3±0.13	11.08±0.20	8±0.18	0±0.0
F6	18.83±0.06	11.5±0.06	15.17±0.06	15.17±0.12	13.94±0.06	14.76±0.06	10.5±0.20	8.5±0.08	0±0.0
OTCL	25.2±0.03	26.6±0.03	0±0.0	24.5±0.03	24±0.06	0±0.0	24.5±0.03	24±0.06	20.5±0.03
OTCBr	25.17±0.03	23.67±0.03	0±0.0	23.5±0.03	23±0.06	0±0.0	23±0.06	22.5±0.09	20.5±0.03
OTCF	25.17±0.03	25.17±0.08	0±0.0	24±0.06	24.5±0.03	0±0.0	25±0.06	22.5±0.03	21±0.07
Code	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**	**B16**	**B17**	
L4	0±0.0	0±0.0	9.57±0.26	0±0.0	0±0.0	0±0.0	10.4±0.22	0±0.0	
L5	0±0.0	13±0.25	0±0.0	11.73±0.23	0±0.0	0±0.0	0±0.0	0±0.0	
L6	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	9.33±0.20	
Br4	9±0.31	0±0.0	9.67±0.24	0±0.0	10±0.14	11.83±0.22	9±0.16	0±0.0	
Br5	11±0.26	0±0.0	16.5±0.13	14.5±0.15	15.83±0.16	14.17±0.12	15.33±0.22	11.67±0.20	
Br6	8.5±0.25	0±0.0	9.33±0.20	11±0.04	10.17±0.16	0±0.0	0±0.0	9.33±0.20	
F4	0±0.0	0±0.0	0±0.0	11.33±0.29	11.83±0.23	0±0.0	0±0.0	0±0.0	
F5	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	10.17±0.16	11.08±0.20	11.23±0.18	
F6	11.58±0.25	11.08±0.33	0±0.0	0±0.0	11.73±0.23	0±0.0	0±0.0	0±0.0	
OTCL	24.5±0.03	21.5±0.03	0±0.0	0±0.0	20.5±0.03	0±0.0	22±0.06	23.67±0.59	
OTCBr	24±0.06	22±0.0	0±0.0	0±0.0	22±0.06	0±0.0	22±0.13	23.67±0.59	
OTCF	22±0.06	22.5±0.03	0±0.0	0±0.0	21±0.06	0±0.0	21.5±0.03	23.67±0.59	

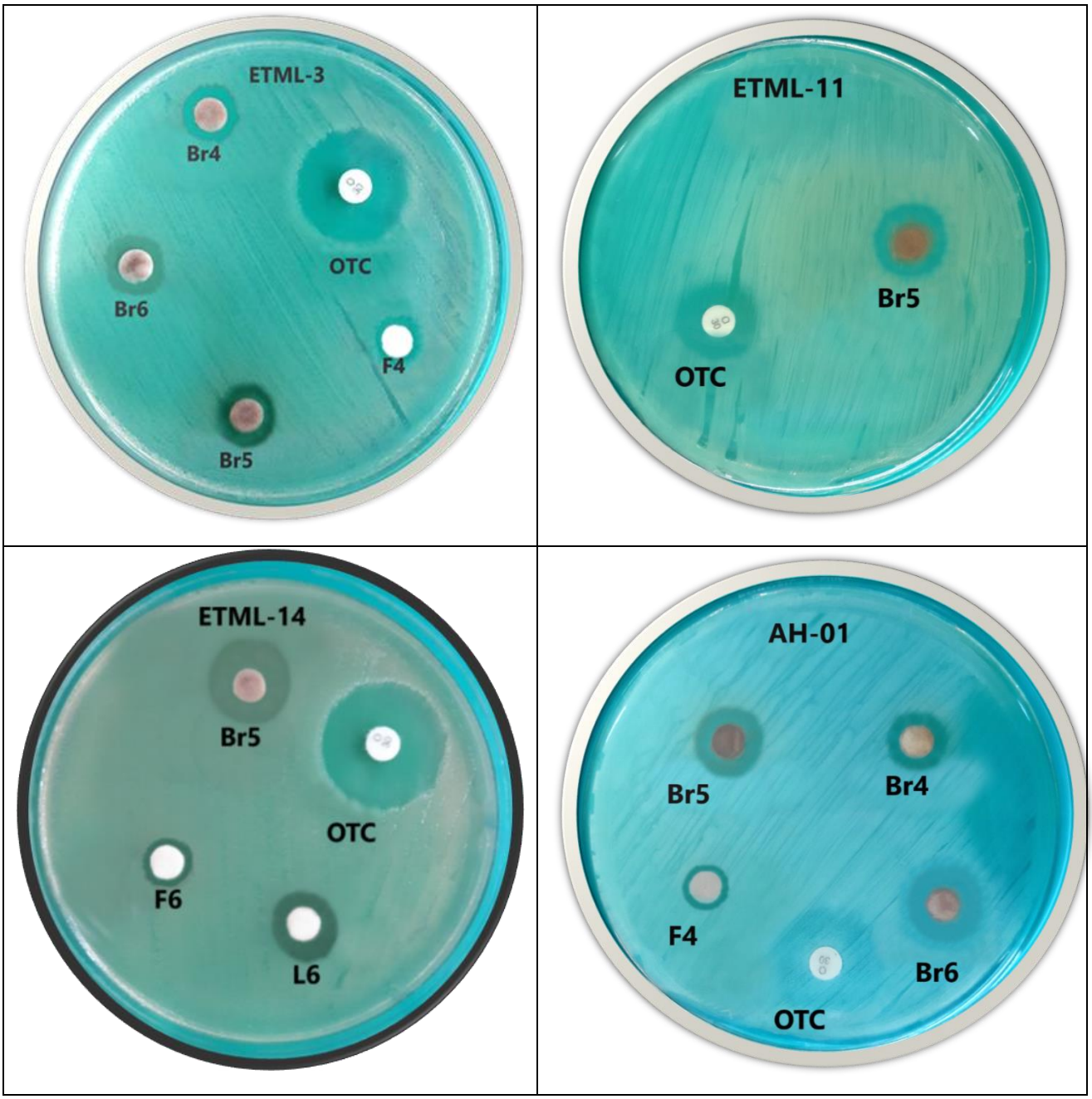
Values are represented as Mean± SE, n=3; **L4:** Acetone extract of arjuna leaf; **L5:** Ethanol extract of arjuna leaf; **L6:** Methanol extract of arjuna leaf; **Br4:** Acetone extract of arjuna bark; **Br5:** Ethanol extract of arjuna bark; **Br6:** Methanol extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **F5:** Ethanol extract of arjuna fruit; **F6:** Methanol extract of arjuna fruit; **OTCL:** Oxytetracycline in leaf extract**; OTCBr:** Oxytetracycline in bark extract**; OTCF:** Oxytetracycline in fruit extract**.**

Table 5b. The minimum inhibitory concentration (mg L^{-1}) values of bacterial strains v/s solvent extracts

	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**
L2	19±0.07	19±0.07	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
L3	15±0.09	15±0.09	23±0.06	22±0.0	22±0.0	22±0.0	21±0.07	23±0.06	23±0.06
L4	11±0.13	11±0.13	19±0.07	19±0.07	19±0.07	19±0.07	19±0.07	19±0.07	17±0.25
L5	9±0.16	9±0.16	11±0.13	10±0.28	11±0.13	11±0.13	13±0.11	11±0.39	12±0.47
L6	11±0.13	11±0.13	10±0.28	10±0.28	12±0.47	12±0.47	13±0.33	13±0.33	20±0.14
L7	20±0.14	20±0.14	25±0.06	25±0.06	25±0.06	25±0.06	25±0.06	25±0.06	0±0.00
Br2	18±0.16	18±0.16	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Br3	13±0.10	13±0.11	23±0.06	22±0.00	22±0.00	22±0.0	21±0.07	23±0.06	23±0.06
Br4	8±0.00	8±0.00	19±0.07	16±0.18	10±0.28	11±0.39	15±0.09	19±0.07	20±0.14
Br5	7±0.20	7±0.20	11±0.13	9±0.16	10±0.28	9±0.16	9±0.16	15±0.28	15±0.09
Br6	9±0.16	9±0.16	10±0.28	10±0.28	12±0.47	12±0.47	12±0.24	16±0.18	17±0.25
Br7	19±0.07	19±0.07	25±0.05	25±0.05	25±0.06	25±0.06	25±0.06	25±0.06	0±0.00
F2	18±0.16	18±0.16	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
F3	13±0.10	13±0.11	23±0.06	22±0.00	22±0.0	22±0.0	21±0.07	23±0.06	23±0.06
F4	9±0.16	9±0.16	18±0.16	16±0.18	19±0.07	19±0.07	19±0.07	19±0.07	21±0.07
F5	9±0.16	9±0.16	10±0.28	11±0.13	11±0.13	11±0.13	11±0.13	15±0.28	19±0.07
F6	9±0.16	9±0.16	11±0.13	14±0.40	10±0.28	12±0.47	12±0.24	16±0.18	19±0.07
F7	19±0.07	19±0.07	23±0.06	25±0.05	25±0.06	17±0.25	25±0.06	25±0.06	0±0.00
	B10	**B11**	**B12**	**B13**	**B14**	**B15**	**B16**	**B17**	
L2	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.0	0±0.0	0±0.0	
L3	23±0.06	23±0.06	17±0.42	17±0.42	0±0.00	0±0.0	0±0.0	0±0.0	
L4	20±0.00	20±0.00	16±0.0	16±0.0	0±0.00	20±0.14	20±0.14	20±0.14	
L5	19±0.07	9±0.16	18±0.0	12±0.47	19±0.07	18±0.16	18±0.16	18±0.16	
L6	21±0.07	21±0.07	21±0.07	21±0.07	21±0.07	21±0.07	21±0.07	15±0.09	
L7	23±0.06	23±0.06	23±0.06	23±0.06	0±0.00	0±0.00	0±0.00	22±0.13	
Br2	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	
Br3	23±0.06	23±0.06	21±0.07	14±0.20	0±0.0	0±0.0	0±0.00	0±0.00	
Br4	17±0.23	20±0.14	14±0.20	17±0.08	14±0.20	20±0.14	12±0.47	20±0.14	
Br5	12±0.47	19±0.07	10±0.28	11±0.39	12±0.47	11±0.39	10±0.57	18±0.16	
Br6	15±0.28	21±0.07	19±0.22	14±0.40	15±0.28	21±0.07	13±0.54	12±0.24	
Br7	23±0.06	23±0.06	23±0.06	23±0.06	0±0.00	0±0.00	0±0.00	22±0.13	
F2	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	
F3	23±0.06	23±0.06	21±0.07	21±0.20	0±0.00	0±0.00	0±0.00	0±0.00	
F4	17±0.25	20±0.14	14±0.20	13±0.54	13±0.33	20±0.14	20±0.14	20±0.14	
F5	18±0.16	19±0.07	14±0.20	19±0.07	17±0.08	12±0.24	18±0.16	11±0.39	
F6	13±0.33	10±0.28	19±0.22	19±0.07	15±0.28	21±0.07	15±0.09	13±0.33	
F7	23±0.06	23±0.06	23±0.06	23±0.06	0±0.00	0±0.00	22±0.13	22±0.13	

Values are represented as Mean± SE, n=3; **L2:** Ethyl acetate extract of arjuna leaf; **L3:** Chloroform extract of arjuna leaf; **L4:** Acetone extract of arjuna leaf; **L5:** Ethanol extract of arjuna leaf; **L6:** Methanol extract of arjuna leaf; **L7:** Distilled water extract of arjuna leaf; **Br2:** Ethyl acetate extract of arjuna bark; **Br3:** Chloroform extract of arjuna bark; **Br4:** Acetone extract of arjuna bark; **Br5:** Ethanol extract of arjuna bark; **Br6:** Methanol extract of arjuna bark; **Br7:** Distilled water extract of arjuna bark; **F2:** Ethyl acetate extract of arjuna fruit; **F3:** Chloroform extract of arjuna fruit; **F4:** Acetone extract of arjuna fruit; **F5:** Ethanol extract of arjuna fruit; **F6:** Methanol extract of arjuna fruit; **F7:** Distilled water extract of arjuna fruit.

PLATE 1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F4
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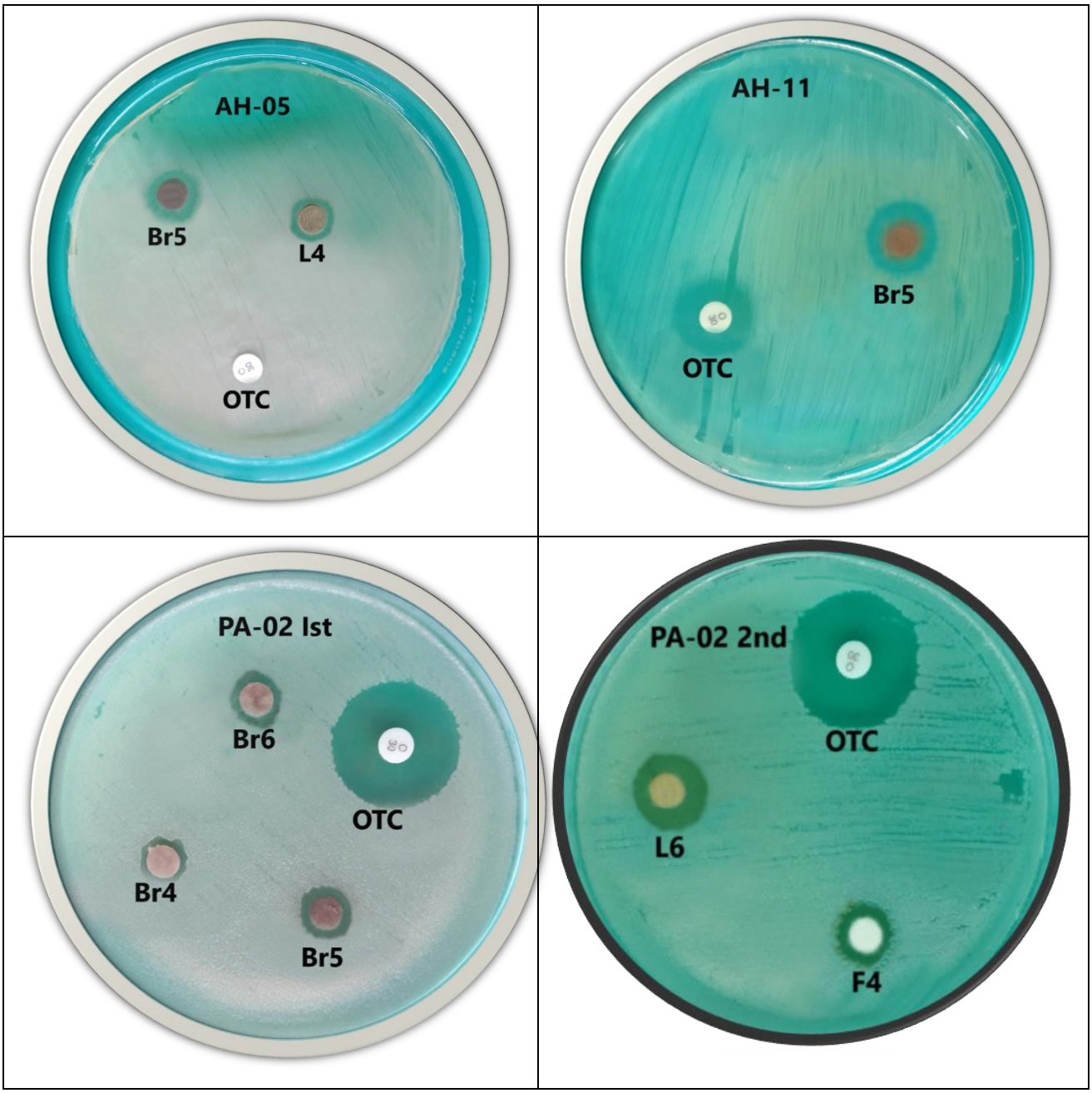
F4
Br6
OTC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Br5
L4
OTC
AH-11
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OTC
PA-02 Ist
Br6
OTC
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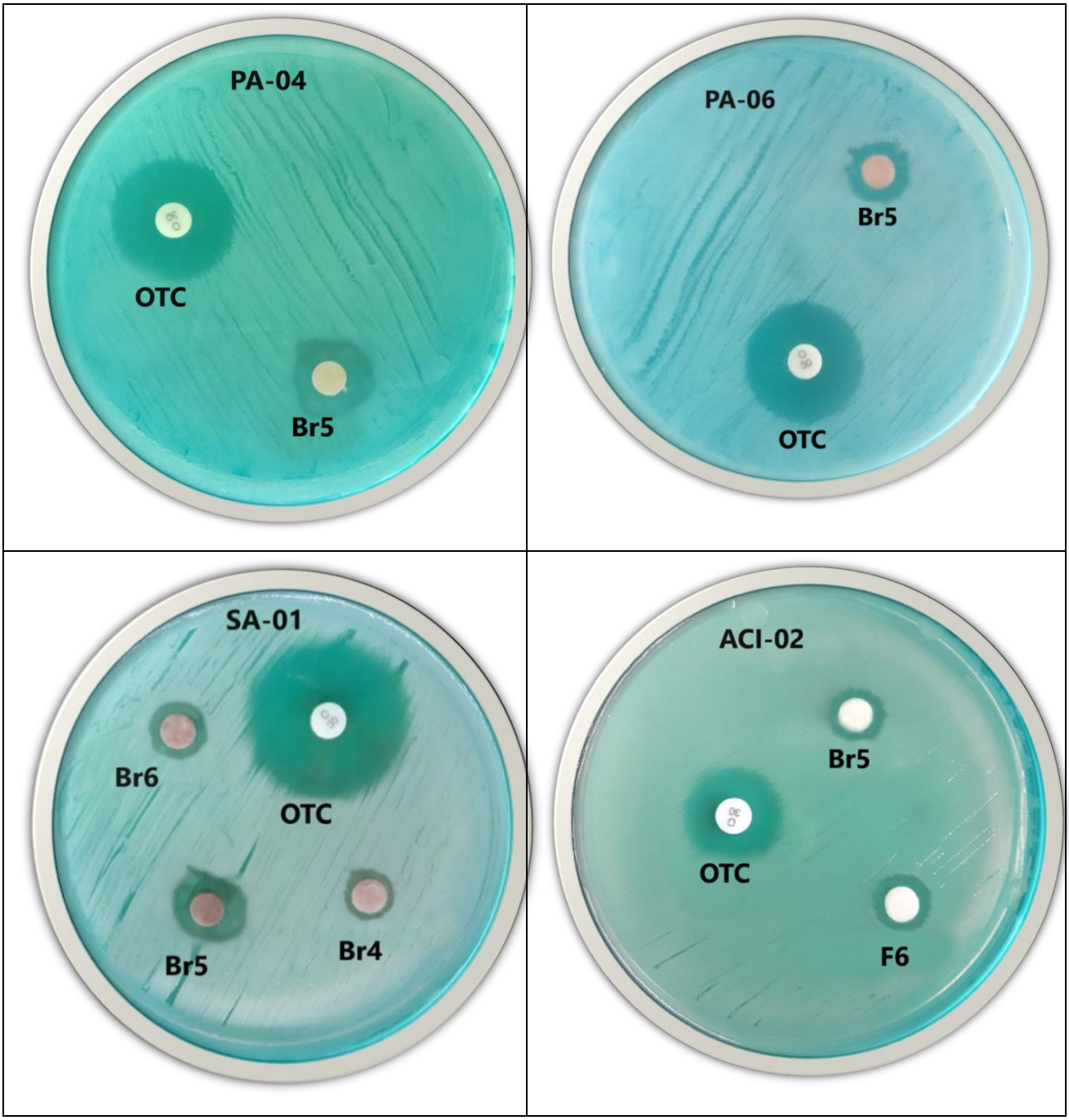
PA-02 2nd
OTC
L6
F4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OTC
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Br5
Br4
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Br5
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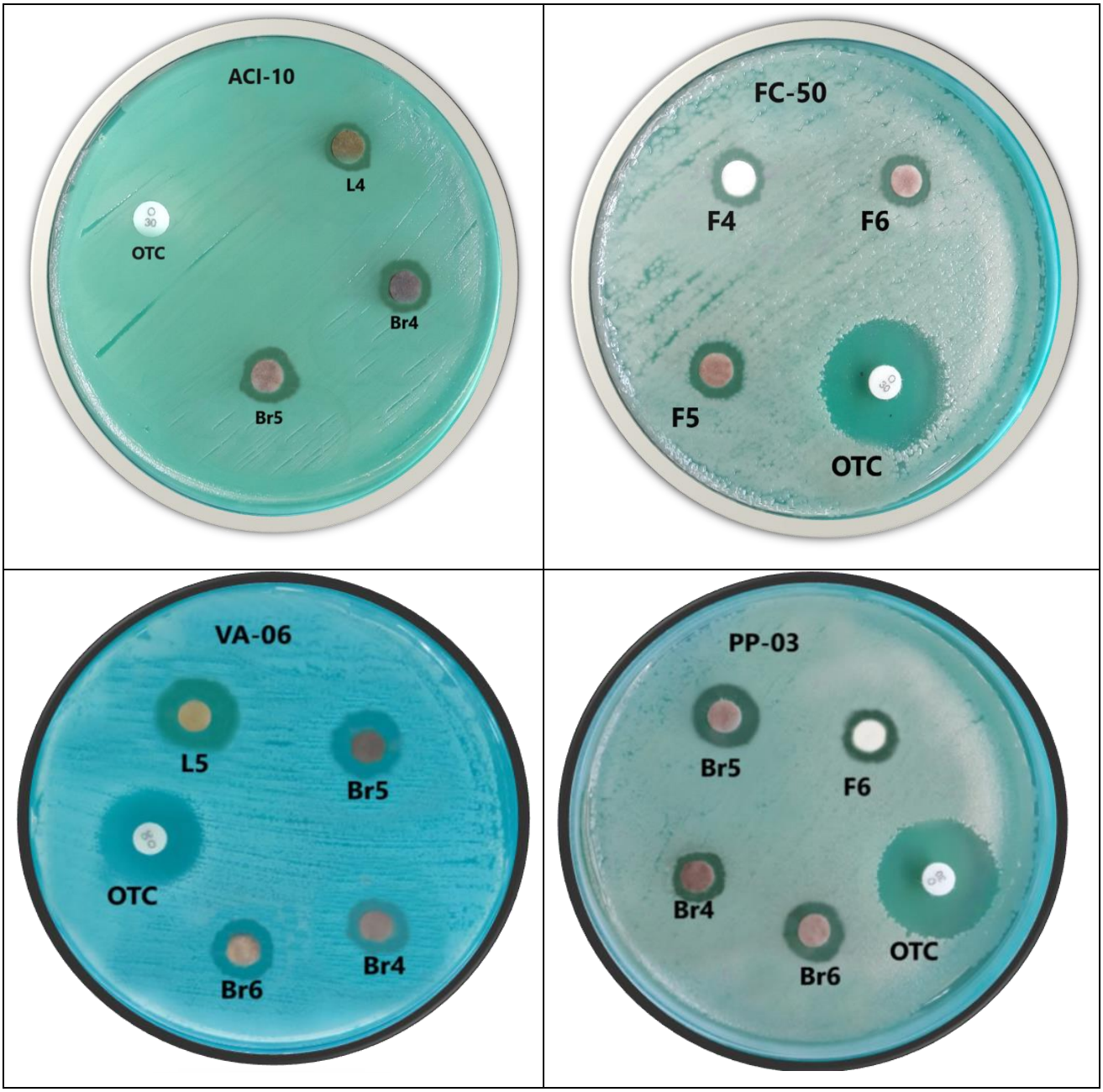
F6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Br5
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Br4
PP-03
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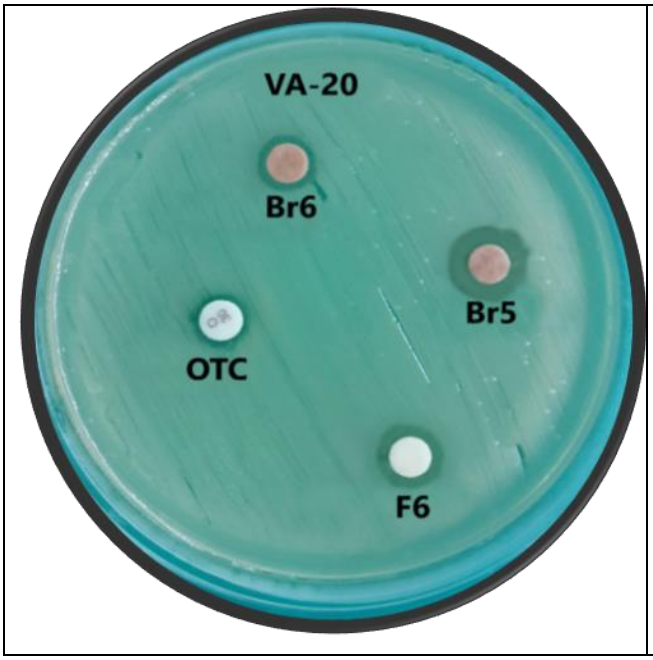
F6
Br4
Br6
OTC



***Aeromonas hydrophila*-AH-01; *Aeromonas hydrophila*-AH-04; *Aeromonas hydrophila*-AH-11*; Aeromonas hydrophila*-AH-05; *Acinetobater pittii*-ACI-10; *AcinetobaterJunii*-ACI-02; *Edwardsiella tarda*-ETML-3; *Edwardsiella tarda*-ETML-14; *Flavobacterium columnare*-FC-50; *Pseudomonas aeruginosa*-PA-06; *Pseudomonas aeruginosa*-PA-02; *Pseudomonas putida*-PP-03; *Pseudomonas putida*-PP-04; *Staphylococcus aureus*-SA-01; *Staphylococcus aureus*-SA-05; *Vibrio anguillarum* VA-20; *Vibrio anguillarum*-VA-06.**

Plate 1. Showing zone of inhibition of solvent extracts of *T. arjuna* against specific bacterial isolates

4.1.2. Antifungal activity

The ethanolic extracts of bark and leaf exhibited the antibiogram as, 10± 0.36 mm and 13± 0.53 mm, respectively towards *Aphanomyces invadans* (Plate 2). Other solvent extracts did not show any zone of inhibition for fungus.

4.1.3. Antiparasitic activity

4.1.3.1. *In-vitro* screening of selected solvent extracts and efficacy study of effective solvent extracts

The solvent extracts F4, Br4, Br5, Br6, and F6 was selected as best five showing efficacy against *A. bengalensis,* thus were taken as test solvent extract for further studies.

PLATE 2

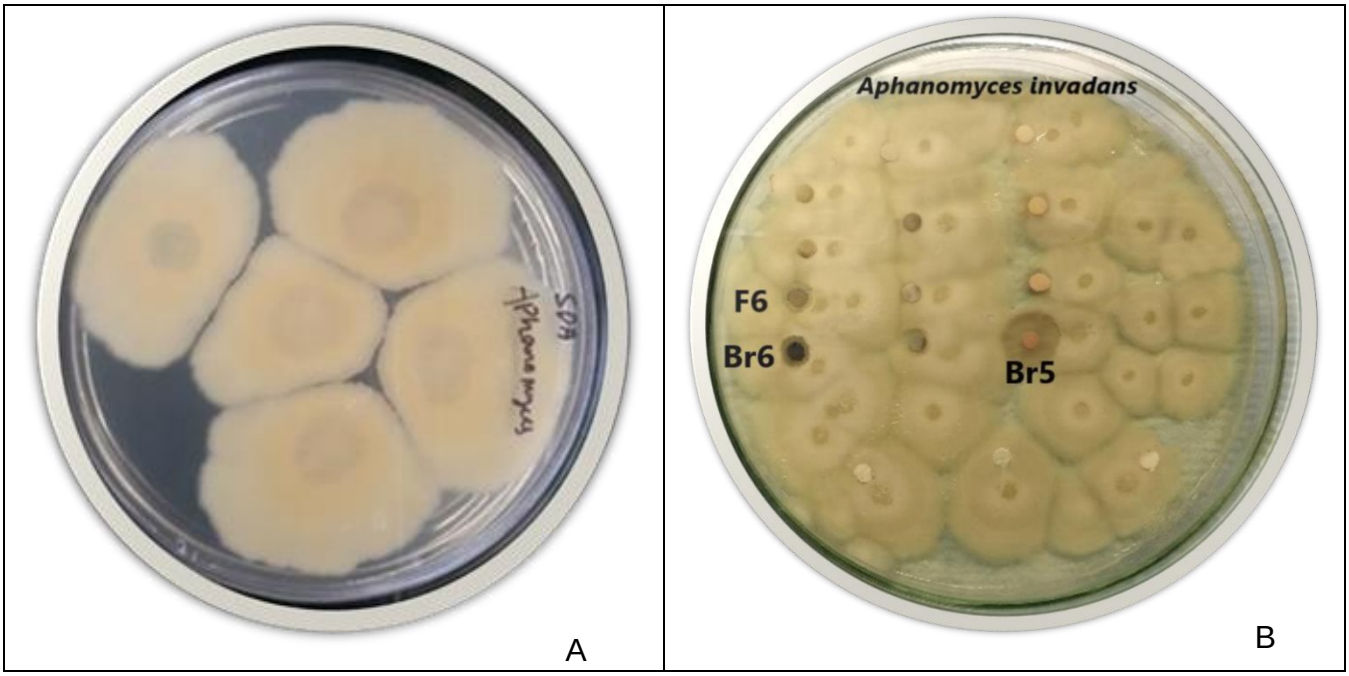


Plate 2. Showing A) growing, *Aphanomyces invadans* on SDB plate and B) zone of inhibition of effective solvent extracts against *A. invadans*.

4.1.3.2. Estimation of LC_{50} of solvent extracts against *L. rohita* and *Argulus bengalensis* under *in-vivo* and *in-vitro* experiments

The result of *in-vivo* toxicity study of five solvent extract of *T. arjuna* showed that Br4 showed least toxicity as indicated by LC_{50} value (854.12 mg L^{-1}) followed by F6 (442.56 mg L^{-1}), F4 (288.35 mg L^{-1}), Br6 (228.43 mg L^{-1}) and Br5 (213.21 mg L^{-1}) at 12 h time interval while minimum values were reported at 60 h time interval as 67.67, 78.13, 79.12, 156.47 and 256.43 mg L^{-1} for Br5, Br6, F4, F6 and Br4, respectively (Table 6).

For all five extracts, LC_{50} value was decreasing as time of exposure increases and maximum LC_{50} was reported in Br4-IN (218.93 mg L^{-1}) at 1 h interval and LC_{50} at 5 h interval was minimum for each solvent extract and reported least in Br5-IN (19.14 mg L^{-1}) and maximum in Br4-IN (22.56. 8 mg L^{-1}).

4.1.3.3. Estimation of Relative survival percentage (RPS) of *L. rohita* and anti-parasitic efficacy (AE) percentage of solvent extracts against *Argulus bengalnesis*

Under *in-vivo* condition, the AE in bath treatment was found to be higher at 36, 48 and 60 h intervals for Br5-50BA, at 48 and 60 h for F4-50BA, Br6-50BA and F4-50BA.The solvent extracts at different time interval and concentration showed a diverse pattern of distribution of AE *i.e.* 100% AE was showed by Br- 40 at 4 and 5 h; F4-40 at 5 h; Br6-40 at 5 h; Br4- 50 at 5 h; TF4-50 at 4 and 5 h; Br5-50 at 2, 3, 4 and 5 h; Br6-50 at 4 and 5 h and F6-50 at 5 h (Table 7a & b).

The LC_{50} value of each extract in immersion study was higher side as compared to the bath treatment. The trend of LC_{50} values for both treatments at every time interval following the order as Br4>F6>F4>Br6>Br5. Minimum LC_{50} values in immersion and bath treatments was reported in Br5-BA (27.92 mg L^{-1}) and Br5-IM (33.6 mg L^{-1}) at 60 h interval while maximum LC_{50} was reported in Br4-IM (554.72 mg L^{-1}) and Br4-BA (359.52 mg L^{-1}) at 12 h interval.

Comparative RPS of *L. rohita* under *in-vivo* study showed that in bath treatment RPS varied from >65% - ≥ 90 % and in immersion treatment, it was approximately ranged between 50 % - 85 %. F4-40BA (60 h), Br5-50BA (48 h) and F6-50BA (60 h) showed maximum RPS (≥ 80-90%) in bath treatment while in the case of immersion it was maximum in Br5-50IM (85%) at 60 h followed by Br4-50IM (≤ 80%) at 60 h Br4-50BA, Br5-50BA and TF4-50BA exhibited the RPS overlapping at 12, 24 and 36 h time interval whereas Br4-40IM, Br5-40IM, F4-30IM etc., were found to overlap at 12 and 24 h interval.

Table 6. Showing LC_{50} (mg L^{-1}) values of effective solvent extracts against *L. rohita* and against *A. bengalensis* under *in-vitro* and *in-vivo* treatments

Treatment	Solvent concentration	10-50 mg L^{-1}	10-50 mg L^{-1}	10-50 mg L^{-1}	10-50 mg L^{-1}	10-50 mg L^{-1}
***In-vivo* treatment (IN)**	Solvent extract/LC_{50}	**1 h**	**2 h**	**3 h**	**4 h**	**5 h**
	(DMSO mlL^{-1})	1560.23±12.45	1293±13.65	1068±15.64	989±16.18	876±15.87
	Br4-IN	218.93±8.45	163.21±9.38	100.62±11.83	88.55± 9.78	75.8±12.69
	F4-IN	129.04±7.67	100.67±10.45	54.06±6.98	39.05±9.37	34.96±6.79
	Br5-IN	80.51±11.23	28.55±4.59	16.31±4.13	14.9±3.68	13.14±3.79
	Br6-IN	123.15±12.56	78.85±8.45	31.05±6.64	25.07±2.67	22.55±2.69
	F6-IN	133.49±12.24	109.23±6.78	80.39±7.34	59.5±6.49	43.11±5.98
***In-vitro* (Immersion), IM**	Solvent extracts/LC_{50}	**12 h**	**24 h**	**36 h**	**48 h**	**60 h**
	(DMSO mlL^{-1})	2255.23±22.45	2089±18.69	1768±15.64	1489±16.18	1176±15.87
	Br4-IM	554.72±9.53	342.23±9.87	290.92±11.23	233.49±9.76	165.79±7.29
	F4-IM	164.21±8.91	117.96±7.84	67.48±8.97	52.65±8.48	34.37±8.95
	Br5-IM	107.41±5.46	94.51±5.72	62.17±4.67	45.06±5.27	33.6±7.58
	Br6-IM	125.82±9.49	99.4±7.23	77.96±6.28	57.32±7.34	50.77±5.89
	F6-IM	342.89±11.28	237.08±9.47	137.74±7.78	122.85±8.37	83.48±5.94
***In-vitro* (bath), BA**	Solvent extracts/LC_{50}	**12 h**	**24 h**	**36 h**	**48 h**	**60 h**
	(DMSO mlL^{-1})	1967±24±45	1745.34±37	1546.67±12.56	1342±15.56	1080.23±12.67
	Br4-BA	359.72±10.23	232.34±12.26	136.599±13.47	63.74±16.12	49.48±17.65
	F4-BA	119.22±12.56	107.49±14.23	57.07±9.34	53.18±13.23	41.75±12.27
	Br5-BA	95.28±9.45	79.89±6.89	57.57±8.95	53.32±7.34	27.92±9.56
	Br6-BA	115.12±8.25	97.89±6.78	68.35±7.34	46.47±7.28	36.33±7.45
	F6-BA	229.26±6.78	196.11±7.98	112.76±6.24	51.57±8.58	44.36±8.23
***In-vitro* (Fish)**	Solvent extracts/LC_{50}	**12 h**	**24 h**	**36 h**	**48 h**	**60 h**
	Br4-BA	854.12±15.56	482.21±6.78	382.22±7.89	287.12±7.23	265.43±8.93
	F4-BA	288.35±6.28	249.55±8.95	187.48±9.67	138.52±13.28	79.12±17.68
	Br5-BA	213.21±9.68	168.53±11.45	136.67±15.67	113.07±14.67	67.67±12.59
	Br6-BA	228.43±14.27	186.42±15.83	165.24±13.85	145.34±13.96	78.13±14.17

Values are represented as Mean± SE, n=3; **Br4:** Acetone extract of arjuna bark; **Br5:** Ethanol extract of arjuna bark; **Br6:** Methanol extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **F6:** Methanol extract of arjuna fruit.

Table 7a. Showing values of *in vivo* study of solvent extracts of *T. arjuna* against *A. bengalensis*

Solvent Extract/ time	Relative Percentage Survival of *L. rohita*					Mean Surviving *Argulus bengalensis*					Anti-parasitic efficacy (%)			
	12 h	24 h	36 h	48 h	60 h	12 h	24 h	36 h	48 h	60 h	24 h	36 h	48 h	60 h
CT BA	55	55	55	55	54	100	95	97	93	87	0	0	0	0
Br4-10BA	67	67	67	67	67	99	95	93	88	79	5	7	12	21
F4-10BA	67	70	73	77	80	94	87	82	75	69	13	18	25	31
Br5-10BA	70	73	77	80	83	91	83	74	69	63	17	26	31	37
Br6-10BA	67	70	73	77	80	93	85	77	72	68	15	23	28	32
TF6-10BA	67	67	70	70	73	96	91	87	81	74	9	13	19	26
Br4-20BA	70	70	73	73	73	92	83	79	74	69	12	17	20	24
F4-20BA	73	73	73	80	80	82	56	54	43	38	32	44	45	56
Br5-20BA	77	77	77	80	83	76	47	41	33	29	40	53	59	66
Br6-20BA	73	73	73	80	80	79	54	44	38	34	36	46	56	61
F6-20BA	70	70	73	73	77	88	66	64	53	48	27	34	35	46
Br4-30BA	70	73	77	77	77	87	81	78	72	69	19	21	27	30
F4-30BA	77	77	80	80	80	68	55	45	34	24	45	55	66	76
Br5-30BA	80	83	83	83	87	54	45	32	24	13	55	68	76	87
Br6-30BA	77	80	83	83	87	63	48	36	28	17	52	64	72	83
F6-30BA	70	73	77	77	77	78	63	56	44	33	37	43	56	66
Br4-40BA	73	77	80	80	80	77	71	68	62	59	28	31	37	39
F4-40BA	80	80	83	83	83	46	33	17	9	0	67	83	91	100
Br5-40BA	83	83	87	87	90	38	24	6	0	0	76	94	100	100
Br6-40BA	80	80	83	83	87	42	29	13	4	0	71	87	96	100
F6-40BA	73	77	80	83	83	52	42	22	17	6	58	78	83	94
Br4-50BA	77	80	80	83	83	57	41	38	32	23	59	61	67	70
F4-50BA	83	83	83	87	87	20	15	0	0	0	85	100	100	100
Br5-50BA	83	83	87	90	90	8	0	0	0	0	100	100	100	100
Br6-50BA	83	83	83	87	90	14	8	0	0	0	92	100	100	100
F6-50BA	77	80	83	87	87	36	19	4	0	0	81	96	100	100
CT- IM	52	52	51	50	50	100	97	95	92	85	0	0	0	0
Br4-10IM	50	50	53	53	53	99	96	94	90	82	4	6	10	18
F4-10IM	50	50	57	60	60	95	89	85	78	71	11	15	22	29
Br5-10IM	57	57	60	63	67	92	85	77	71	65	15	23	29	35
Br6-10IM	53	53	53	60	60	94	88	81	76	70	12	19	24	30
F6-10IM	50	50	53	57	60	97	93	91	85	79	7	9	15	21
Br4-20IM	50	53	57	57	60	94	86	81	77	72	14	17	19	25
F4-20IM	53	53	57	63	63	84	65	59	52	43	10	13	18	20

Br5-20IM	57	57	63	63	67	79	61	49	41	36	39	50	56	61
Br6-20IM	53	53	57	60	60	82	63	53	45	41	37	45	52	56
F6-20IM	50	53	57	57	60	86	71	66	58	51	29	32	39	46
Br4-30IM	57	57	60	63	67	90	83	81	75	71	17	17	23	26
F4-30IM	57	60	60	67	67	72	60	52	41	32	19	24	30	37
Br5-30IM	60	67	70	73	73	59	51	41	32	18	49	58	67	80
Br6-30IM	57	60	63	70	73	70	55	42	34	21	45	57	65	77
F6-30IM	57	57	60	63	67	82	68	62	53	40	32	36	45	57
Br4-40IM	57	60	63	67	67	82	75	71	68	60	23	26	29	35
F4-40IM	57	63	63	67	70	51	38	24	21	7	61	74	77	90
Br5-40IM	63	70	70	77	80	41	31	18	14	0	68	80	84	100
Br6-40IM	60	60	67	70	77	44	34	23	17	0	65	75	81	100
F6-40IM	57	60	63	67	70	56	47	29	24	13	52	68	74	84
Br4-50IM	57	60	67	70	73	61	47	42	25	17	52	55	62	63
F4-50IM	63	67	70	77	80	32	25	11	7	4	74	87	93	97
Br5-50IM	67	73	77	80	87	24	13	0	0	0	86	100	100	100
Br6-50IM	63	67	70	77	83	29	21	9	0	0	78	89	100	100
F6-50IM	63	67	70	73	77	41	23	14	0	0	76	84	100	100

CT: Control; **Br4:** Acetone extract of arjuna bark; **Br5:** Ethanol extract of arjuna bark; **Br6:** Methanol extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **F6:** Methanol extract of arjuna fruit; **BA:** Bath treatment with solvent extracts; **IM:** Immersion treatment with solven extracst; 10, 20, 30 40, and 50 indicate the concentration of the solvent extracts in mg L^{-1}; H: indicate time intervals in hour.

Table 7b. Showing values of *in-vitro* study of solvent extracts of *T. arjuna* against *A. bengalensis*

Solvent Extract/time	Mean Surviving *A. bengalensis*					Anti-parasitic efficacy percentage				
	1 h	2 h	3 h	4 h	5 h	1 h	2 h	3 h	4 h	5 h
CT- IN	99	96	93	89	83	0	0	0	0	0
Br4-10IN	94	88	82	76	70	6	12	16	22	28.6
F4-10IN	86	80	72	62	52	14	20	27	37	46.9
Br5-10IN	80	68	56	42	32	20	32	43	57	67.4
Br6-10IN	84	76	66	50	44	16	24	33	49	55.1
F6-10IN	88	84	76	70	62	12	16	22	29	36.7
Br4-20IN	90	84	76	66	60	10	14	22	31	37.5
F4-20IN	82	72	64	56	46	18	27	35	42	52.1
Br5-20IN	70	60	50	32	20	30	39	49	67	79.2
Br6-20IN	76	66	54	38	30	24	33	45	60	68.8
F4-20IN	86	78	72	64	54	14	20	27	33	43.8
Br4-30IN	78	66	58	52	46	22	33	41	47	53.1
F4-30IN	64	50	42	34	22	36	49	57	65	77.6
Br5-30IN	54	42	32	10	0	46	57	67	90	100
Br6-30IN	60	52	38	18	6	40	47	61	82	93.9
F6-30IN	70	58	50	42	28	30	41	49	57	71.4
Br4-40IN	62	54	44	34	22	38	45	55	65	77.6
F4-40IN	52	42	34	14	0	48	57	65	86	100
Br5-40IN	36	22	18	0	0	64	78	82	100	100
Br6-40IN	42	30	26	0	0	58	69	73	100	100
F6-40IN	58	50	38	26	6	42	49	61	73	93.9
Br4-50IN	46	34	24	14	0	54	65	76	86	100
F4-50IN	32	22	14	0	0	68	78	86	100	100
Br5-50IN	16	0	0	0	0	84	100	100	100	100
Br6-50IN	22	10	6	0	0	78	90	94	100	100
F6-50IN	38	30	18	6	0	62	69	82	94	100

The relative percentage survival is not applicable at 1, 2, 3, 4, and 5 h time inervals. Therefore, it has not provided in table 7b. Here, IN-*in-vitro*; 10, 20, 30, 40, and 50 indicate the concentration in mg L^{-1}; 10, 20, 30 40, and 50 indicate the concentration of the solvent extracts in mg L^{-1}; H: indicate time intervals in hour.

4.1.3.4. Estimation of Therapeutic Index (TI)

The values of therapeutic index for *in-vivo* and *in-vitro* treatments are given in Fig. 5. TI value is an index of efficacy of the solvent extracts against treated material

and exhibits an inverse relationship with LC_{50} of the corresponding solvent extracts. TI value ranged from 1.1 to 4.6. Maximum TI value was exhibited by Br4-IM (4.6)> F4-IM (3.9) > and Br4 (3.3), whereas, minimum value was recorded in Br5-BA (1.1) followed by Br5-IN (1.2) < Br6-BA (1.3).

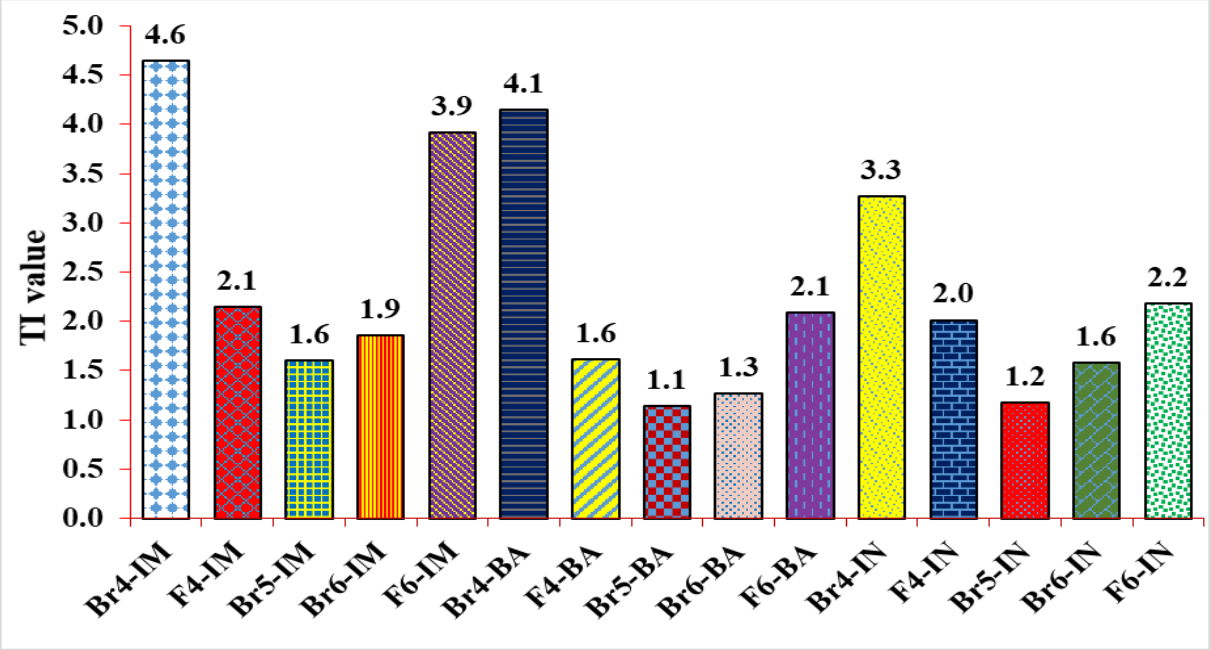


Fig. 5. Comparative TI value for the corresponding solvent extracts against *A. bengalensis*

4.2. Assessment of toxicity of herbal extract using *Artemia salina* model

4.2.1. Estimation of LD_{50}, absolute LC_{50}, absolute LC_{50} %, relative LD_{50} %, and fixing the toxicity level

The figure 6, demonstrate parameters of toxicity studies of mother solvents carried in BSLA. The maximum LC_{50} of was recorded in distilled water (1081.30 µg mL^{-1}) followed by dimethylsulfoxide (1029.00 µg mL^{-1}), and lowest by chloroform (93.36 µg mL^{-1}). The distilled water and DMSO ascertained maximum value at 95 percentage fiducial class intervals. The correlation R^2 value varied from 0.93 to 0.97 with being maximum value (0.97) in three solvents, hexane, ethyl acetate and ethanol.

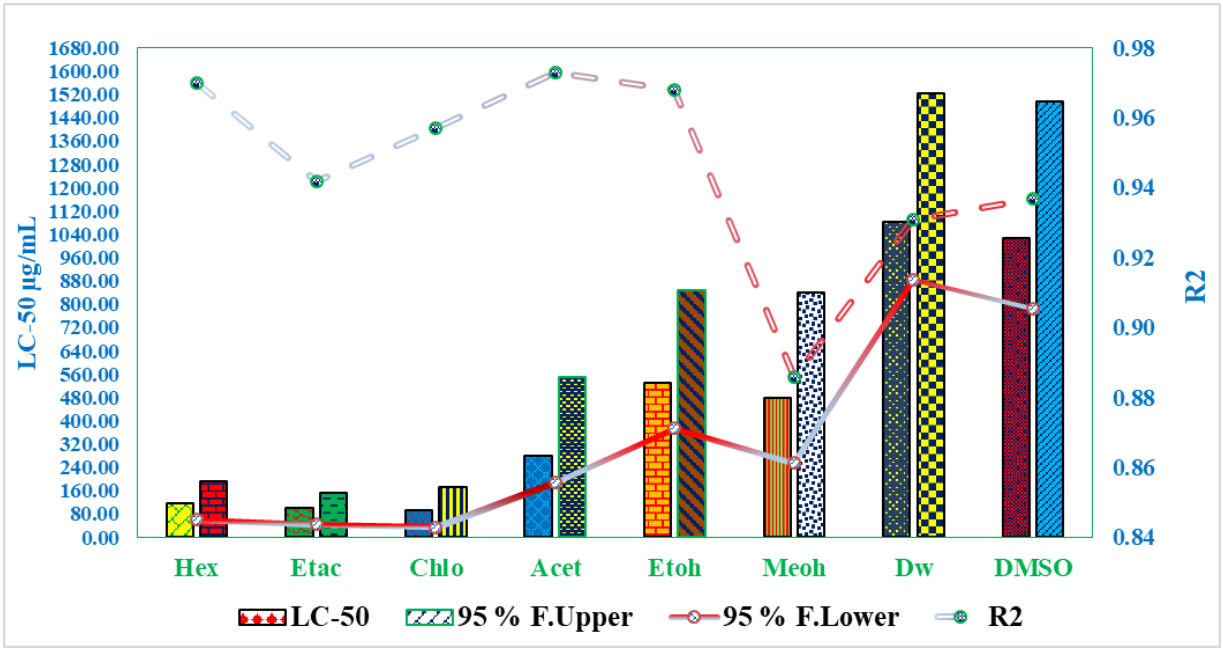


Fig. 6. Showing comparative toxicological parameters of mother solvent extracts and DMSO solvent extracts. The mother solvents and DMSO are being plotted on X-axis while primary Y-1 axis and primary Y-2 axis showing LC_{50} value and R2 values for the respective solvent systems.

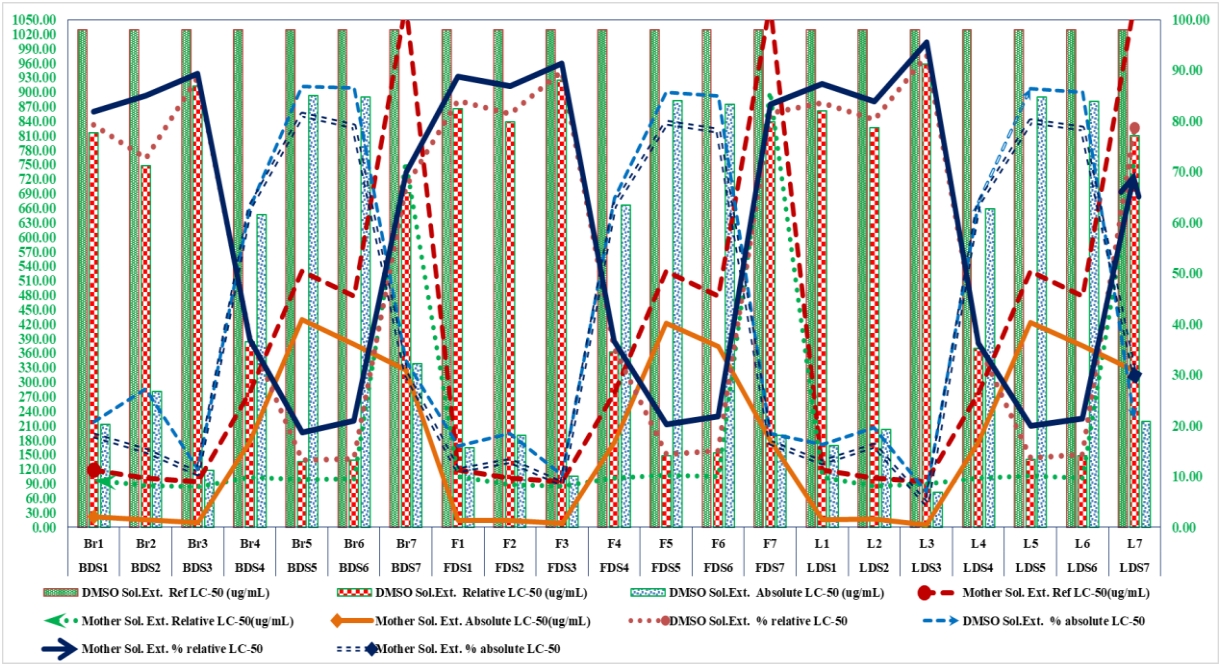


Fig. 7. Demonstrate toxicological parameters of mother solvent extracts and DMSO solvent extracts in BSLA.

The figure 7 and table 8, demonstrate parameters of toxicity studies of DMSO and mother solvent extracts performed in BSLA. The maximum LC_{50} of was recorded in LDS3 (957.15 µg mL^{-1}) followed by FDS3 (924.07 µg mL^{-1}), and lowest by BDS5 (135.99 µg mL^{-1}). Among mother solvent extracts, Br3 recorded maximum LC_{50}

(83.45 µg mL^{-1}). The relative LC_{50} (%) was recorded maximum in L3 (95.51 µg mL^{-1}). The LDS7 ascertained maximum value (1290.23 µg mL^{-1}) at 95 percentage fiducial class intervals. While, Br5 and BDS5 ascertained the lowest value (75.03 µg mL^{-1})

Table 8. Showing toxicological parameters of mother solvents extracts and DMSO solvent extracts

Sample code	Ref. LC_{50}	% Rel. LC_{50}	Rel. LC_{50}	% Absolute L_{C50}	Absolute LC_{50}
BDS1	1029.08	79.28	815.90	20.72	*213.17
BDS2	1029.08	72.72	748.34	27.28	*280.74
BDS3	1029.08	88.65	912.32	11.35	*116.75
BDS4	1029.08	37.24	383.22	62.76	**645.86
BDS5	1029.08	13.21	135.99	86.79	**893.091
BDS6	1029.08	13.51	139.01	86.49	**890.069
BDS7	1029.08	67.16	691.14	32.84	*337.94
FDS1	1029.08	84.05	864.94	15.95	*164.14
FDS2	1029.08	81.50	838.73	18.50	*190.35
FDS3	1029.08	89.80	924.07	10.20	*105.08
FDS4	1029.08	35.24	362.63	64.76	**666.45
FDS5	1029.08	14.28	146.99	85.72	**882.09
FDS6	1029.08	15.00	154.37	85.00	**874.71
FDS7	1029.08	81.54	839.10	18.46	*189.98
LDS1	1029.08	83.65	860.78	16.35	*168.29
LDS2	1029.08	80.34	826.76	19.66	*202.32
LDS3	1029.08	93.01	957.15	6.99	*71.93
LDS4	1029.08	35.93	369.78	64.07	**659.29
LDS5	1029.08	13.60	139.94	86.40	**889.14
LDS6	1029.08	14.38	147.95	85.62	**881.13
LDS7	1029.08	78.73	810.22	21.27	*218.87
Br1	118.51	81.93	97.10	18.07	*21.41
Br2	101.75	84.97	86.46	15.03	*15.29
Br3	93.36	89.39	83.45	10.61	*9.91
Br4	278.32	36.96	102.87	63.04	*175.45
Br5	528.78	18.66	98.65	81.34	**430.13
Br6	477.67	20.97	100.16	79.03	**377.51
Br7	1081.30	69.88	755.60	30.12	*325.71
F1	118.51	88.83	105.27	11.17	*13.24
F2	101.75	86.94	88.46	13.06	*13.29
F3	93.36	91.38	85.31	8.62	*8.05
F4	278.32	36.69	102.13	63.31	**176.19
F5	528.78	20.31	107.41	79.69	**421.37
F6	477.67	21.85	104.36	78.15	*373.31
F7	1081.30	83.32	900.94	16.68	*180.36
L1	118.51	87.27	103.42	12.73	*15.08
L2	101.75	83.88	85.35	16.12	*16.40
L3	93.36	95.51	89.16	4.49	*4.19
L4	278.32	36.25	100.89	63.75	**177.44
L5	528.78	20.00	105.73	80.00	**423.05
L6	477.67	21.39	102.19	78.61	**375.48
L7	1081.30	70.10	757.99	29.90	*323.31

4.3. Assessment of the antioxidant potential

4.3.1. Qualitative screening of phytochemicals

A total of 21 solvent extract combination showed different qualitative phytochemical profiling. Presence and absence of different phytochemicals in different solvent extracts is mentioned (Table 9) and it is seen that solvent extract Br5 had maximum tannins, phenols, flavonoids and steroids. However, other solvent extracts like Br4, Br6, L4, L5, L6, F5 and F6 also showed high amounts of phytochemicals. Different phytochemicals in different solvent extracts of *T. arjuna* showed variation in colors (Plate 3, 4 and 5).

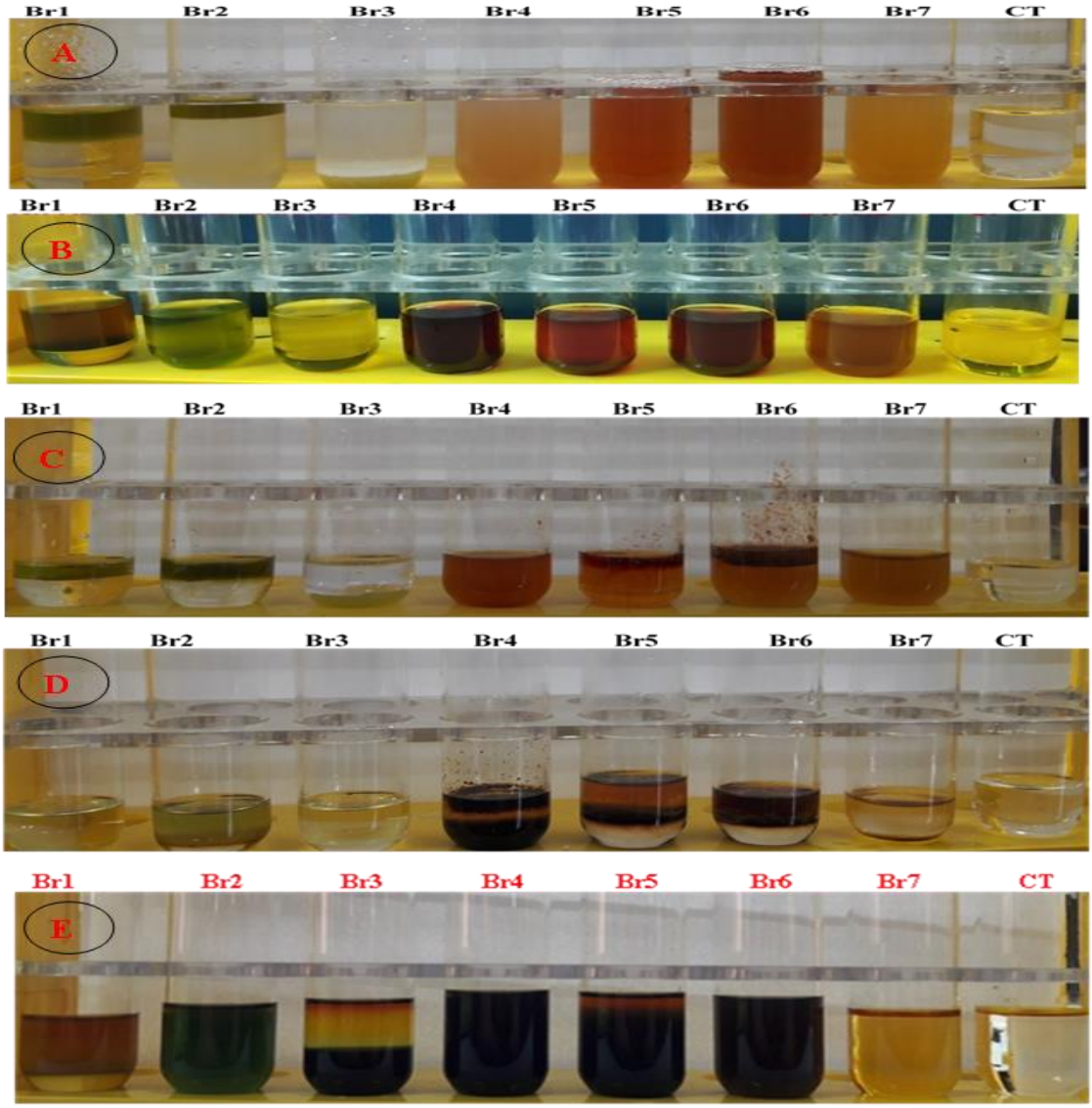


Plate 3. Phytochemical profiling of solvent extract of *T. arjuna* bark

A: Saponins; B: Alkaloids; C: Flavonoids D: Steroids; E: Tannins

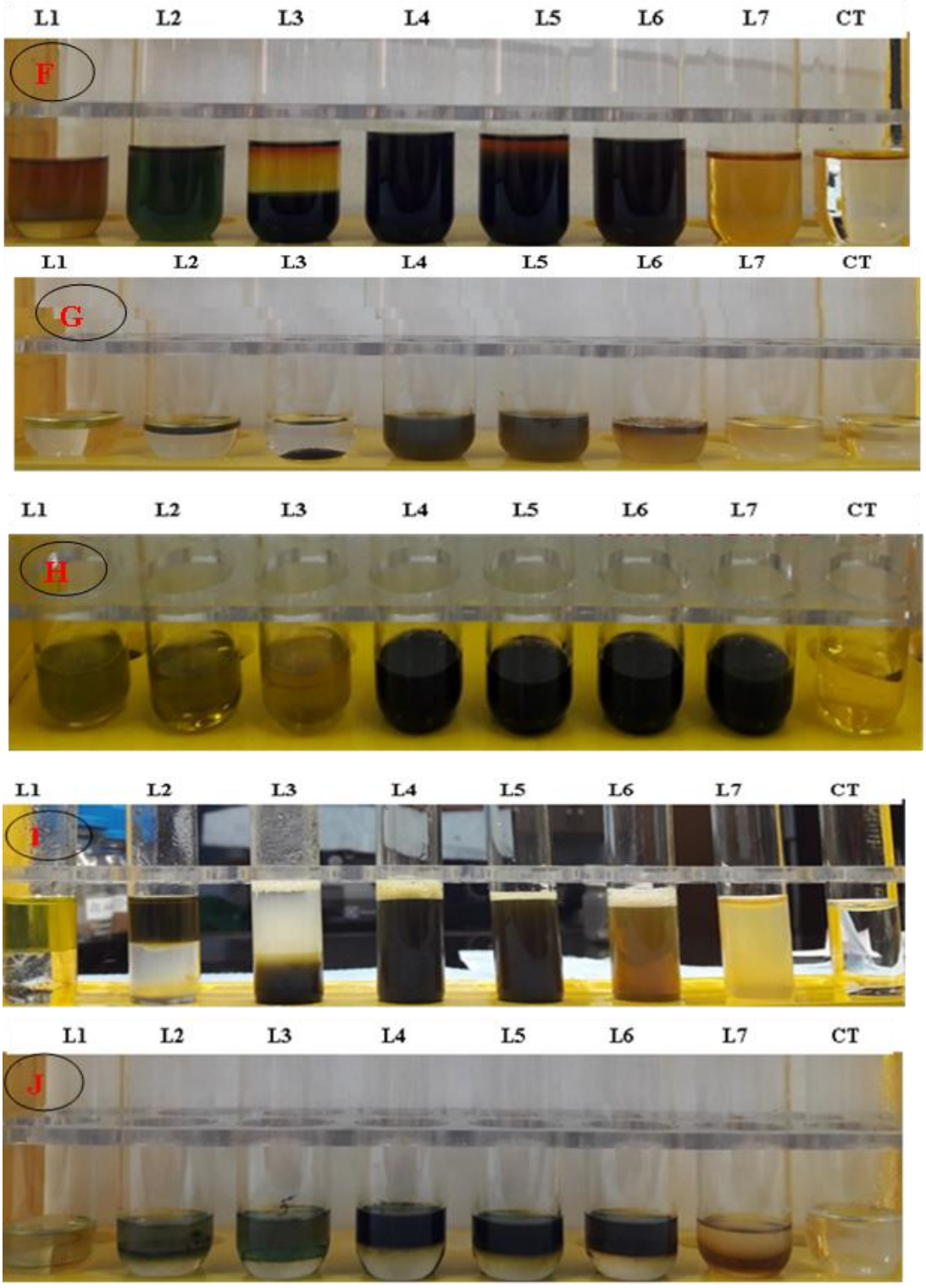


Plate 4. Phytochemical profiling of solvent extracts of *T. arjuna* leaf

F: Alkaloids; G: Flavonoids; H: Tannins; I: Saponins; J: Steroids

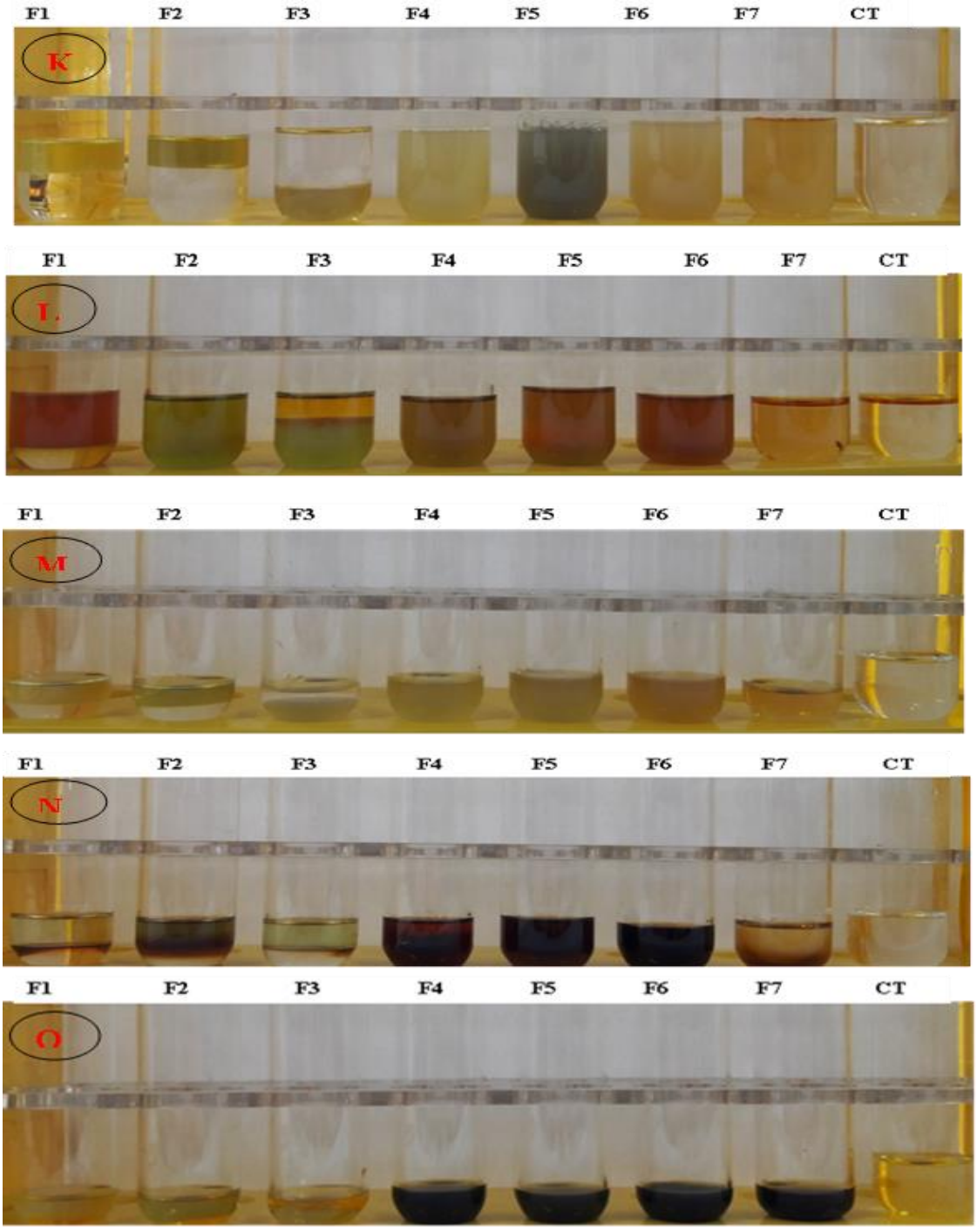


Plate 5. Phytochemical profiling of solvent extracts of *T. arjuna* fruit

K: Saponins; L: Alkaloids; M: Flavonoids; N: Steroids; O: Tannins

Table 9. Qualitative phytochemical analysis of solvent extracts of *T. arjuna*

Solvent/ phytochemical	Tannin and phenolics	Steroids	Alkaloids	Flavonoids	Saponin
Br1	-	-	-	-	-
F1	-	+	+++	-	-
L1	-	-	+	-	-
Br2	-	+	++	+	-
F2	-	++	-	-	-
L2	+	-	-	-	-
Br3	-	-	-	-	-
F3	-	+	+	-	-
L3	+	-	+	-	+
Br4	+++	+++	++	+	+
F4	++	++	+	+	+
L4	+++	+	ND	++	+++
Br5	+++	+++	++	+++	++
F5	+++	+++	++	++	++
L5	+++	++	+	++	++
Br6	+++	+++	+	++	++
F6	+++	+++	++	++	+
L6	+++	++	ND	++	+++
Br7	++	+	-	+	+
F7	+	-	-	+	+
L7	++	ND	+	-	+

+++ high concentration; ++ moderate concentration; + low concentration; ND-not detected; - absent

4.3.2. *In-vitro* antioxidant activity

4.3.2.1. Estimation of ferric reducing antioxidant power (FRAP)

The maximum FRAP values were recorded in bark extracts followed by leaf extracts and fruit extracts. Among bark extracts, ethanolic bark (Br5) extract exhibited the maximum value (2.5-34 µM Fe (II)/g) with corresponding to the concentration of the samples (25-1250 µg mL^{-1}) (Fig. 8).

Among leaf and fruit extracts, the maximum value were recorded in methanol. The values of methanol fruit and methanol leaf extracts were, 0.98-9.9 µM Fe (II)/g and 1.1-21.4 µM Fe (II)/g, respectively. Among solvents, ethanol was found to be most effective and solvent of choice for extraction from bark whereas methanol can

be used for extraction of bioactive principles from leaf and fruit of *T. arj*una. In figure 8, the shapes inside the bar starting from down to upward represent ascending concentration of the extracts sample.

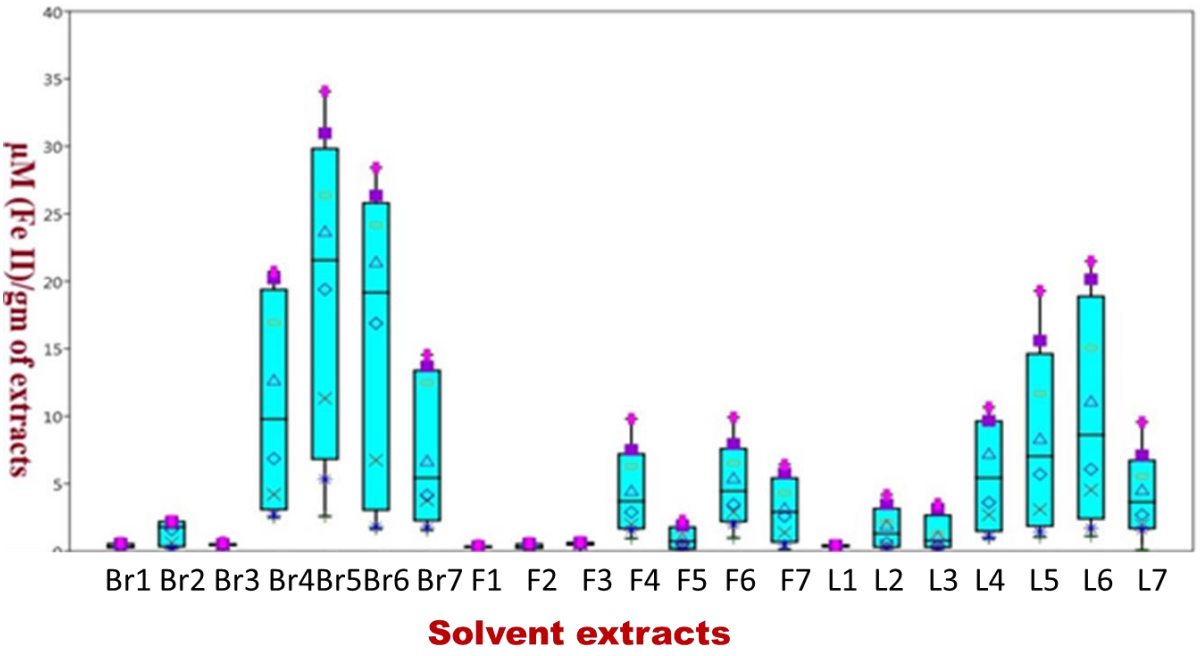


Fig. 8. Comparative FRAP potential of solvent extracts of *T. arjuna*

4.3.2.2. Estimation of total phenolic contents (TPC)

The TPC value exhibited different order of prominence as compared with FRAP. The maximum TPC values were recorded in bark extracts followed by fruit extracts and leaf extracts. Among bark extracts, ethanolic bark extract exhibited the maximum value (144.67-1794 µg mL^{-1} gallic acid equivalent) with corresponding to the concentration of the samples (25-1250 µg mL^{-1}) (Fig. 9). Among fruit extracts, maximum values were recorded in ethanol.

Among leaf and fruit extracts, the maximum value were recorded in ethanol. The values of ethanol fruit (F5) and ethanol leaf (L5) extracts were, 103-1403 µg mL^{-1} gallic acid equivalent, and 128-1303 µg mL^{-1} gallic acid equivalent, respectively. 1 Among solvents, ethanol was found to be most effective and solvent of choice for extraction of phenolic constituents from bark, from leaf and fruit of *T. arj*una. In figure

9, the shapes inside the bar starting from down to upward represent ascending concentration of the extracts sample.

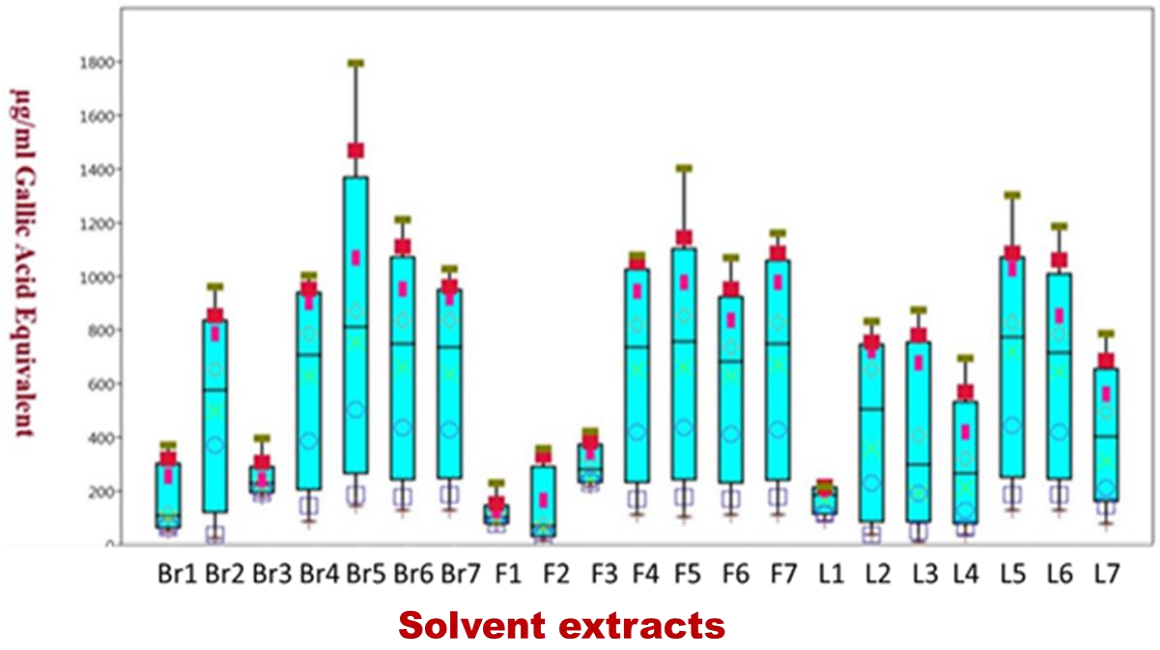


Fig. 9. Comparative TPC content of solvent extracts of *T. arjuna*

4.3.2.3. Estimation of total flavonoids contents (TFC)

In figure 10, the shapes inside the bar starting from down to upward represent ascending concentration of the extracts sample. The TFC value exhibited almost same order of prominence like FRAP. The maximum TFC values were recorded in bark extracts followed by leaf extracts and fruit fruit extracts. Among bark extracts, ethanolic bark (Br5) extract exhibited the maximum value (52.7-382.7 µg mL^{-1} quercetin equivalent) with corresponding to the concentration of the samples (25-1250 µg mL^{-1}) (Fig. 10).

Among leaf and fruit extracts, the maximum value were recorded in ethanol. The values of ethanol fruit (F5) and ethanol leaf (L5) extracts were, 44.37-304.46 µg mL^{-1} quercetin equivalent, and 49.37-284.74 µg mL^{-1} quercetin equivalent, respectively. Among solvents, ethanol was found to be most effective and solvent of choice for extraction of flavonoids constituents from bark, leaf and fruit of *T. arj*una.

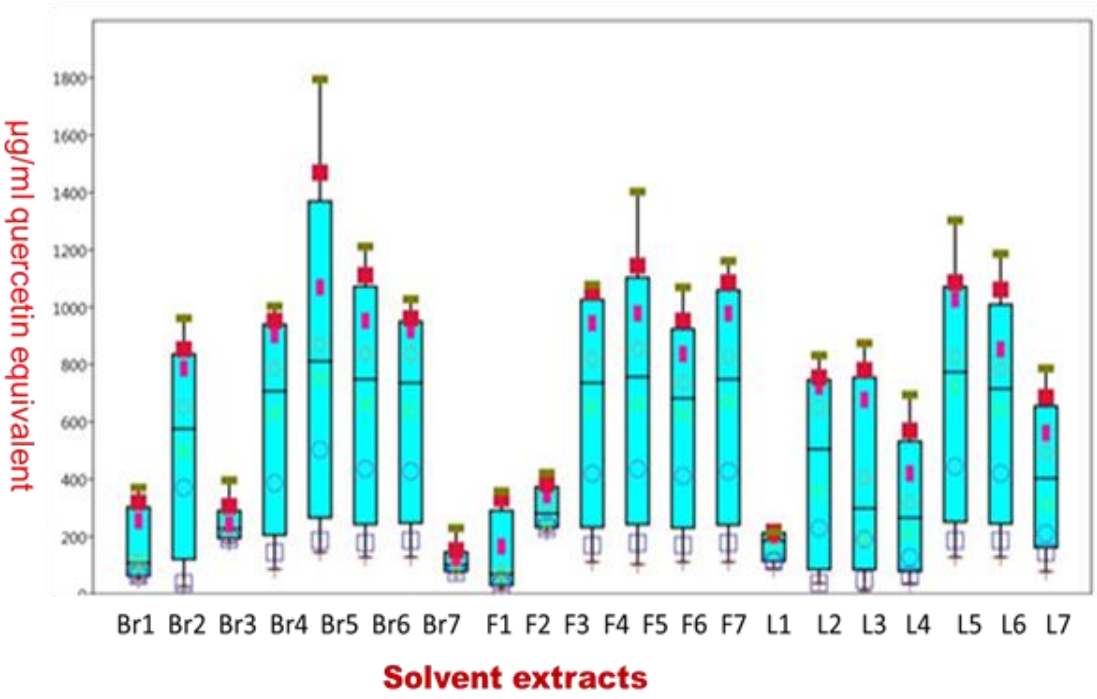


Fig. 10. Comparative TFC of solvent extracts of *T. arjuna* inhibition activity

4.3.2.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH assay)

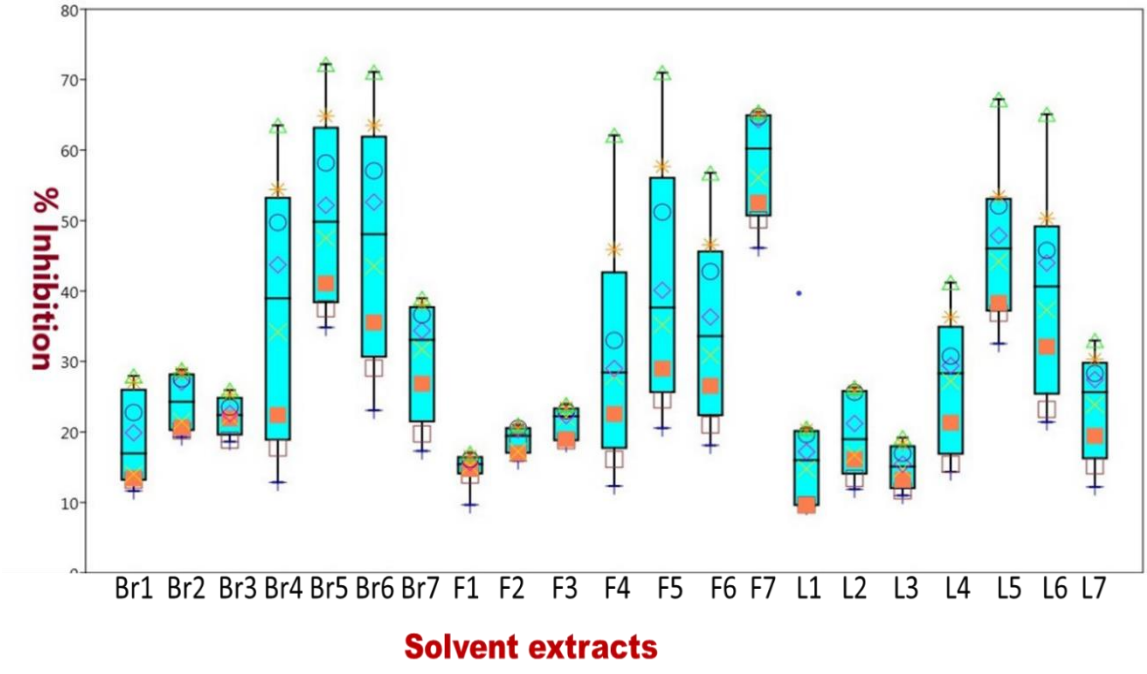


Fig. 11. Comparative DPPH inhibition (%) potential of solvent extracts of *T. arjuna*

In figure 11, the shapes inside the bar starting from down to upward represent ascending concentration of the extracts sample. The DPPH inhibition (%) value exhibited almost same order of prominence like TPC. The maximum TFC values were recorded in bark extracts followed by leaf extracts and fruit fruit extracts. Among bark extracts, ethanolic bark (Br5) extract exhibited the maximum value (36.44-73.78%) with corresponding to the concentration of the samples (25-1250 µg mL^{-1}) (Fig. 11).

Among fruit extracts, the maximum value were recorded in ethanolic fruit extract, F5 (22.35-72.84%). While, among leaf extracts, methanolic leaf extract (L6) showed maximum DPPH inhibition value (24.46-71.1%). Among solvents, ethanol was found to be most effective and solvent of choice for extraction of bioactive compounds from bark, and fruit whereas methanol can be used for extraction from leaf of *T. arj*una.

4.3.2.5. 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS assay)

In figure 12, the shapes inside the bar starting from down to upward represent ascending concentration of the extracts sample. The ABTS inhibition (%) value exhibited almost same order of prominence like DPPH. The maximum TFC values were recorded in bark extracts followed by leaf extracts and fruit extracts. Among bark extracts, ethanolic bark (Br5) extract exhibited the maximum value (34.31-71.64%) with corresponding to the concentration of the samples (25-1250 µg mL^{-1}) (Fig. 12).

Among fruit extracts, the maximum value were recorded in ethanolic fruit extract, F5 (20.8-71.24%). While, among leaf extracts, ethanolic leaf extract (L5) showed maximum ABTS inhibition value (32.85-67.52%). Among solvents, ethanol was found to be most effective and solvent of choice for extraction of bioactive compounds from bark, and fruit leaf of *T. arj*una.

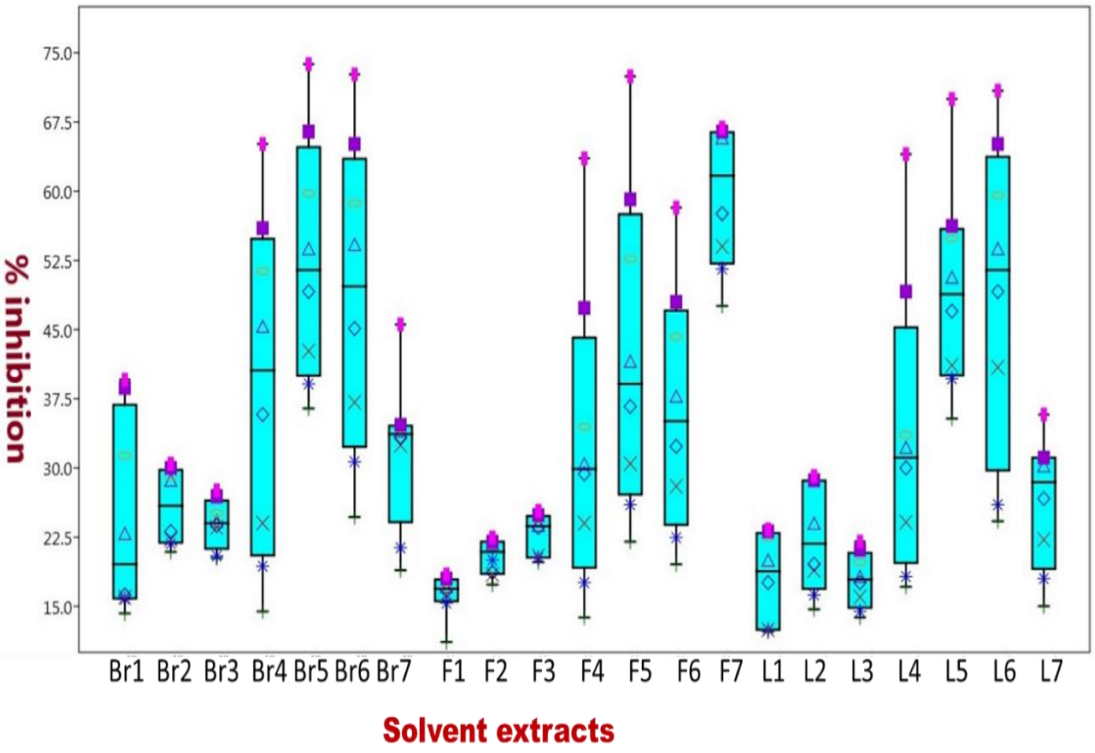


Fig. 12. Comparative ABTS inhibition (%) potential of solvent extracts of *T. arjuna*

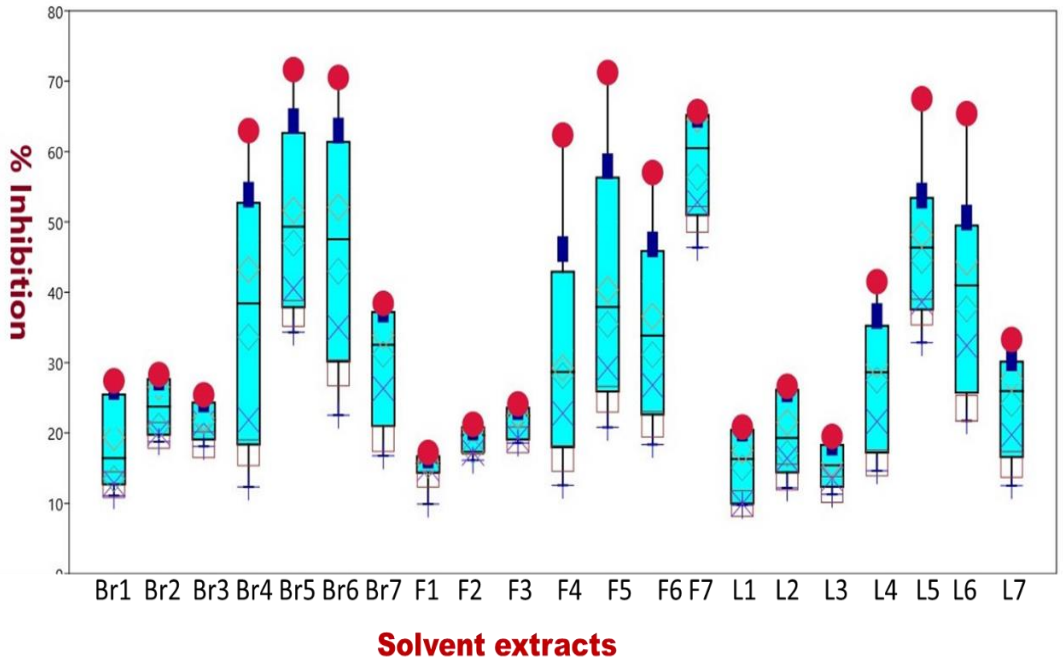


Fig. 13. Comparative NO inhibition (%) potential of solvent extracts of *T. arjuna*

4.3.2.6. Nitric Oxide assay (NO Assay)

In figure 13, the shapes inside the bar starting from down to upward represent ascending concentration of the extracts sample. The NO inhibition (%) value exhibited almost same order of prominence like ABTS. The maximum No inhibition values were recorded in bark extracts followed by leaf extracts and fruit extracts. Among bark extracts, ethanolic bark (Br5) extract exhibited the maximum value (39.64-76.97%) with corresponding to the concentration of the samples (25-1250 µg mL^{-1}) (Fig. 13).

Among fruit extracts, the maximum value were recorded in ethanolic fruit extract, F5 (24.89-75.35%). While, among leaf extracts, ethanolic leaf extract (L5) showed maximum ABTS inhibition value (38.53-72.25%). Among solvents, ethanol was found to be most effective and solvent of choice for extraction of bioactive compounds from bark, and fruit leaf of *T. arj*una.

4.3.3. DNA scission inhibition

The gallic acid exhibited maximum DNA scission inhibition activity among the standard antioxidants followed by ascorbic acid, BHT and BHA. Apparently from the gel it can be inferred that, Br5, Br6 and Br4 has maximum activity which was followed by F5, F4, L3, L2 etc., and exhibited relatively same free radicals scavenging activity in densitometry analyses of plasmid pBR322 (Table 10).

The important variable of densitometry analysis are: relative quantity, relative front, lane and band percentages. Relative front value exhibited no significance ($p>0.05$), among the different solvent extracts as compare with standard antioxidants with being maximum value among solvent extract in ethanolic bark extract (Br5). The other variables such as relative quantity, lane percentage and band percentage showed same order of activity.

Among solvent extract, Br5 and Br7 exhibited a substantial difference ($p<0.05$) when compared in relation to relative quantity. The Br5 exhibited maximum lane

percentage and band percentage as compared with other solvent extracts. As a trend, Br5 showed maximum values for all densitometry variables as compared with other solvent extracts, and the values of polar solvent extracts such as Br5, Br6, Br4 are significantly different($p<0.05$) from non-polar, polar aprotic solvent extracts (Fig. 14).

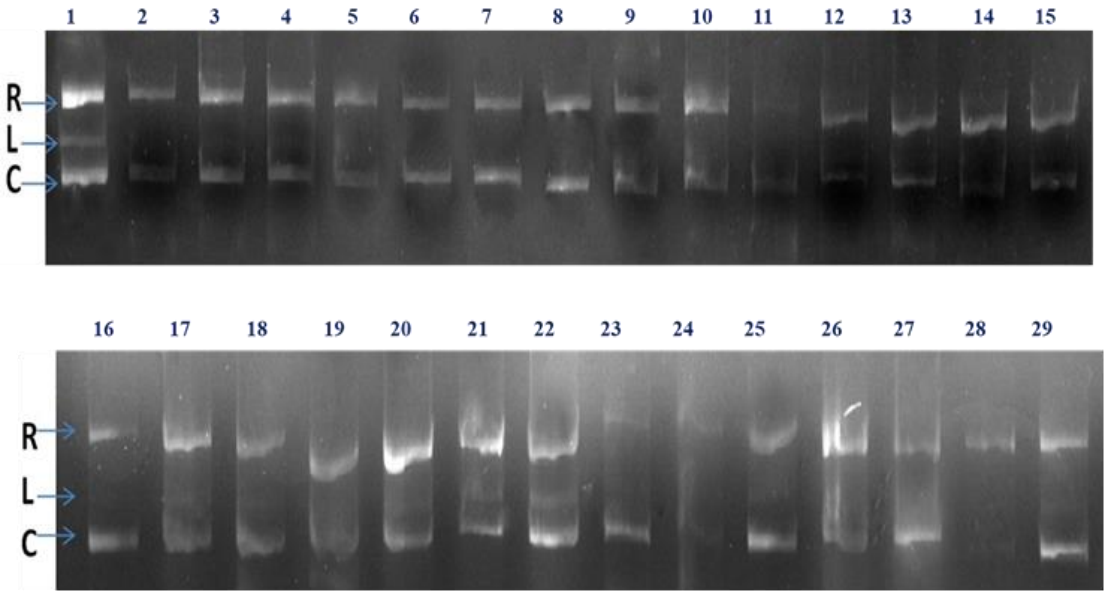


Fig.14. Showing comparative electrophoretic pattern of pBR322 DNA scission inhibition activity of solvent extracts of *T. arjuna.*

Here, lanes 1-29 are represented as: 1-Plasmid DNA; 2-H_2O_2 +Plasmid DNA; 3-THF+Plasmid DNA; 4-THF+H_2O_2+Plasmid DNA; 5-BHA+ H_2O_2+Plasmid DNA; 6- BHT+ H_2O_2+Plasmid DNA; 7- Ascorbic acid+ H_2O_2+Plasmid DNA; 8- Gallic acid + H_2O_2+Plasmid DNA; 9-Br1+H_2O_2+PlasmidDNA; 10-Br2+H_2O_2+Plasmid DNA; 11-Br3+ H_2O_2+Plasmid DNA; 12-Br4+H_2O_2+Plasmid DNA; 13-Br5+H_2O_2+Plasmid DNA; 14-Br6+H_2O_2+Plasmid DNA; 15-Br7+H_2O_2+Plasmid DNA; 16-L1+H_2O_2+Plasmid DNA; 17-L2+ H_2O_2+Plasmid DNA; 18-L3+ H_2O_2+Plasmid DNA; 19-L4+ H_2O_2+PlasmidDNA; 20-L5+H_2O_2+Plasmid DNA; 21-L6+H_2O_2+Plasmid DNA; 22-L7+H_2O_2+Plasmid DNA; 23-F1+H_2O_2+Plasmid DNA; 24-F2+H_2O_2+Plasmid DNA; 25-F3+H_2O_2+Plasmid DNA; 26-F4+H_2O_2+Plasmid DNA; 27-F5+H_2O_2+Plasmid DNA; 28-F6+H_2O_2+Plasmid DNA; 29-F7+H_2O_2+Plasmid D

Table 10. Densitometry study of a gel picture of DNA scission inhibition by *T. arjuna* solvent extracts

Gel Coding	Abbreviation	Rf	RQ (ng)	Band (%)	Lane (%)
L1	Plasmid DNA	0.87 ± 0.13^{d}	1.32 ± 0.26^{c}	56.8 ± 0.02^{i}	28.3 ± 0.04^{h}
L2	H_2O_2+Plasmid DNA	0.55 ± 0.17^{a}	0.33 ± 1.05^{a}	3.9 ± 0.32^{a}	4.2 ± 0.22^{a}
L3	THF+ Plasmid DNA	0.61 ± 0.15^{a}	0.8 ± 0.39^{ab}	35.7 ± 0.03^{bcd}	14.4 ± 0.06^{de}
L4	H_2O_2+THF++Plasmid DNA	0.57 ± 0.17^{a}	0.35 ± 1.05^{a}	4.1 ± 0.32^{a}	4.4 ± 0.22^{a}
L5	BHA+H_2O_2+Plasmid DNA	$0.63\pm0.15^{a,c}$	0.83 ± 0.39^{ab}	37.7 ± 0.03^{a}	16.4 ± 0.06^{de}
L6	BHT+H_2O_2+Plasmid DNA	0.66 ± 0.15^{c}	0.89 ± 0.39^{ab}	38.5 ± 0.03^{abc}	17.2 ± 0.06^{de}
L7	Ascorbic acid+H_2O_2+Plasmid	0.73 ± 0.15^{b}	0.95 ± 0.39^{ab}	52.6 ± 0.03^{bci}	22.2 ± 0.06^{de}
L8	Gallic acid+H_2O_2+Plasmid DNA	0.84 ± 0.15^{a}	1.26 ± 0.39^{ab}	$54.7\pm0.03^{a,d,i}$	24.2 ± 0.06^{de}
L9	H_2O_2+Plasmid DNA +Br1	$0.56\pm0.16^{c,d}$	0.32 ± 1.05^{a}	4.9 ± 0.25^{a}	3.3 ± 0.28^{a}
L10	H_2O_2+Plasmid DNA +Br2	$0.59\pm0.14^{c,d}$	0.83 ± 0.39^{b}	37.3 ± 0.03^{cde}	13.2 ± 0.07^{c}
L11	H_2O_2+Plasmid DNA +Br3	0.32 ± 0.14^{b}	$0.2\pm0.39^{a,c}$	21.8 ± 0.03^{bc}	3.2 ± 0.10^{b}
L12	H_2O_2+Plasmid DNA +Br4	0.62 ± 0.14^{c}	0.97 ± 0.34^{b}	39.7 ± 0.03^{ij}	16.8 ± 0.06^{de}
L13	H_2O_2+Plasmid DNA +Br5	0.68 ± 0.13^{a}	1.23 ± 0.29^{b}	49 ± 0.03^{j}	22.9 ± 0.05^{gh}
L14	H_2O_2+Plasmid DNA +Br6	0.66 ± 0.11^{a}	1.16 ± 0.30^{b}	44.9 ± 0.03^{ij}	19.2 ± 0.06^{fg}
L15	H_2O_2+Plasmid DNA +Br7	0.61 ± 0.14^{a}	0.84 ± 0.37^{a}	36.8 ± 0.03^{hi}	15.6 ± 0.04^{jkl}
L16	H_2O_2+Plasmid DNA +L1	0.63 ± 0.13^{a}	1.01 ± 0.27^{b}	45.1 ± 0.03^{j}	20.8 ± 0.04^{jkl}
L17	H_2O_2+Plasmid DNA +L2	0.63 ± 0.14^{a}	0.86 ± 0.37^{ab}	34 ± 0.04^{b}	13.6 ± 0.07^{cd}
L18	H_2O_2+Plasmid DNA +L3	0.61 ± 0.15^{a}	1.09 ± 0.29^{b}	42 ± 0.03^{h}	19.9 ± 0.05^{ijkl}
L19	H_2O_2+Plasmid DNA +L4	0.59 ± 0.15^{a}	0.95 ± 0.33^{b}	38.7 ± 0.03^{f}	14.5 ± 0.06^{de}
L20	H_2O_2+Plasmid DNA + L5	0.65 ± 0.14^{a}	1.03 ± 0.31^{b}	41 ± 0.03^{gh}	20 ± 0.05^{ijkl}
L21	H_2O_2+Plasmid DNA +L6	0.54 ± 0.14^{a}	0.97 ± 0.32^{b}	37.3 ± 0.01^{def}	13.9 ± 0.04^{d}
L22	H_2O_2+Plasmid DNA +L7	0.61 ± 0.15^{a}	0.82 ± 0.38^{ab}	39.6 ± 0.03^{fg}	18.5 ± 0.05^{hi}
L23	H_2O_2+Plasmid DNA +F1	0.61 ± 0.15^{a}	0.92 ± 0.34^{b}	35.3 ± 0.03^{bc}	15.6 ± 0.06^{ef}
L24	H_2O_2+Plasmid DNA +F2	0.60 ± 0.15^{a}	0.81 ± 0.39^{ab}	38.3 ± 0.03^{ef}	19.3 ± 0.05^{ij}
L25	H_2O_2+Plasmid DNA +F3	0.60 ± 0.14^{a}	0.89 ± 0.35^{ab}	37.9 ± 0.03^{bc}	13.3 ± 0.07^{cd}
L26	H_2O_2+Plasmid DNA +F4	0.61 ± 0.14^{a}	0.95 ± 0.37^{ab}	38.8 ± 0.03^{def}	19.3 ± 0.05^{fg}
L27	H_2O_2+Plasmid DNA +F5	0.64 ± 0.14^{a}	1.01 ± 0.31^{b}	45.4 ± 0.03^{j}	20.8 ± 0.04^{jkl}
L28	H_2O_2+Plasmid DNA +F6	0.59 ± 0.14^{a}	0.85 ± 0.33^{b}	37.6 ± 0.03^{f}	14.7 ± 0.04^{jkl}
L29	H_2O_2+Plasmid DNA +F7	0.62 ± 0.15^{a}	0.99 ± 0.32^{b}	39.4 ± 0.03^{fg}	19.8 ± 0.05^{ijk}

Values are represented as Mean + SE, n=3, different superscript in same column indicate significant/ substantial difference at $p<0.05$.

4.4. Mineral profiling of dry powders of bark, leaf and fruit and solvent extracts of *Terminalia arjuna*

Mineral profiling of the selected solvent extracts for both types of extraction methods has completed for 14 important minerals. The arjuna fruit powder possesses Zn in the highest quantity (29.314±0.001 mg L^{-1}) followed by its bark powder (27.394±0.001 mg L^{-1}) and leaf powder (23.16±0.002 mg L^{-1}), respectively. Extraction by both methods resulted in low minerals in ethanolic bark extracts as compared to the powder from and other bark solvent extracts. The highest Zn was recorded in methanolic fruit extracts (45.816±0.001 mg L^{-1} and 47.021±0.001 mg L^{-1}) for serial (Table 11a and b) and individual (Table 12a and b) fractions, respectively. Over all mineral profiling of powder forms of three parts revealed that arjuna fruit has better profiling followed by bark and leaf. While after extraction, the pattern has changed and the overall mineral profiling of powder forms and solvent extracts can be represented in ascending order as follows: F6>AF≥Br6>AB>L5≥B4>AL> Br5> F5≥ L7> L4>F7>Br7>L6.

4.4.1. Calculation of extract yield (%)

The yield % of both selected solvent extracts (individual and serial fraction) exhibits the narrow differences in yield % and varies from the nature of the parts used. Irrespective of solvents extracts the maximum yield % was recorded in bark extracts for both fractions, individual and serial, and the highest value was recorded for ethanolic extract of bark (23.6±0.026, 22.23±0.017) followed by methanolic extract (22.7±0.016, 22.10±0.004), acetone (11.36±0.005,11.11±0.005) and distilled water (4.1±0.048, 3.75±0.047), for individual and serial fractions, respectively. The trend showing that bark extracts has maximum yield followed by leaf extracts and fruit extracts for polar protic solvents and pattern, and bark extract followed by fruit extract and leaf extracts was different in case of polar protic and polar aprotic, respectively (Table 13). It showed the comparative yield % of selected solvent extracts from arjuna. The yield is a function of polarity and nature of solvents, and nature and physical structure of the herbal material to be extracted.

Table 11a. Showing comparative mineral concentration (mg L^{-1}) of serial fractions of *T. arjuna* solvent extracts

Sample /Mineral	Li	Be	V	Cr	Mn	Co	Ni
AF	0.071±0.007	0.006±0.001	0.224±0.004	0.069±0.011	10.815±0.037	0.600±0.025	1.401±0.011
AL	0.055±0.009	0.004±.002	0.142±0.007	0.450±0.017	6.837±0.006	0.468±0.032	1.040±0.014
AB	0.052±0.010	0.006±0.069	0.166±0.006	0.486±0.015	9.653±0.004	0.437±0.034	1.802±0.008
Br4	0.051±0.010	0.002±0.154	0.115±0.006	0.441±0.017	8.198±0.005	0.373±0.040	1.036±0.014
F4	0.032±0.015	0.006±0.069	0.136±0.007	0.417±0.018	6.422±0.006	0.350±0.043	0.665±0.023
L4	0.039±0.013	0.004±0.102	0.137±0.007	0.386±0.019	8.377±0.005	0.326±0.046	0.745±0.020
Br5	0.036±0.014	0.004±0.096	0.134±0.007	0.386±0.019	7.570±0.005	0.399±0.038	0.727±0.021
F5	0.042±0.012	0.003±0.141	0.146±0.007	0.427±0.018	6.432±0.006	0.360±0.042	0.675±0.022
L5	0.042±0.012	0.003±0.120	0.176±0.006	0.480±0.016	7.982±0.005	0.411±0.037	1.422±0.011
Br6	0.058±0.009	0.004±0.099	0.155±0.006	0.473±0.016	4.816±0.008	0.471±0.032	1.774±0.008
F6	0.047±0.011	0.004±0.100	0.178±0.006	0.556±0.013	4.830±0.008	0.390±0.039	1.651±0.009
L6	0.063±0.008	0.046±0.009	0.169±0.006	0.439±0.015	5.084±0.008	0.612±0.025	1.871±0.008
Br7	0.074±0.007	0.005±0.008	0.178±0.006	0.273±0.027	7.416±0.005	0.662±0.023	1.890±0.008
F7	0.056±0.009	0.004±0.091	0.136±0.007	0.464±0.016	8.416±0.005	0.761±0.020-	1.252±0.012
L7	0.048±0.010	0.079±0.005	0.164±0.006	0.470±0.016	9.116±0.004	0.580±0.026	1.356±0.011

Values are Mean± SE, n=3;

AF: arjuna fruit powder; **AL**: arjuna leaf powder; **AB**: arjuna bark powder; **Br4:** Acetone extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **L4:** Acetone extract of arjuna leaf; **Br5:** Ethanol extract of arjuna bark; **F5:** Ethanol extract of arjuna fruit; **L5:** Ethanol extract of arjuna leaf; **Br6:** Methanol extract of arjuna bark; **F6:** Methanol extract of arjuna fruit; **L6:** Methanol extract of arjuna leaf; **Br7:** Distilled water extract of arjuna bark; **F7:** Distilled water extract of arjuna fruit; **L7:** Distilled water extract of arjuna leaf.

Table 11b. Showing comparative mineral concentration (mg L^{-1}) of serial fractions of *T. arjuna* solvent extracts

Sample /Mineral	Li	Be	V	Cr	Mn	Co	Ni
AF	5.145±0.002	29.314±0.001	0.025±0.259	0.152±0.043	0.837±0.022	0.261±0.058	0.017±0.030
AL	5.214±0.002	23.16±0.002	0.013±0.009	0.084±0.077	0.717±0.025	0.40±0.028	0.009±0.055
AB	3.148±0.003	25.114±0.002	0.016±0.040	0.112±0.058	0.775±0.023	0.169±0.089	0.015±0.033
Br4	2.709±0.004	27.394±0.001	0.0141±0.046	0.090±0.072	0.707±0.025	0.158±0.095	0.009±0.055
F4	3.508±0.003	18.967±0.002	0.0031±0.208	0.060±0.108	0.844±0.021	0.172±0.087	0.014±0.037
L4	2.928±0.004	19.307±0.002	0.0113±0.057	0.108±0.060	0.729±0.025	0.148±0.101	0.007±0.068
Br5	2.940±0.004	22.138±0.002	0.0113±0.058	0.146±0.045	0.687±0.026	0.123±0.122	0.009±0.055
F5	3.518±0.003	18.977±0.002	0.0131±0.049	0.070±0.093	0.854±0.021	0.182±0.082	0.024±0.021
L5	4.463±0.002	26.411±0.002	0.0131±0.050	0.084±0.077	0.972±0.019	0.186±0.081	0.011±0.046
Br6	5.886±0.002	28.523±0.001	0.0159±0.041	0.170±0.038	1.401±0.017	0.033±0.457	0.033±0.015
F6	4.868±0.002	45.816±0.001	0.0185±0.035	0.149±0.044	0.874±0.021	0.029±0.523	0.018±0.028
L6	4.146±0.003	12.106±0.003	0.0333±0.020	0.130±0.050	0.774±0.023	0.033±0.455	0.014±0.036
Br7	4.846±0.002	17.417±0.002	0.0185±0.035	0.111±0.059	0.667±0.027	0.031±`0.478	0.018±0.028
F7	3.885±0.003	19.528±0.002	0.0203±0.032	0.143±0.045	0.860±0.021	0.038±0.393	0.013±0.039
L7	4.502±0.002	20.493±0.002	0.0185±0.035	0.130±0.050	0.775±0.023	0.048±0.315	0.013±0.039

Values are Mean± SE, n=3;

AF: arjuna fruit powder; **AL**: arjuna leaf powder; **AB**: arjuna bark powder; **Br4:** Acetone extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **L4:** Acetone extract of arjuna leaf; **Br5:** Ethanol extract of arjuna bark; **F5:** Ethanol extract of arjuna fruit; **L5:** Ethanol extract of arjuna leaf; **Br6:** Methanol extract of arjuna bark; **F6:** Methanol extract of arjuna fruit; **L6:** Methanol extract of arjuna leaf; **Br7:** Distilled water extract of arjuna bark; **F7:** Distilled water extract of arjuna fruit; **L7:** Distilled water extract of arjuna leaf.

Table 12a. Showing comparative mineral concentration (mg L^{-1}) of individual fractions of *T. arjuna* solvent extracts

Sample/Mineral	Li	Be	V	Cr	Mn	Co	Ni
AF	0.076±0.065	0.076±0.002	0.226±0.005	0.697±0.017	11.238±0.002	0.600±0.025	1.387±0.001
AL	0.056±0.018	0.056±0.003	0.l153±0.069	0.454±0.025	6.899±0.003	0.468±0.032	1.027±0.001
AB	0.053±0.019	0.053±0.053	0.166±0.063	0.445±0.026	8.260±0.003	0.373±0.040	1.022±0.001
Br4	0.073±0.014	0.073±0.002	0.201±0.052	0.780±0.015	10.466±0.002	0.472±0.032	1.833±0.001
F4	0.054±0.019	0.054±0.002	0.170±0.062	0.711±0.016	7.234±0.003	0.385±0.039	0.696±0.002
L4	0.061±0.017	0.061±0.002	0.171±0.061	0.680±0.017	9.189±0.002	0.361±0.042	0.776±0.002
Br5	0.057±0.018	0.057±0.002	0.169±0.062	0.680±0.017	8.382±0.003	0.434±0.035	0.759±0.002
F5	0.064±0.016	0.064±0.003	0.180±0.058	0.721±0.016	7.244±0.003	0.395±0.038	0.706±0.002
L5	0.064±0.016	0.064±0.003	0.211±0.050	0.774±0.015	8.794±0.003	0.446±0.034	1.454±0.001
Br6	0.080±0.013	0.080±0.002	0.190±0.055	0.767±0.015	5.629±0.004	0.506±0.030	1.805±0.001
F6	0.068±0.015	0.068±0.002	0.212±0.050	0.850±0.014	5.642±0.004	0.425±0.035	1.682±0.001
L6	0.085±0.012	0.085±0.000	0.203±0.052	0.787±0.015	5.896±0.004	0.647±0.023	1.903±0.001
Br7	0.096±0.010	0.096±0.002	0.212±0.050	0.567±0.020	8.229±0.003	0.697±0.022	1.921±0.001
F7	0.077±0.013	0.077±0.002	0.170±0.062	0.758±0.015	9.229±0.002	0.796±0.019	1.283±0.001
L7	0.070±0.014	0.070±0.003	0.199±0.053	0.764±0.015	9.929±0.002	0.615±0.024	1.403±0.001

Values are Mean± SE, n=3;

AF: arjuna fruit powder; **AL**: arjuna leaf powder; **AB**: arjuna bark powder; **Br4:** Acetone extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **L4:** Acetone extract of arjuna leaf; **Br5:** Ethanol extract of arjuna bark; **F5:** Ethanol extract of arjuna fruit; **L5:** Ethanol extract of arjuna leaf; **Br6:** Methanol extract of arjuna bark; **F6:** Methanol extract of arjuna fruit; **L6:** Methanol extract of arjuna leaf; **Br7:** Distilled water extract of arjuna bark; **F7:** Distilled water extract of arjuna fruit; **L7:** Distilled water extract of arjuna leaf.

Table 12b. Showing comparative mineral concentration (mg L^{-1}) of individual fractions of *T. arjuna* solvent extracts

Sample/Mineral	Cu	Zn	Ga	Se	Rb	Mo	Ag
AF	5.176±0.004	29.233±0.007	0.019±0.006	0.151±0.040	0.835±0.019	0.262±0.063	0.0164±0.006
AL	5.245±0.004	23.161±0.002	0.013±0.008	0.083±0.072	0.715±0.022	0.541±0.030	0.0087±0.011
AB	2.740±0.007	21.399±0.002	0.014±0.008	0.089±0.067	0.705±0.023	0.160±0.103	0.0087±0.011
Br4	3.207±0.006	26.319±0.001	0.019±0.006	0.134±0.045	0.785±0.020	0.197±0.084	0.019±0.005
F4	3.567±0.006	20.172±0.002	0.006±0.019	0.083±0.073	0.854±0.019	0.200±0.082	0.0171±0.006
L4	2.987±0.007	20.512±0.002	0.014±0.008	0.130±0.046	0.739±0.022	0.177±0.093	0.0109±0.009
Br5	2.998±0.007	23.343±0.001	0.014±0.008	0.168±0.036	0.697±0.023	0.152±0.109	0.013±0.008
F5	3.577±0.006	20.182±0.002	0.016±0.007	0.093±0.065	0.864±0.019	0.210±0.078	0.027±0.004
L5	4.521±0.004	27.616±0.001	0.016±0.007	0.106±0.056	0.982±0.016	0.214±0.077	0.0145±0.007
Br6	5.945±0.003	29.728±0.001	0.018±0.006	0.192±0.031	1.051±0.015	0.061±0.269	0.037±0.003
F6	4.927±0.004	47.021±0.001	0.021±0.005	0.171±0.035	0.884±0.018	0.057±0.289	0.0216±0.0046
L6	4.204±0.005	13.311±0.003	0.036±0.003	0.153±0.039	0.784±0.020	0.061±0.268	0.017±0.006
Br7	4.905±0.004	18.622±0.002	0.021±0.005	0.134±0.045	0.677±0.024	0.060±0.276	0.0216±0.005
F7	3.944±0.005	20.733±0.002	0.023±0.005	0.165±0.036	0.870±0.018	0.067±0.248	0.0163±0.006
L7	4.560±0.004	21.698±0.002	0.021±0.005	0.153±0.039	0.785±0.020	0.076±0.217	0.0164±0.006

Values are Mean± SE, n=3;

AF: arjuna fruit powder; **AL**: arjuna leaf powder; **AB**: arjuna bark powder; **Br4:** Acetone extract of arjuna bark; **F4:** Acetone extract of arjuna fruit; **L4:** Acetone extract of arjuna leaf; **Br5:** Ethanol extract of arjuna bark; **F5:** Ethanol extract of arjuna fruit; **L5:** Ethanol extract of arjuna leaf; **Br6:** Methanol extract of arjuna bark; **F6:** Methanol extract of arjuna fruit; **L6:** Methanol extract of arjuna leaf; **Br7:** Distilled water extract of arjuna bark; **F7:** Distilled water extract of arjuna fruit; **L7:** Distilled water extract of arjuna leaf.

Table 13. Yield (%) of serial and individual fractions of solvent extracts of *T. arjuna*

Solvent	Avg. yield Ind.	Avg. yield ser.	Solvent	Avg. yield Ind.	Avg. yield ser.
Br1	2.64±0.04	2.64±0.01	Br4	11.36±0.01	11.11±0.01
F1	1.8±0.31	1.51±0.38	F4	2.08±0.02	1.72±0.03
L1	1.14±0.07	0.91±0.01	L4	11.68±0.03	11.11±0.01
Br2	3.36±0.08	2.73±0.09	Br5	23.6±0.02	22.23±0.21
L2	1.52±0.034	1.11±0.01	F5	2.92±0.01	2.37±0.54
F2	1.36±0.023	1.11±0.04	L5	5.16±0.03	5.05±0.06
Br3	1.92±0.03	1.21±0.01	Br6	22.7±0.01	22.10±0.04
F3	1.62±0.03	1.21±0.01	F6	3.96±0.04	3.63±0.07
L3	2.3±0.11	2.12±0.03	L6	7.12±0.01	7.07±0.02
Br4	11.36±0.05	11.11±0.01	Br7	4.1±0.05	3.75±0.47
L7	3.98±0.05	3.63±0.06	F7	1.62±0.05	1.51±0.02

Values are Mean± SE, n=6;
L1: Hexane extract of arjuna leaf; **L2:** Ethyl acetate extract of arjuna leaf; **L3:** Chloroform extract of arjuna leaf; **L4:** Acetone extract of arjuna leaf; **L5:** Ethanol extract of arjuna leaf; **L6:** Methanol extract of arjuna leaf; **L7:** Distilled water extract of arjuna leaf; **Br1:** Hexane extract of arjuna bark; **Br2:** Ethyl acetate extract of arjuna bark; **Br3:** Chloroform extract of arjuna bark; **Br4:** Acetone extract of arjuna bark; **Br5:** Ethanol extract of arjuna bark; **Br6:** Methanol extract of arjuna bark; **Br7:** Distilled water extract of arjuna bark; **F1:** Hexane extract of arjuna fruit; **F2:** Ethyl acetate extract of arjuna fruit; **F3:** Chloroform extract of arjuna fruit; **F4:** Acetone extract of arjuna fruit; **F5:** Ethanol extract of arjuna fruit; **F6:** Methanol extract of arjuna fruit; **F7:** Distilled water extract of arjuna fruit.

4.5. Characterization of the compounds

4.5.1. Collection of fractions through column chromatography

A total of 34 fractions were collected through column chromatography of *T. arjuna* bark extract. Maximum fraction (5) was collected with ethanol, chloroform and water, 80:20 and methanol, hexane, 90:10+ 1 mL formic acid. The minimum fraction (2) was collected with Methanol and chloroform, 50:50. Number of fractions collected from different solvent extracts is represented (Table 14).

Table 14. Showing fractions collected through column chromatography

Sl.No.	Total elution volume (mL)	Name of solvent elution	Fractions collected
1	100	Methanol and chloroform, 50:50	2
2	100	Methanol, hexane, 90:10+ 1 mL formic acid	5
3	100	Ethanol, hexane, 90:10+ 1 mL formic acid	4
4	200	Methanol and chloroform, 80:20	4
5	150	Methanol, chloroform and water,80:20:3+1 mL fromic acid	3
6	200	Methanol chloroform and water, 80:20:5+ I mL formic acid	4
7	150	Ethanol, chloroform and water, 80:20:5+ I mL formic acid	3
8	150	Ethanol, chloroform and water, 80:20:3+ I mL formic acid	4
9	150	Ethanol, chloroform and water, 80:20	5

4.5.2. Standardization of solvent systems for TLC finger printing

A total of four solvent systems were found functional. Among these four solvent systems optimized, Ethanol: chloroform in a ratio of 80:20 with 1.0 mL formic acid was found to be most effective in detecting the presence of antioxidant, flavonoid and phenolic compounds (Plate 6).

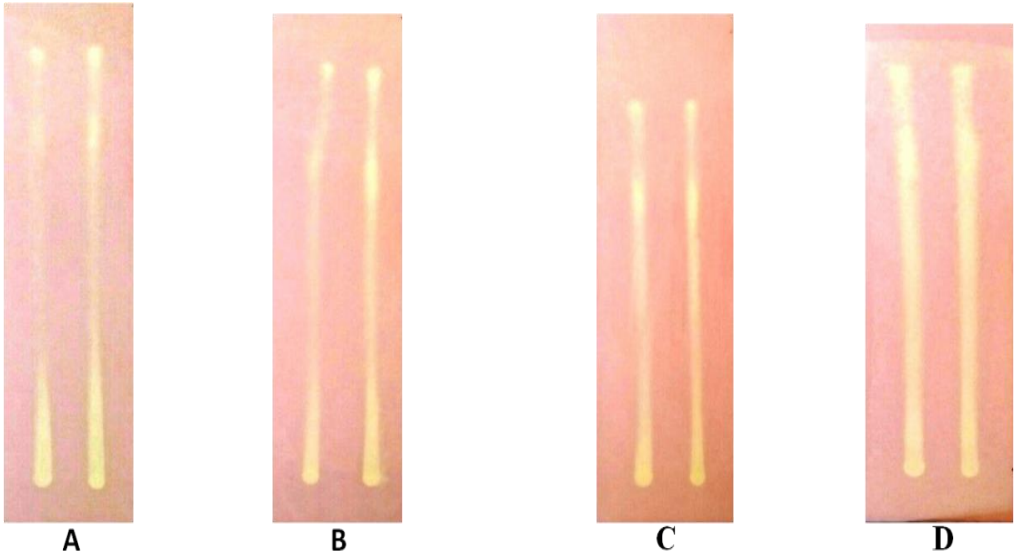


Plate 6. Showing standardization of solvent systems for bioactive constituents

A) Ethanol: Hexane 90:10 (mL) with 1.0 mL of formic acid;
B) Ethanol: Chloroform: Water 80:20:3 (mL) with 1.0 mL of formic acid;
C) Ethanol: Chloroform: Water 80:20:5 (mL) with 1.0 mL of formic acid;
D) Ethanol: Chloroform: Water 80:20:7 (mL) with 1.0 mL of formic acid.

4.5.2.1 Screening of antioxidant constituents

The qualitative screening of the presence of antioxidants in the extracts can be done with DPPH reagent. The presence of antioxidant in TLC plate can be confirmed with visualisation of yellow colour spot upon bleaching with purple coloured DPPH reagents. The Rf value for the spot was estimated as to be 0.93 (Plate 7A).

4.5.2.2 TLC analysis of flavonoid constituents

The presence of flavonoids in TLC plate can be confirmed with visualisation of blue colour spot upon bleaching with purple coloured DPPH reagents and anisaldehyde sulphuric acid. The Rf value for the spot was estimated as to be 0.88 (Plate 7B).

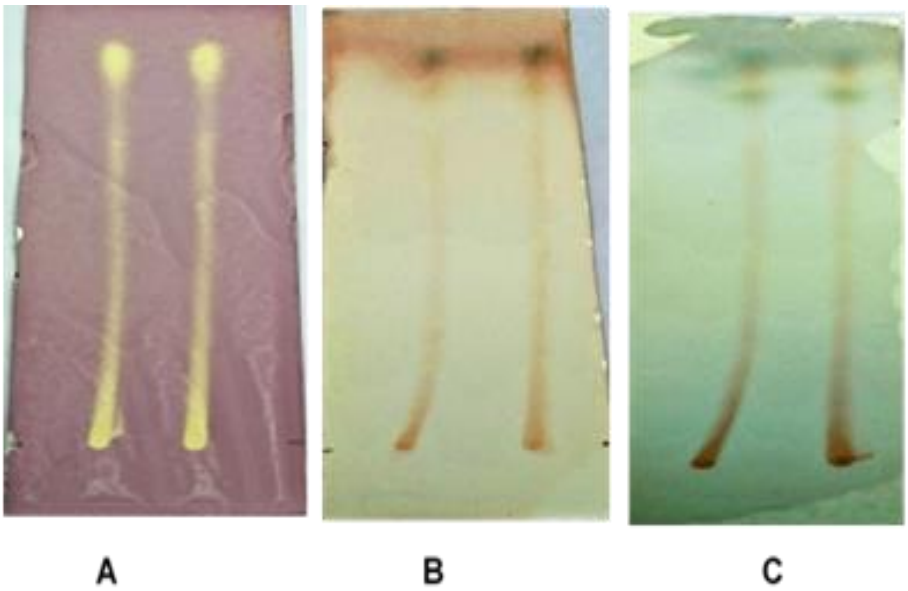


Plate 7. Showing TLC analysis for A) antioxidant; B) flavonoid and C) phenol constituents

4.5.2.3. TLC analysis of phenol constituents

The presence of phenolic constitutes in TLC plate can be confirmed with visualisation of red colour spot upon bleaching with purple coloured DPPH reagents and anisaldehyde sulphuric acid and vanillin sulphuric acid. The Rf value for the spot was estimated as to be 0.83 (Plate 7C).

4.5.3. High resonance liquid chromatography coupled to electrospray orbitrap mass spectrometry (HRLCMS)

The HRLCMS analyse showed that gallic acid and ellagic acid were commonly found bioactive compounds across the facile matrix extract of *T. arjuna*. For instance, out of 6 fruit extract, 3 extracts namely acetone fruit extract (F4), ethanol fruit extract (F5) and methanol fruit extract (F6) exhibited these two metabolites.

Similarly, three leaf extracts, acetone leaf extract (L4), ethanol leaf extract (L5) and methanol leaf extract (L6) displayed these two bioactive principles. While, 4 bark extracts showed these compounds which are as follows: acetone bark extract (Br4), ethanol bark extract (Br5) and methanol bark extract (Br6), and ethyl acetate bark extract (Br2).

The gallic acid comes under hydrolysable tannin groups and ellagic acid is formed due to dimerization of gallic acid. The myricetin and quercetin were among the common flavonoid compounds found in most of the bark and leaf extracts of *T. arjuna*. A comparative HRLCMS analyse and prominent bioactive compounds with their details across solvent extract are being represented (Table 15a, b and c), respectively.

Table15a. Showing bioactive constituents of *T. arjuna* bark across the extract's matrix

Sample Code	Compound Name	Molecular Formula	Molecular Weight	Area
Br1	Cholest-4-en-3-one	$C_{27}H_{44}O$	384.34	237038
	Gallic acid	$C_7H_6O_5$	170.02	190613
	Jasmonic acid	$C_{12}H_{18}O_3$	210.13	154867
Br2	Tropine	$C_8H_{15}NO$	141.12	97383
	Betulin	$C_{30}H_{50}O_2$	442.38	47454
	Ursolic acid	$C_{30}H_{48}O_3$	456.36	977223
	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	6086264
	Ellagic acid	$C_{14}H_6O_8$	302.01	800504
	Gallic acid	$C_7H_6O_5$	170.02	1413705
Br3	Jasmonic acid	$C_{12}H_{18}O_3$	210.13	127262
	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.09	173239
	Ursolic acid	$C_{30}H_{48}O_3$	456.36	343487
	Betulin	$C_{30}H_{50}O_2$	442.38	535267
	Jasmonic acid	$C_{12}H_{18}O_3$	210.13	440310
	Ursolic acid	$C_{30}H_{48}O_3$	456.36	1065991
Br4	Gallic Acid	$C_7H_6O_5$	170.02	3751314
	Ellagic acid	$C_{14}H_6O_8$	302.01	6240864
	(-)-Epigallocatechin	$C_{15}H_{14}O_7$	306.07	4666503
	Catechin	$C_{15}H_{14}O_6$	290.08	830681
	Catechin gallate	$C_{22}H_{18}O_{10}$	442.09	534767
	Quercetin	$C_{15}H_{10}O_7$	302.04	298464
	Epicatechin	$C_{15}H_{14}O6$	290.08	1191073
	Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	458.08	785517
	Myricetin	$C_{15}H_{10}O_8$	318.04	424585
	Ursolic acid	$C_{30}H_{48}O_3$	456.36	366622
Br5	Catechin	$C_{15}H_{14}O_6$	290.08	485673
	Ellagic acid	$C_{14}H_6O_8$	302.01	1417504
	Gallic acid	$C_7H_6O_5$	170.02	555546
	(-)-Epigallocatechin	$C_{15}H_{14}O_7$	306.07	2348791
	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	6672616
Br6	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	1130958
	Ellagic acid	$C_{14}H_6O_8$	302.01	3237724
	Gallic acid	$C_{22}H_6O_5$	170.02	858510
	Jasmonic acid	$C_{12}H_{18}O_3$	210.13	100145
	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.10	59251
Br7	Hesperetin	$C_{16}H_{14}O_6$	302.08	1673957
	4-Hydroxycoumarin	$C_9H_6O_3$	162.03	386621
	4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	160.05	150602
	Quinine	$C_{20}H_{24}N_2O_2$	324.18	661869

Table15b. Showing bioactive constituents of *T. arjuna* leaf across the extract's matrix

Sample Code	Compound Name	Molecular Formula	Molecular Weight	Area
L1	Vitexin	C_{21} H_{20} O_{10}	432.12	2593852
	Ursolic acid	C_{30} H_{48} O_3	456.36	90132
	Jasmonic acid	C_{12} H_{18} O_3	210.13	979318
	Andrographolide	C_{20} H_{30} O_5	350.21	463489
	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	470.34	179361
L2	Quercetin-3β-D-glucoside	C_{21} H_{20} O_{12}	464.10	525241
	Orientin	C_{21} H_{20} O_{11}	448.10	245718
	Tropine	C_8 H_{15} NO	141.12	103342
	Jasmonic acid	C_{12} H_{18} O_3	210.13	978653
	Gallic acid	C_7 H_6 O_5	170.02	417073
	Andrographolide	C_{20} H_{30} O_5	350.23	303040
L3	Betulin	C_{30} H_{50} O_2	442.38	22450
	Jasmonic acid	C_{12} H_{18} O_3	210.13	26781
	Ursolic acid	C_{30} H_{48} O_3	456.36	138092
	Cholest-4-en-3-one	C_{27} H_{44} O	384.34	72345
L4	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	470.34	875408
	Gallic acid	C_7 H_6 O_5	170.02	2825765
	Ellagic acid	C_{14} H_6 O_8	302.01	573841
	Orientin	C_{21} H_{20} O_{11}	448.10	219466
	Myricetin	C_{15} H_{10} O_8	318.04	206708
	Jasmonic acid	C_{12} H_{18} O_3	210.13	341528
	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	470.34	240403
L5	Quercetin-3β-D-glucoside	C_{21} H_{20} O_{12}	464.10	793870
	Orientin	C_{21} H_{20} O_{11}	448.10	132684
	Jasmonic acid	C_{12} H_{18} O_3	210.13	387114
	Myricetin	C_{15} H_{10} O_8	318.04	163504
	Gallic acid	C_7 H_6 O_5	170.02	6062407
	Epigallocatechin gallate	C_{22} H_{18} O_{11}	458.08	755144
	Ellagic acid	C_{14} H_6 O_8	302.01	1045378
	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	470.34	140490
L6	18-β-Glycyrrhetinic acid	C_{30} H_{46} O_4	470.34	90373
	Ellagic acid	C_{14} H_6 O_8	302.01	452955
	Gallic acid	C_7 H_6 O_5	170.02	1994162
	Jasmonic acid	C_{12} H_{18} O_3	210.13	251721
	Orientin	C_{21} H_{20} O_{11}	448.10	86588
	Quercitrin	C_{15} H_{10} O_7	448.10	456359

Table 15c. Showing bioactive constituents of *T. arjuna* fruit across the extract's matrix

Sample Code	Compound Name	Molecular Formula	Molecular Weight	Area
F1	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	2623851
	Cholest-4-en-3-one	$C_{27}H_{44}O$	384.34	1979479
	Betulin	$C_{30}H_{50}O_2$	442.38	854009
	Ursolic acid	$C_{30}H_{48}O_3$	456.36	317453
F2	Andrographolide	$C_{20}H_{30}O_5$	350.21	687431
	Betulin	$C_{30}H_{50}O_2$	442.38	365769
	Gallic acid	$C_7H_6O_5$	170.02	2488059
	Ellagic acid	$C_{14}H_6O_8$	302.01	1498877
	Cholest-4-en-3-one	$C_{27}H_{44}O$	384.34	998095
	4-Coumaric acid	$C_9H_8O_3$	164.05	208920
	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	4737974
F3	Ellagic acid	$C_{14}H_6O_8$	302.01	1877676
	Cholest-4-en-3-one	$C_{27}H_{44}O$	384.34	272430
	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	2093683
F4	16-Hydroxyhexadecanoic acid	$C_{16}H_{32}O_3$	272.23	58938
	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	3020794
	3-Coumaric acid	C9 H8 O3	164.05	175761
	Ellagic acid	$C_{14}H_6O_8$	302.01	4409279
	Gallic acid	$C_7H_6O_5$	170.02	5845458
	Quercitrin	$C_{15}H_{10}O_7$	448.10	219912
	Rutin	$C_{27}H_{30}O_{16}$	610.15	79334
	Vitexin	$C_{21}H_{20}O_{10}$	432.11	192322
F5	Tropine	$C_8H_{15}NO$	141.12	3376405
	Quercitrin	$C_{15}H_{10}O_7$	448.10	95564
	Jasmonic acid	$C_{12}H_{18}O_3$	210.13	107683
	Gallic acid	$C_7H_6O_5$	170.02	4977273
	Ellagic acid	$C_{14}H_6O_8$	302.01	1895607
	Deoxycorticosterone 21-glucoside	$C_{27}H_{40}O_8$	492.27	76108
	Betulin	$C_{30}H_{50}O_2$	442.38	116072
	Andrographolide	$C_{20}H_{30}O_5$	350.21	744258
	4-Coumaric acid	$C_9H_8O_3$	164.05	109470
	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	470.34	3024916
F6	Tropine	$C_8H_{15}NO$	141.12	94846
	Tropine	$C_8H_{15}NO$	141.12	1710697
	Rutin	$C_{27}H_{30}O_{16}$	610.15	203795
	Quercitrin	$C_{15}H_{10}O_7$	448.10	196742
	Gallic acid	$C_7H_6O_5$	170.02	4709294
F7	4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	160.05	150602
	Quinine	$C_{20}H_{24}N_2O_2$	324.18	661869

4.5.4. Data analysis and peak extraction

After data acquisition, the raw files (raw) obtained were processed in Thermo Compound discoverer software (Version 2.1 SP1). For compound identification, Max ID workflow was used to Detect and identify all compounds in a single sample (with ddMS2).

This workflow performs unknown compound detection and predicts elemental compositions for all compounds. The compounds were discovered with the use of the mzCloud (ddMS2) search engine. It also uses mzCloud to run a similarity search for all substances having ddMS2 data. The intensity threshold for unknown chemical detection was set to 500000, the mass tolerance was set to 5 ppm, the S/N threshold was set to 3, and the ions were as follows: [M+H]+1; [M+K]+1; [M+Na]+1; [M+NH4]+1; [M-H]-1.

Following parameters were kept for mzCloud database search: Compound Classes: Endogenous Metabolites, Natural Products/Medicine, Steroids/Vitamins/Hormones, Precursor Mass Tolerance: 10 ppm, FT Fragment Mass Tolerance: 10 ppm, IT Fragment Mass Tolerance: 0.4 Da. The representative chromatogram and mass spectra of the compounds are represented (Fig. 15a-c; 16a-c; 17a-b).

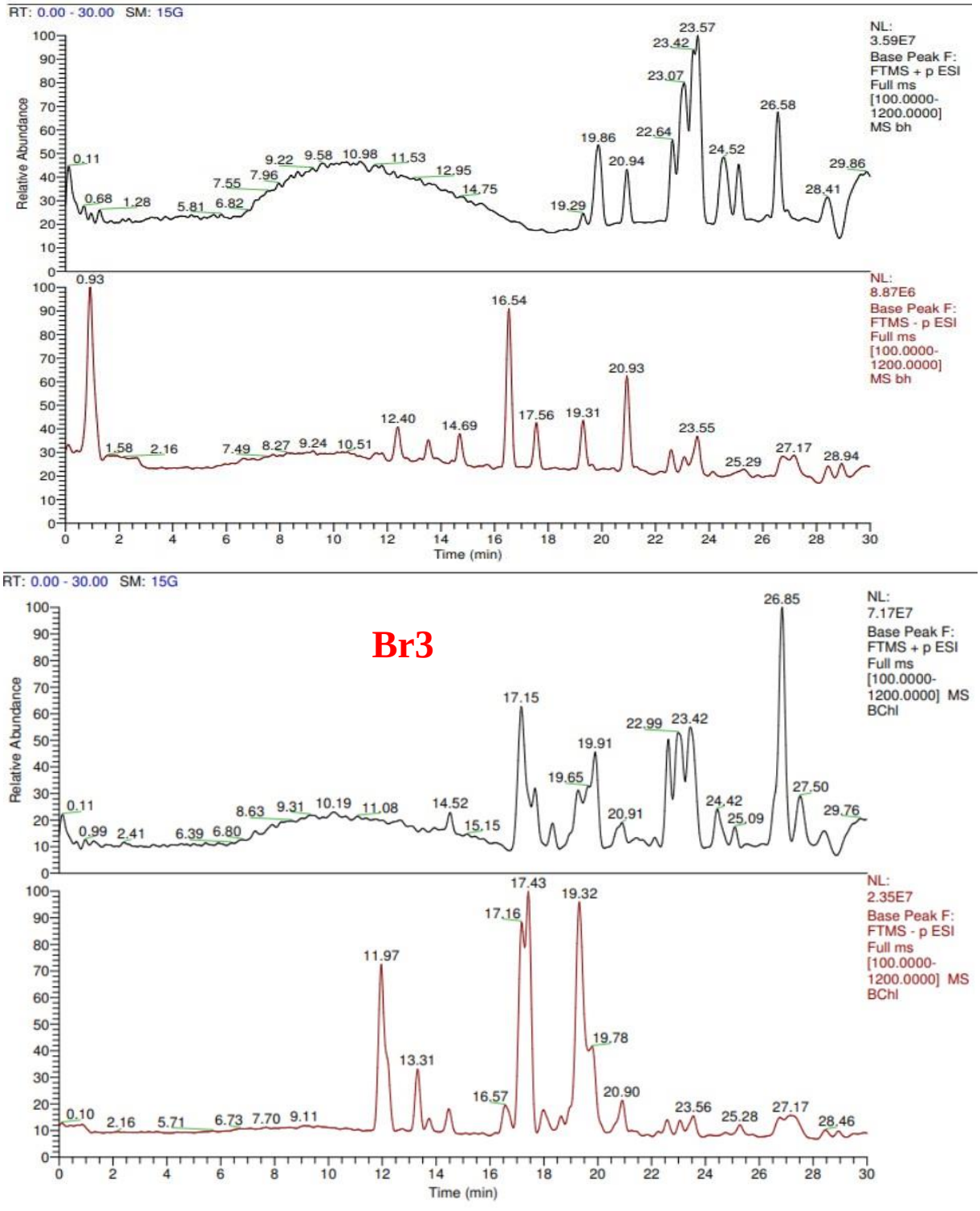
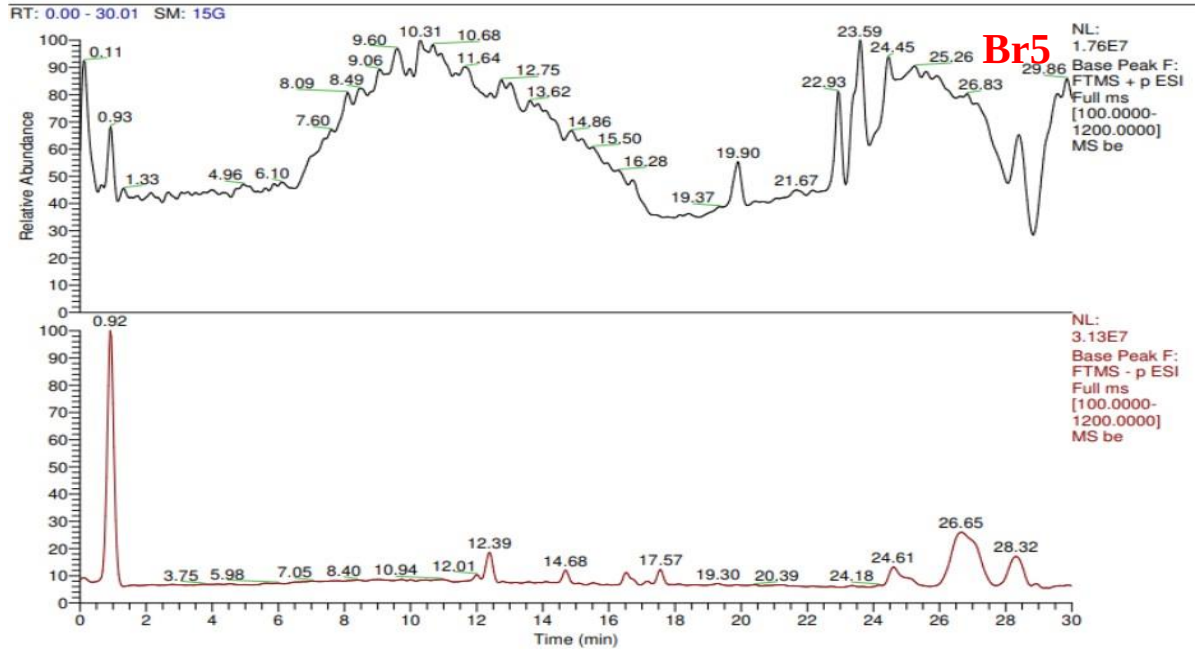
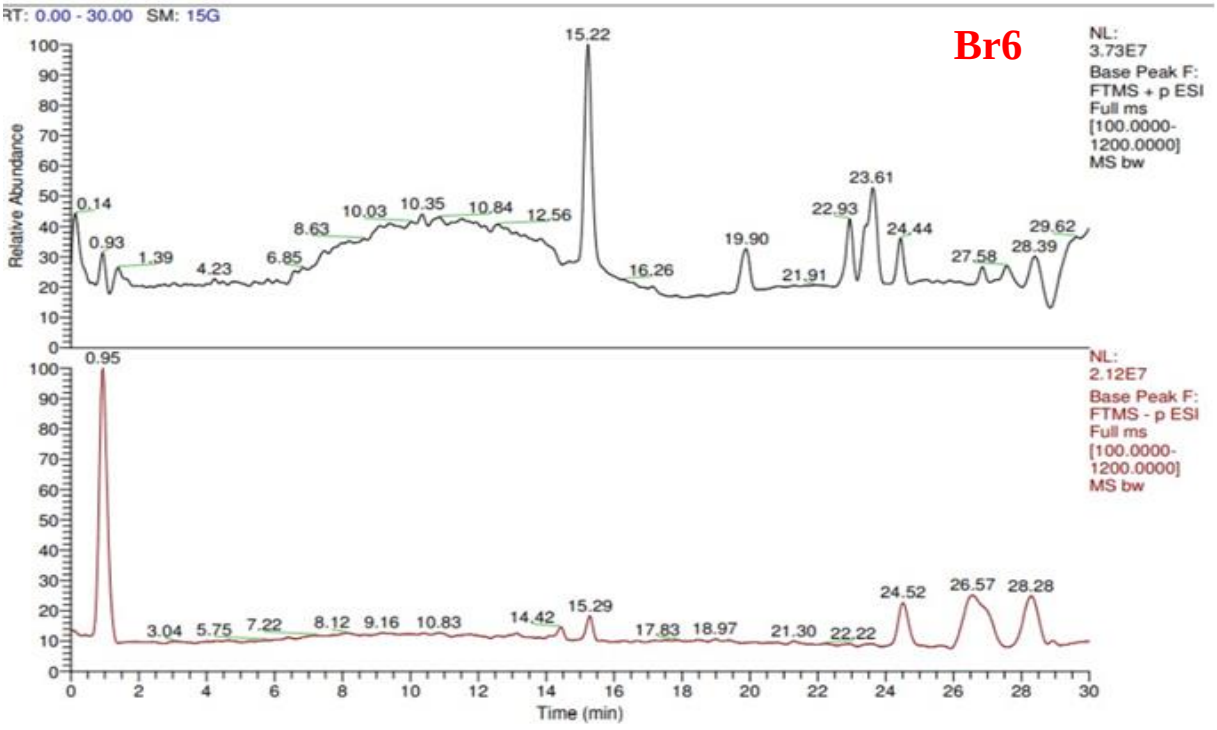


Fig. 15a. Showing chromatogram and mass spectra of Br1 and Br3


RT: 0.00 - 30.01 SM: 15G
Br5
NL:
1.76E7
Base Peak F:
FTMS + p ESI
Full ms
[100.0000-
1200.0000]
MS be
NL:
3.13E7
Base Peak F:
FTMS - p ESI
Full ms
[100.0000-
1200.0000]
MS be
Relative Abundance
Time (min)


RT: 0.00 - 30.00 SM: 15G
Br6
NL:
3.73E7
Base Peak F:
FTMS + p ESI
Full ms
[100.0000-
1200.0000]
MS bw
NL:
2.12E7
Base Peak F:
FTMS - p ESI
Full ms
[100.0000-
1200.0000]
MS bw
Relative Abundance
Time (min)

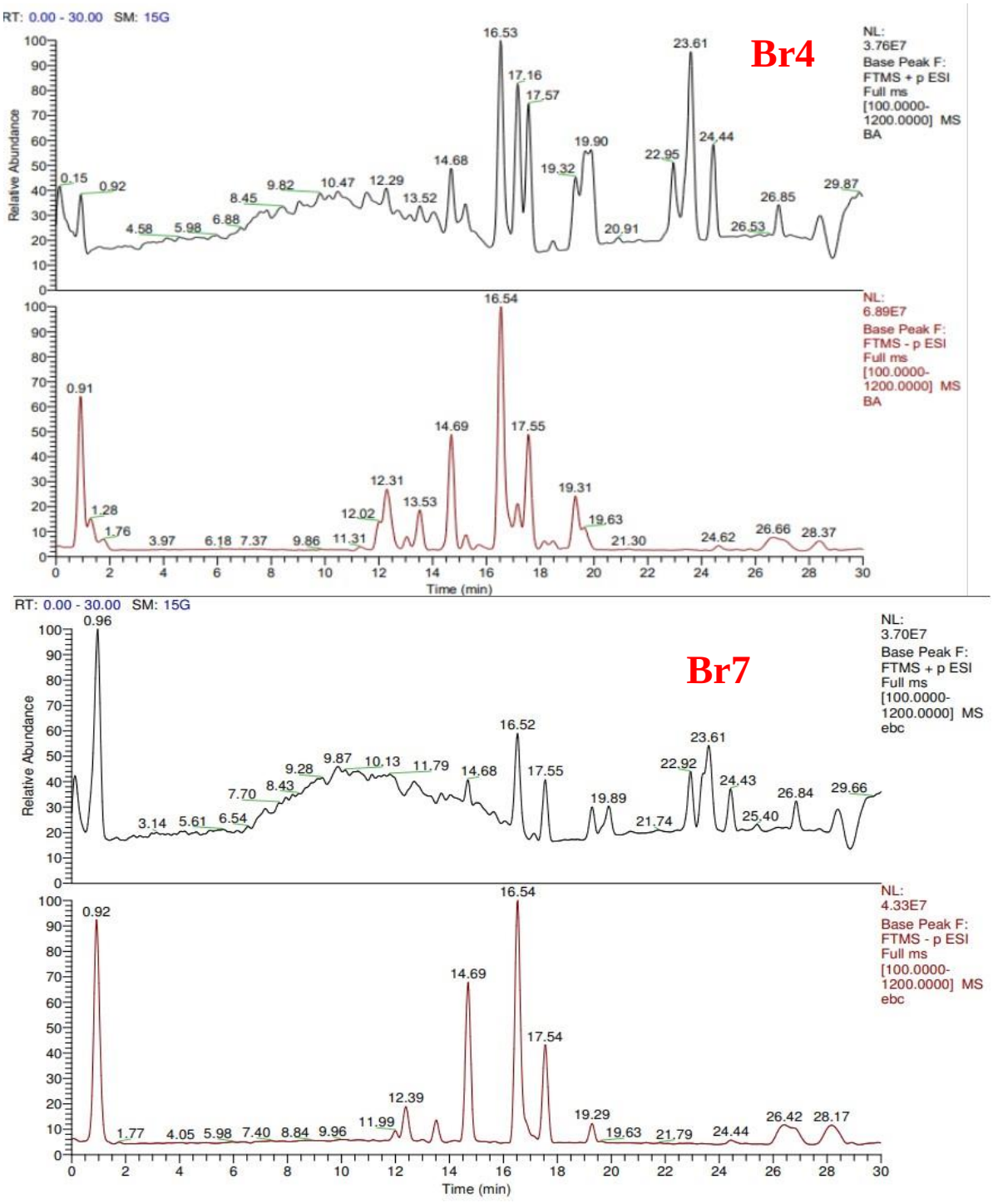
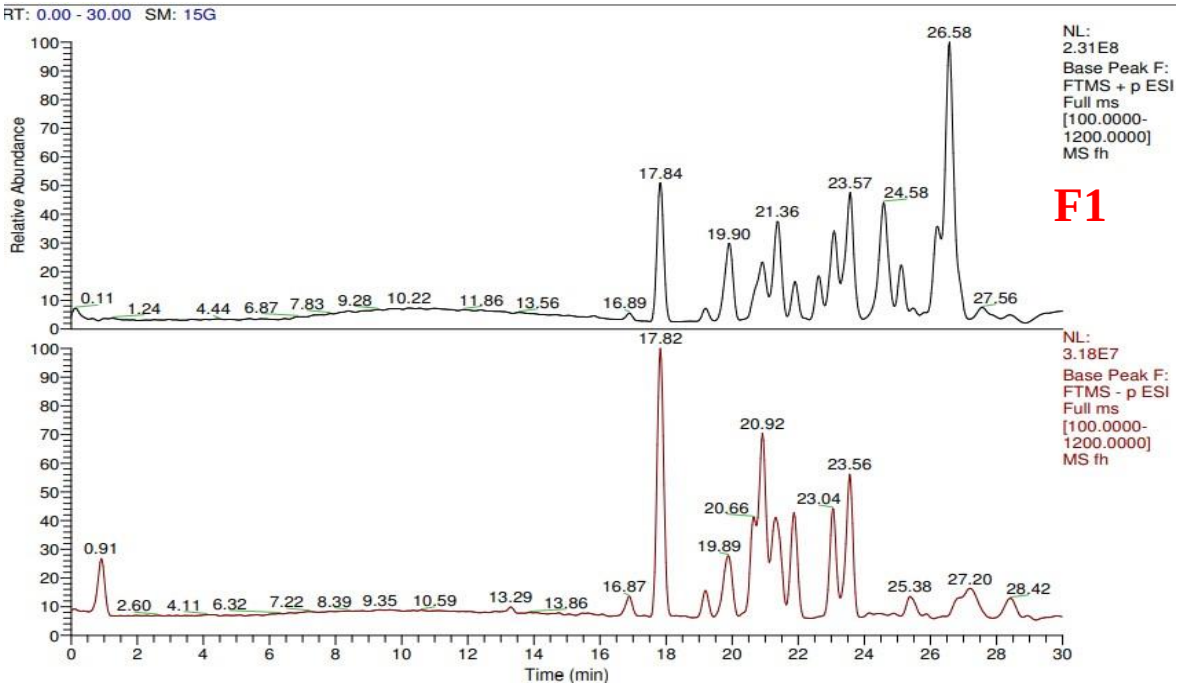


Fig. 15c. Showing chromatogram and mass spectra of Br4 and Br7



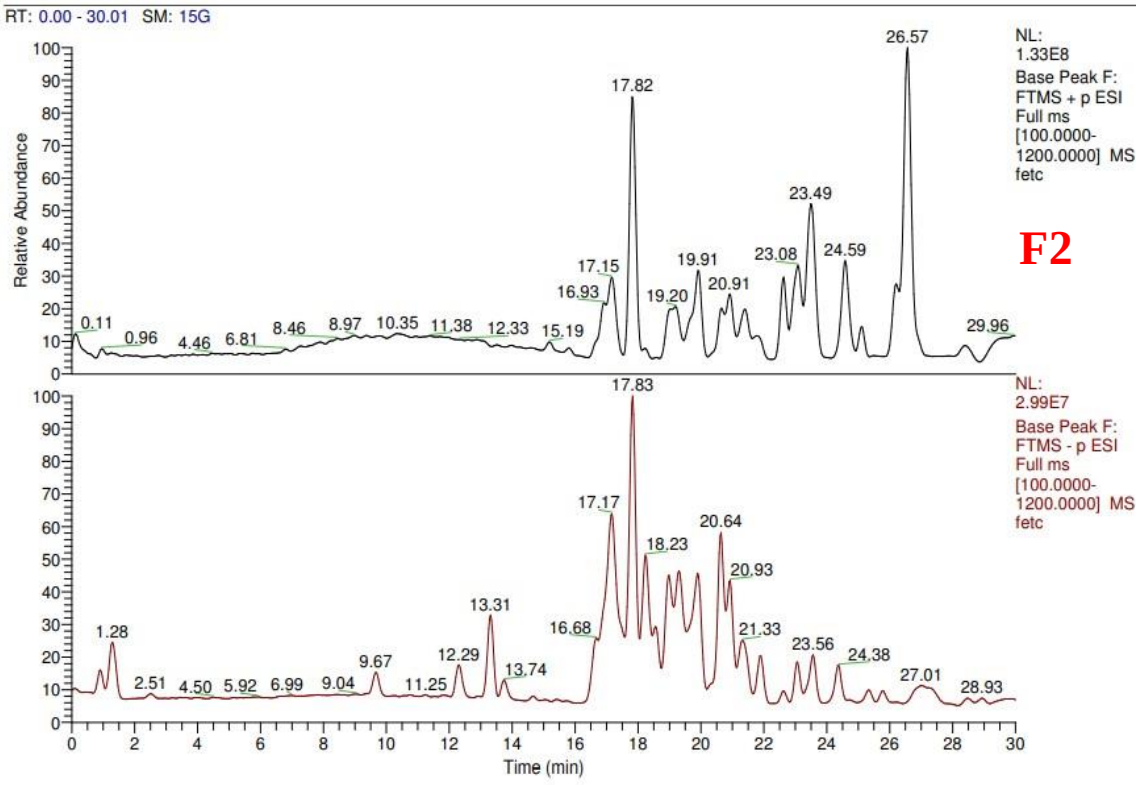


Fig. 16a. Showing chromatogram and mass spectra of F1 and F2

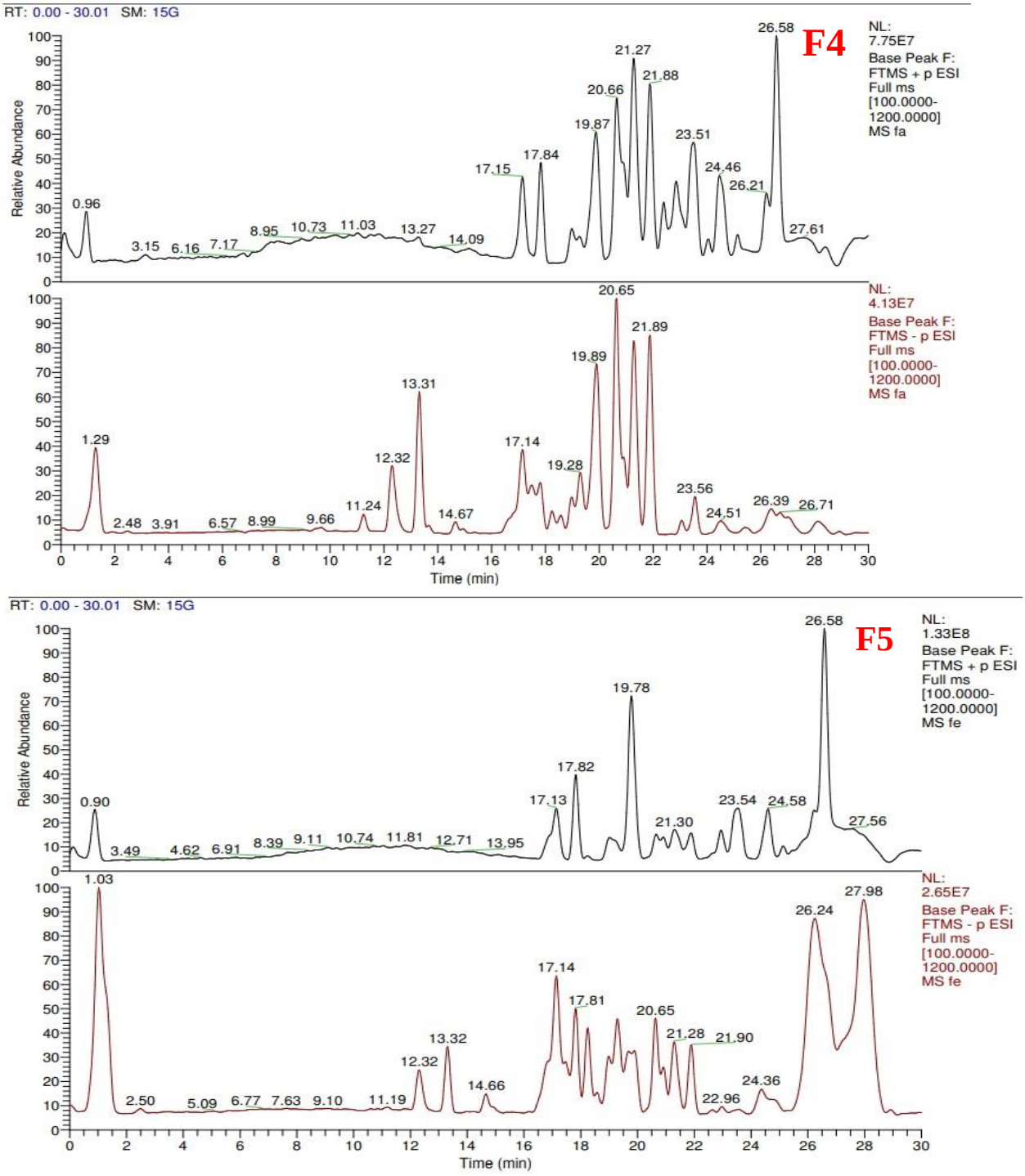


Fig. 16b. Showing chromatogram and mass spectra of F4 and F5

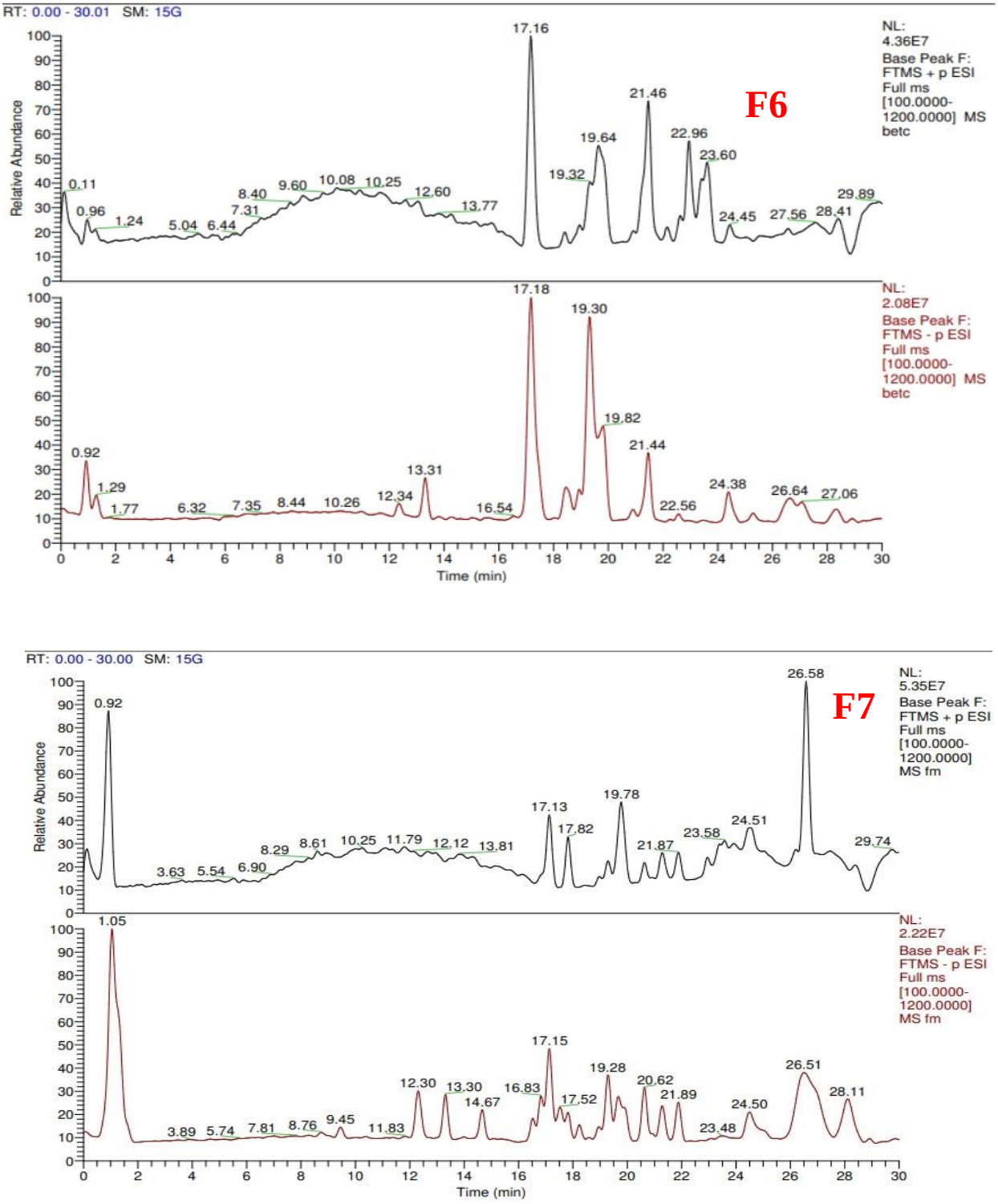


Fig. 16c. Showing chromatogram and mass spectra of F6 and F7

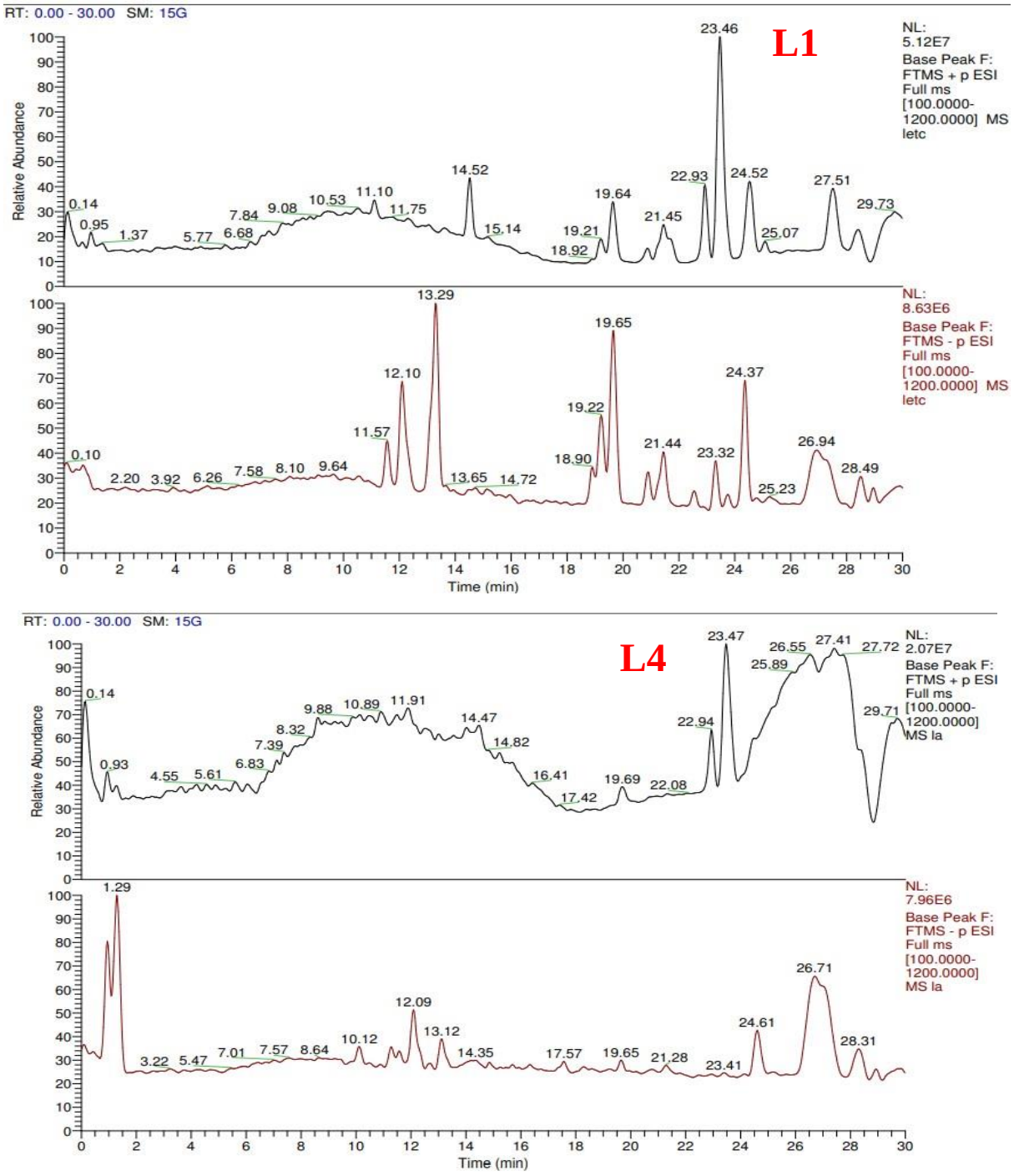


Fig. 17a. Showing chromatogram and mass spectra of L1 and L4

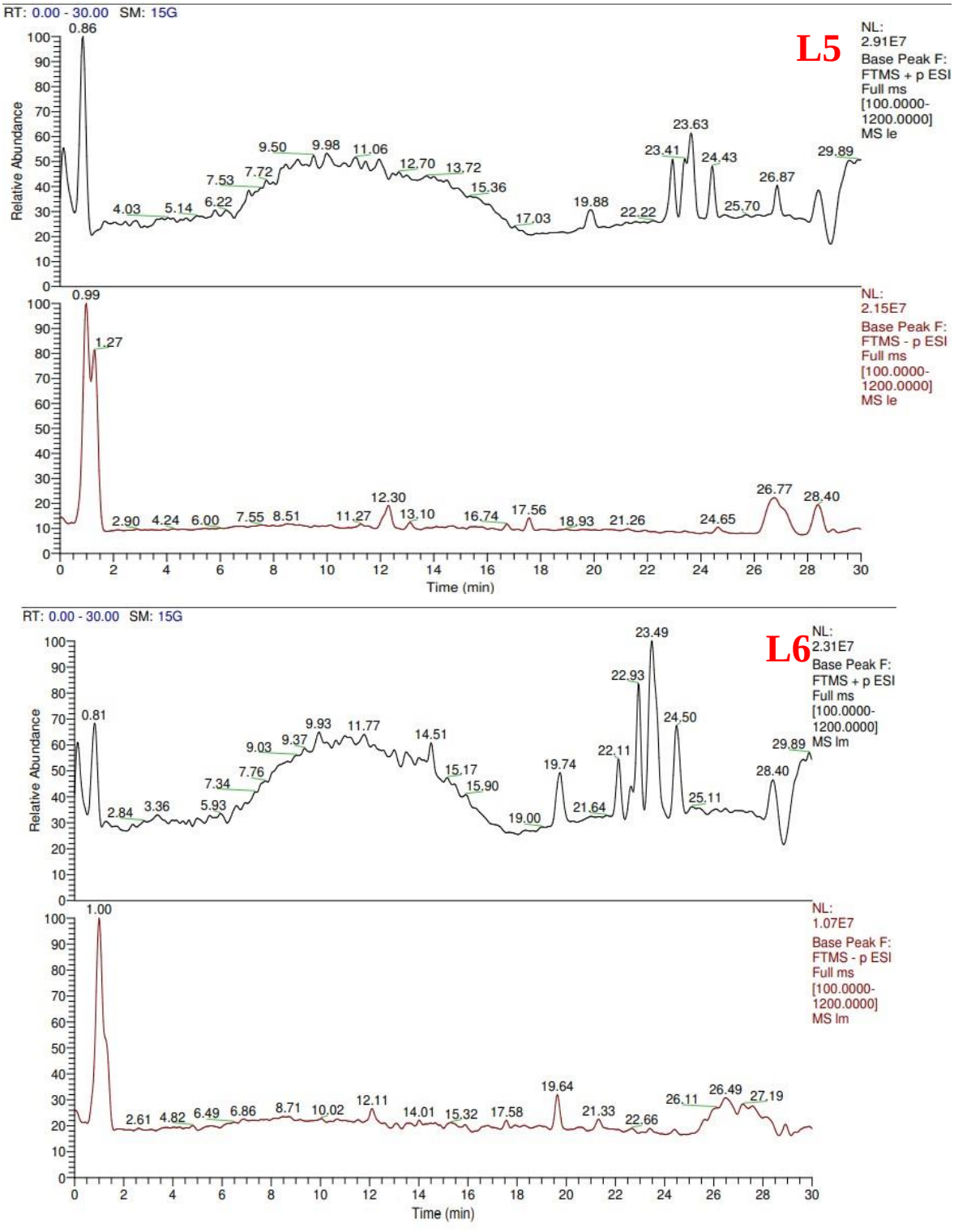


Fig. 17b. Showing chromatogram and mass spectra of L5 and L6

4.5.5. UV-VIS analyses

UV-VIS analyses showed two peaks (200-400 nm) that corresponds to the presence of unsaturated groups and heteroatoms such as S, N, O, which are essential moiety in antioxidants, polyphenols and flavonoids (Fig 18a-18c).

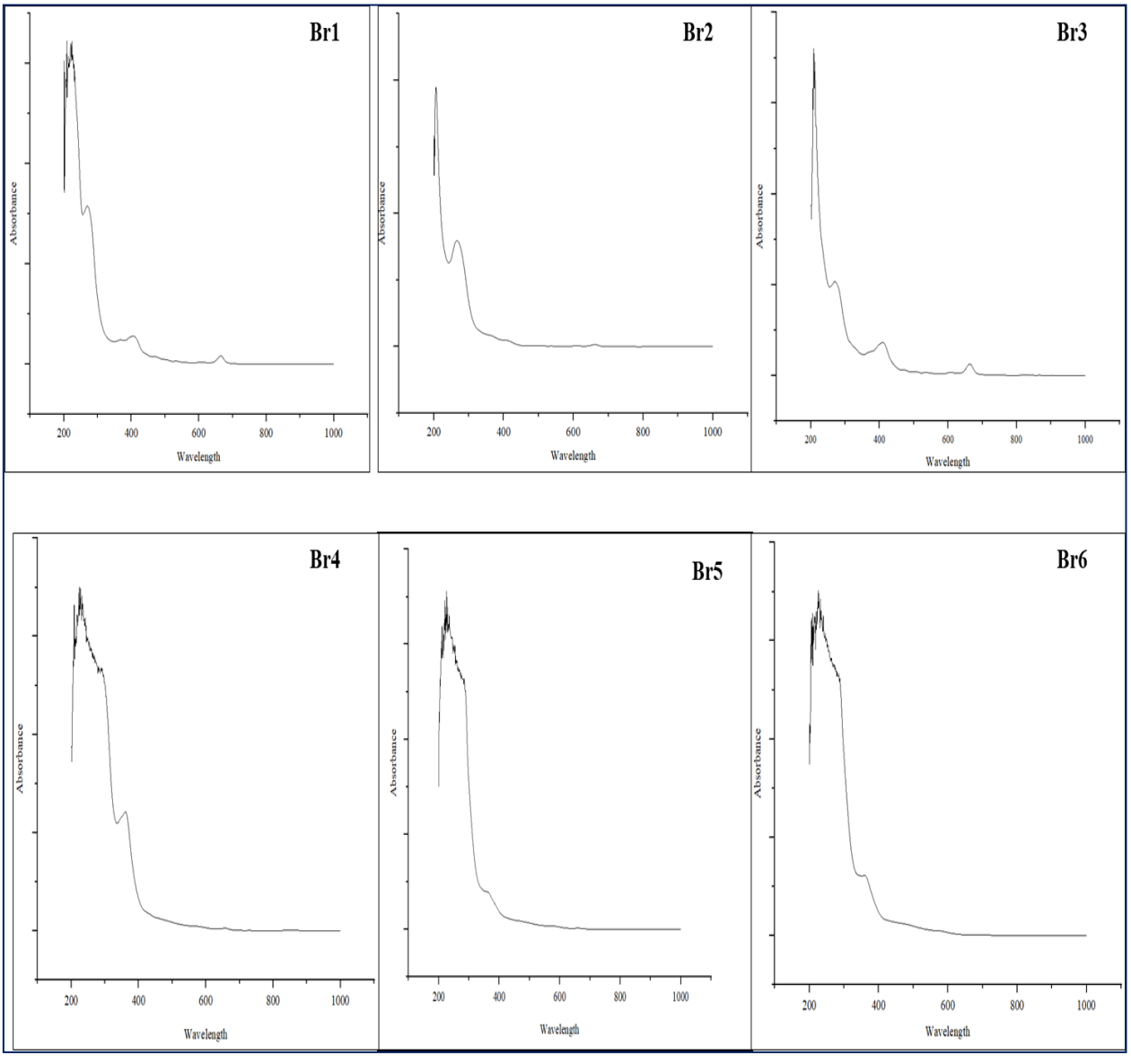


Fig. 18a. Showing UV-VIS spectra solvent extracts of *T. arjuna* bark

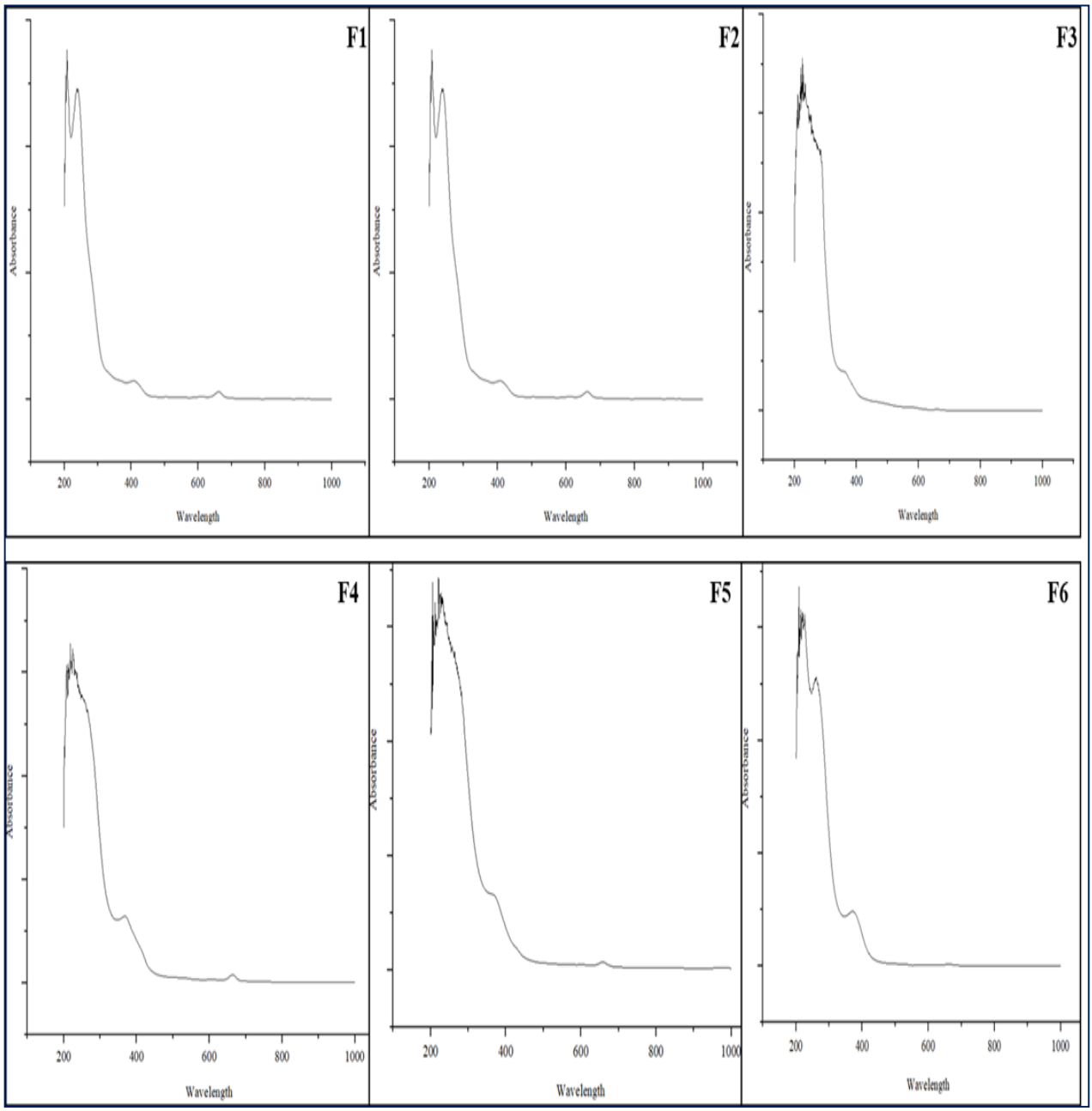


Fig. 18b. Showing UV-VIS spectra of solvent extracts of *T. arjuna* fruit

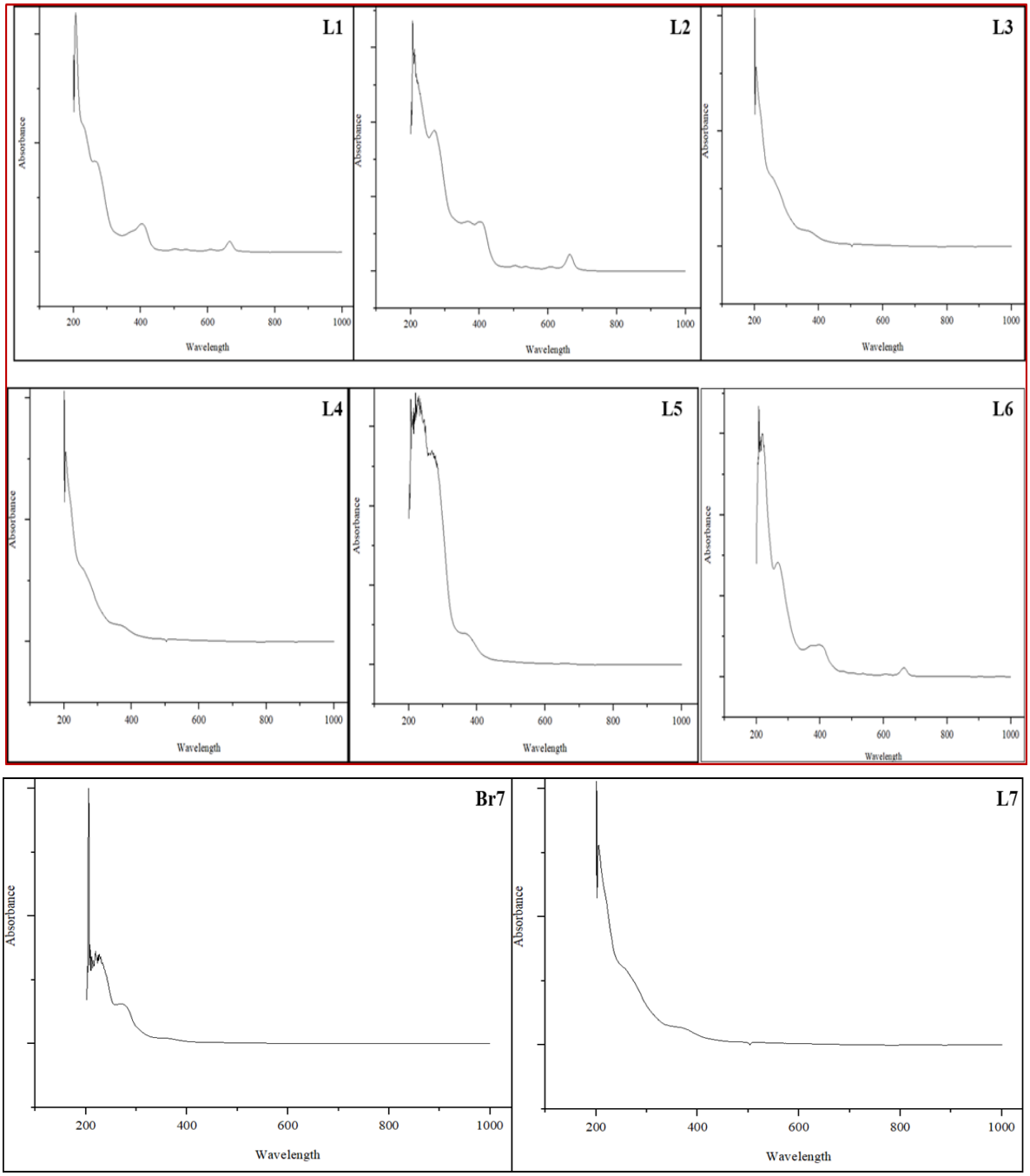


Fig. 18c. Showing UV-VIS spectra of solvent extracts of *T. arjuna* leaf and Br7

4.5.6. FT-IR Data

The FT-IR spectra showed band pattern across the solvent extracts that gives an idea about the functional groups of the bioactive principles in solvent extracts of *T. arjuna* (Fig. 19a-19d). The bending pattern and stretching vibration between functional group at different wave length is represented in Table 16a-c.

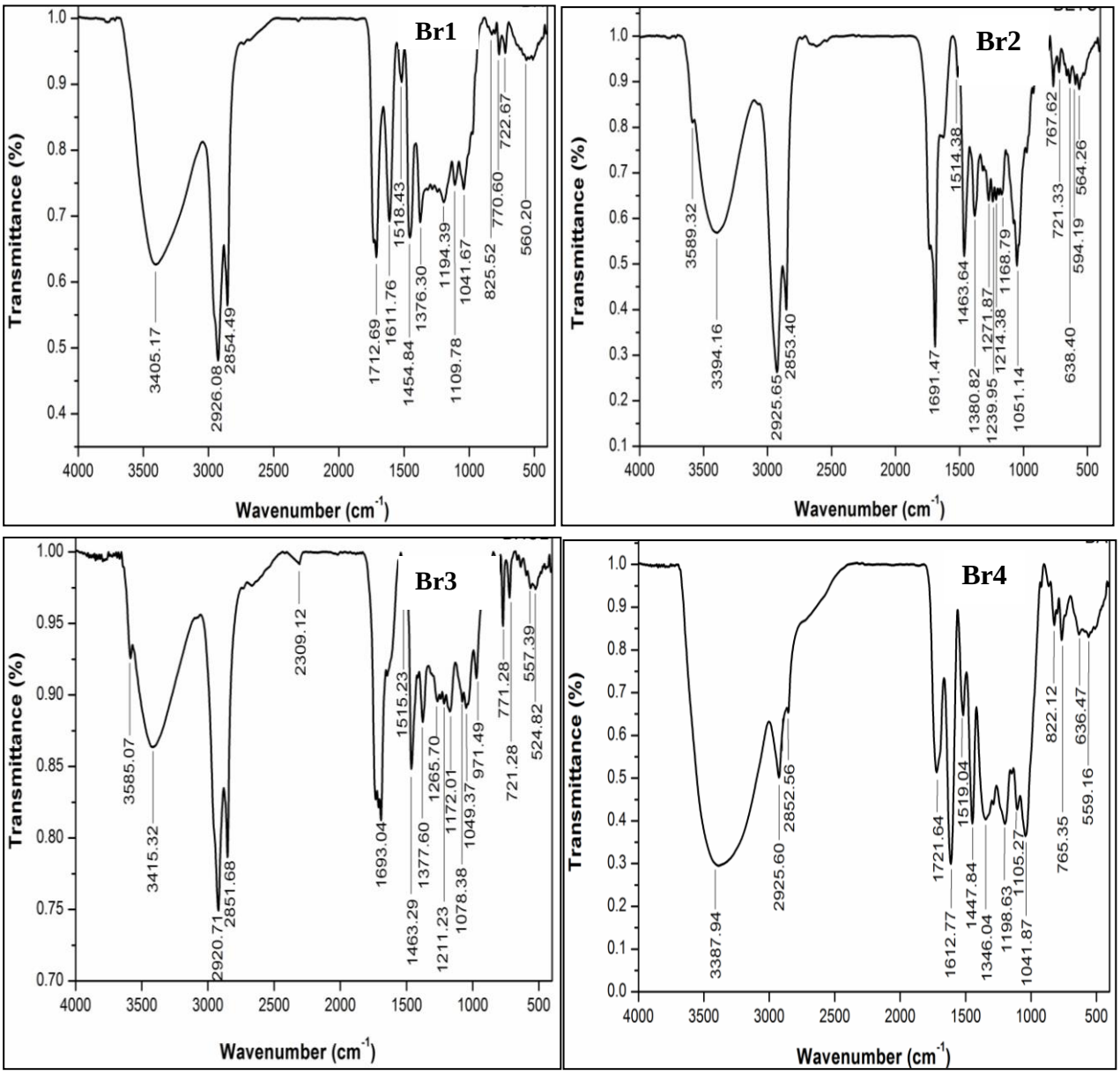
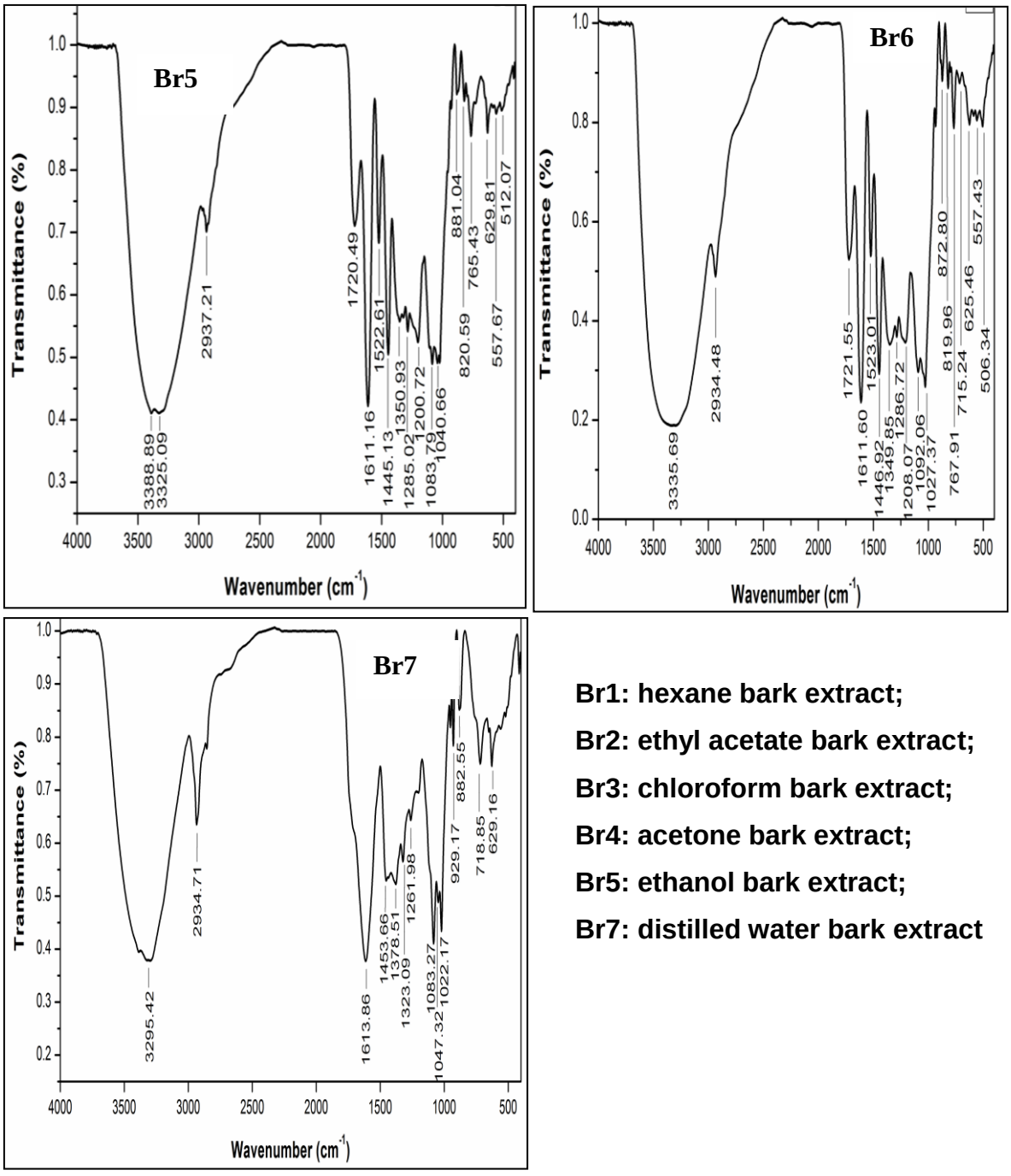


Fig. 19a. FT-IR analyses of band pattern in solvent extracts of *T. arjuna* bark (Br1-Br4)



:

Br1: hexane bark extract;

Br2: ethyl acetate bark extract;

Br3: chloroform bark extract;

Br4: acetone bark extract;

Br5: ethanol bark extract;

Br7: distilled water bark extract

Fig. 19b. FT-IR analyses of band pattern in solvent extracts of *T. arjuna* bark (Br5-Br7)

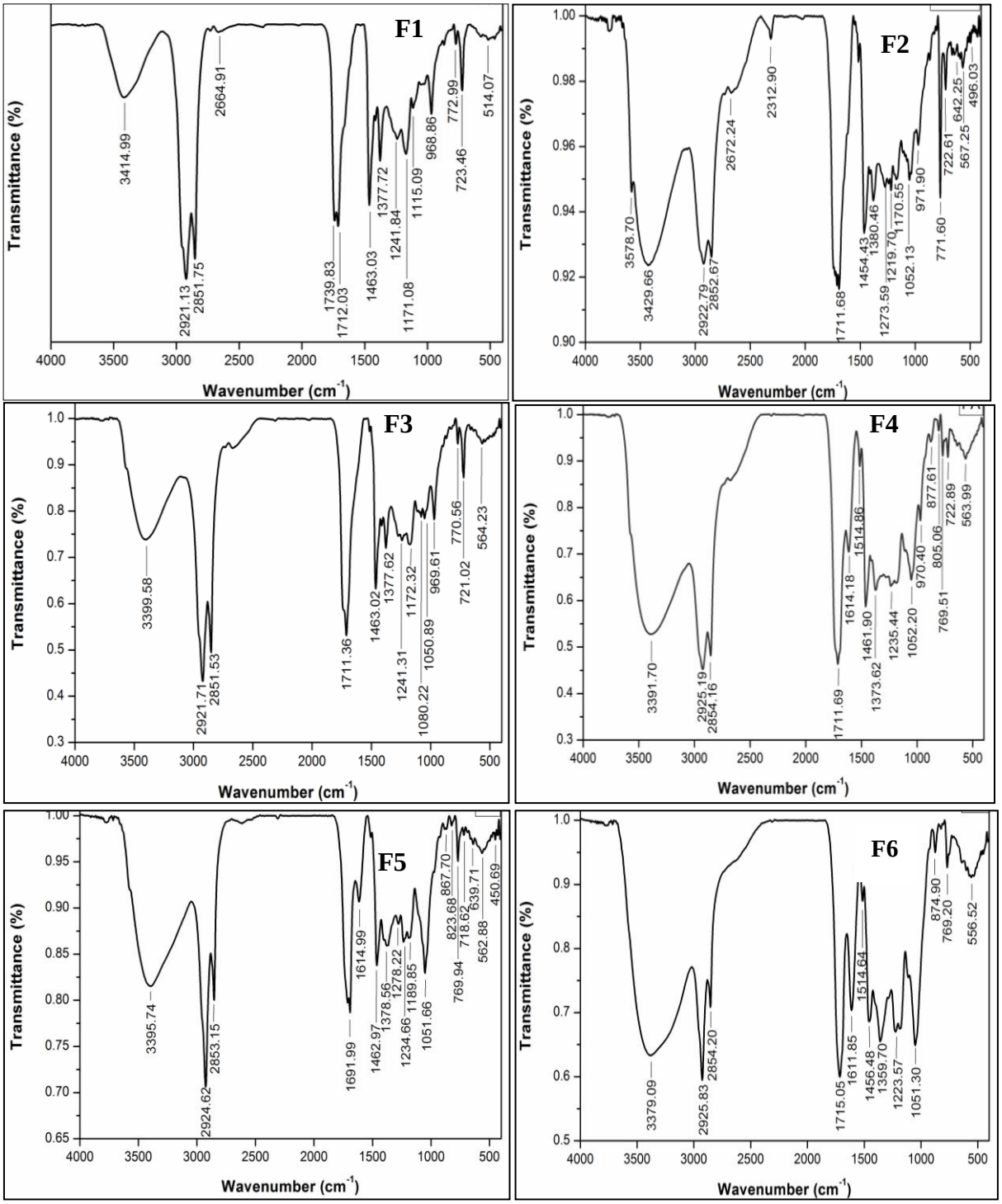


Fig. 19c. FT-IR analyses of band pattern in solvent extracts of *T. arjuna* fruit (F1-F6)

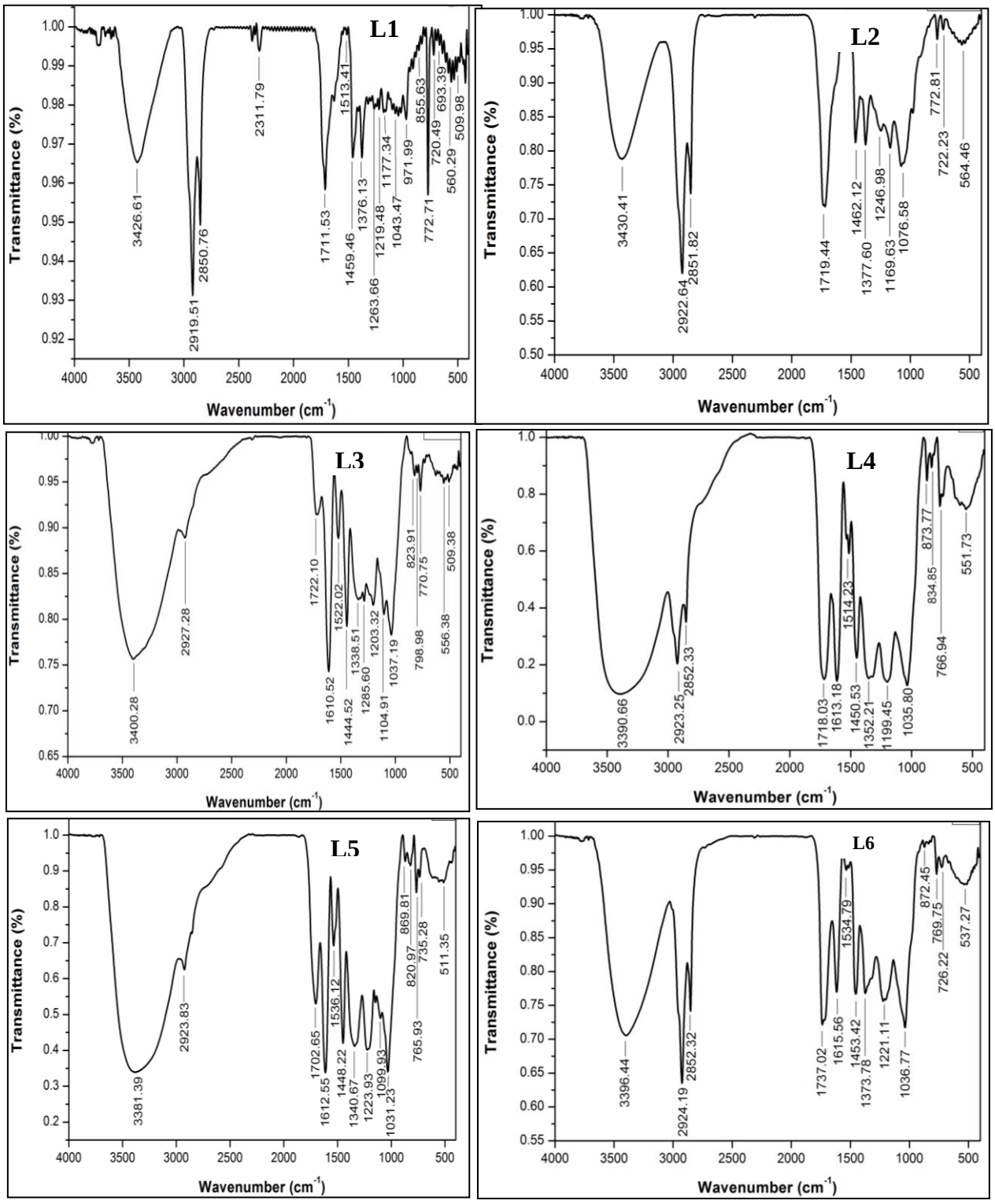


Fig. 19d. FT-IR analyses of band pattern in solvent extracts of *T. arjuna* leaf (L1-L6)

Table 16a. Showing functional groups in bark extracts of *T. arjuna*

Functional grp	Br4	Br5	Br2	Br1	Br3	Br6	Br7
Alcohols (O-H bending)			3589		3585		
	3387	3388	3394	3405	3415		
Alkynes (≡C-H stretching)		3325				3335	
							3295
Alkanes	2925	2937	2925	2926	2920	2934	2934
	2852		2853	2854	2851		
P-H Phosphine					2309		
Aldehydes and Ketones	1721	1720		1712		1721	
Aryl ketones			1691		1693		
1° Amine	1612	1611		1611		1611	1613
2° Amide	1519	1522	1514	1518	1515	1523	
alkanes (CH_2 and CH_3 deformation)	1447	1445	1463	1454	1463	1446	1453
			1380	1376	1377		1378
Alcohols (O-H bending)	1346	1350				1349	
Carboxylic acid		1285	1271		1265	1286	1261
			1239				
			1214		1211	1208	
Alcohols (C-O stretching)	1198	1200		1194			
			1168		1172		
	1105			1109			
		1083			1078	1092	1083
	1041	1040	1051	1041	1049		1047
						1027	1022
Alkenes					971		929
		881				872	882
	822	820		825		819	
Alcohols (O-H bending)	765	765	767	770	771	767	
Alkanes			721	722	721	715	718
Alkynes (≡C-H bending)	636	629	638			625	629
Alkyl halides			594				
	559	557	564	560	557	557	
		512			524	506	

Br1: hexane bark extract; **Br2:** ethyl acetate bark extract; **Br3**: chloroform bark extract; **Br4:** acetone bark extract; **Br5:** ethanol, bark extract; **Br6:** methanol bark extract; **Br7:** ditilled water bark extract.

Table 16b. Showing functional groups in fruit extracts of *T. arjuna*

Functional grp	F4	F3	F5	F2	F1	F6
Alcohols (O-H bending)				3578		
				3429	3414	
	3391	3399	3395			3379
Alkanes and alkyls	2925	2921	2924	2922	2921	2925
	2854	2851	2853	2852	2851	2854
Carboxylic acid (O-H stretching)				2672	2664	
P-H phosphine				2312		
Aldehyde (C=O stretching)					1739	
Alcohols (C-O stretching)	1711	1711		1711	1712	1715
Aryl ketones			1691			
1° Amines	1614		1614			1611
Amides	1514					1514
Alkanes and alkyls	1461	1463	1462		1463	
				1454		1456
	1373	1377	1378	1380	1377	
Alkanes						1359
Carboxylic acids				1273		
	1235	1241	1234	1219	1241	1223
Alcohols (O-H stretching)			1189			
		1172		1170	1171	
					1115	
		1080				
	1052	1050	1051	1052		1051
Alkenes	970	969		971	968	
	877		867			874
	805		823			
Alcohols (O-H bending)	769	770	769	771	772	769
Alkanes -$(CH_2)_n$ bending	722	721	718	722	723	
Alkynes (≡C-H)			639	642		
	563	564	562	567		
Alkyl halides					514	556
			450	496		

F1: hexane fruit extract; **F2:** ethyl acetate fruit extract; **F3:** chloroform fruit extract; **F4:** acetone fruit extract; **F5:** ethanol fruit extract; **F6:** methanol fruit extract; **F7:** distilled water fruit extract.

Table 16c. Showing functional groups in leaf extracts of *T. arjuna*

Functional group	L4	L5	L2	L1	L6
Alcohols			3430	3426	
(O-H bending)	3390	3381			3396
Alkanes	2923	2923	2922	2919	2924
	2852		2851	2850	2852
Phosphine				2311	
Ketones	1718		1719	1711	1737
Carboxylic acid		1702			
1° Amine	1613	1612			1615
2° Amide	1514	1536		1513	1534
Alkanes	1450	1448	1462	1459	1453
Alcohols			1377	1376	1373
(O-H bending)	1352	1340			
Carboxylic acid			1246	1263	
		1223		1219	1221
Alcohols (C-O stretching)	1199		1169	1177	
		1099	1076		
	1035	1031		1043	1036
Alkenes (=C-H bending)				971	
	873	869		855	872
	834	820			
Alcohols (O-H bending)	766	765	772	772	769
Aromatic compound		735			
Alkanes			722	720	726
Alkynes (≡C-H bending)				693	
Alkyl halides	551		564	560	
		511		509	537

L1: hexane leaf extract; **L2:** ethyl acetate leaf extract; **L4:** acetone leaf extract; **L5:** ethanol leaf extract; **L6:** methanol leaf extract.

4.6. Feeding experiment with bark powder and extract of *Terminalia arjuna*

4.6.1. Indoor feed trial of *T. arjuna* bark powder

4.6.1.1 Carcass composition of *L. rohita* fingerlings

In Table 17, the fish carcass configuration is listed. The T3 had the considerably highest ($p<0.05$) moisture content, followed by CT>T1 and T2. T3 had a considerably higher moisture content ($p<0.05$) than the other groups, although T1 and T2 had no significance ($p>0.05$). The crude protein content (CP %) exhibited no

significance across treatment groups (p>0.05). In *L. rohita* fingerlings, crude lipid content is inversely proportional to dietary TABP, with T3 having the lowest fat level (p<0.05) as compared with other treatments. With graded TABP levels demonstrating maximum and minimum values in T1 and T3, respectively and ash content reduced non-significantly (p>0.05) between the groups.

Table 17. Carcass composition of *L. rohita* fingerlings fed different level of *T. arjuna* bark powder (TABP)

Parameters/ Treatments	Initial	CT	T1	T2	T3	p-value
Moisture (%)	71.95+0.95	70.12+0.92^{b}	70.03+0.23^{bc}	69.09+0.53^{c}	75.43+0.35^{a}	**0.005**
Crude protein (%)	16.28±67	16.96±0.89	16.85+0.87	17.26+0.26	15.88+1.48	**0.171**
Crude lipid (%)	6.82+0.78	8.34+0.45^{b}	8.67+0.78^{a,b}	8.89+0.36^{a}	5.54+0.58^{c}	**0.008**
Ash (%)	4.95+0.23	4.58+0.68^{a}	4.39+0.79^{b}	4.34+0.27^{b}	3.45+0.18^{a,c}	**0.005**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly (p <0.05) when compared to each other.

4.6.1.2. Growth performances, nutrient utilisation, and body indices of the *L. rohita*

Dietary TABP considerably (p<0.05) increased weight gain in rohu, with highest and minimum weight gain in T2 and T3, respectively (Table 17); however, there was no substantial (p>0.05) variation between CT and T1 groups (p>0.05). Dietary TABP had a substantial impact on growth rates, such as, WG (%), SGR, FCR, PER, and nutrient utilisation, such as apparent net protein utilisation (ANPU), fat retention (FR), and energy retention (ER). Among the dietary treatments, *L. rohita* fingerlings fed with 10 g kg^{-1} TABP had the highest WG (%), 148.41, SGR (1.51%), and PER (1.31), whereas FCR (1.63) followed the opposite pattern (Table 18).

At 15 g kg^{-1} TABP, growth rates, feed conversion, and nutrient consumption were the lowest. However, CT and T3 had similar growth rates, feed conversion, and

nutrient consumption ($p>0.05$). All treatment groups had equal feed consumption ($p>0.05$). Protein and fat intake, on the other hand, changed in response to dietary TABP ($p<0.05$). Survival (%) was similar in the CT and T1 groups ($p>0.05$), but differed substantially ($p<0.05$) in the T2 and T3 groups. The hepatosomatic index (HSI) was considerably differed between treatments ($p<0.05$). The T2 groups had the highest gastrosomatic index (GaSI) and Craniosomatic index (CSI) values compared with the other treatments ($p<0.05$).

4.6.1.3. Relative percentage survival

The RPS of the feed experiment, followed by the challenge study, revealed a substantial variations ($p<0.05$) between the groups (Fig. 20). The T3 is not statistically different from CT or T1 ($p>0.05$). T2 had the highest RPS (81.25%) following infection with *A. hyrophila*, while T3 had the lowest (51.11) after infection with *E. tarda*. The RPS can be represented as follows, T2>T1>T3>CT.

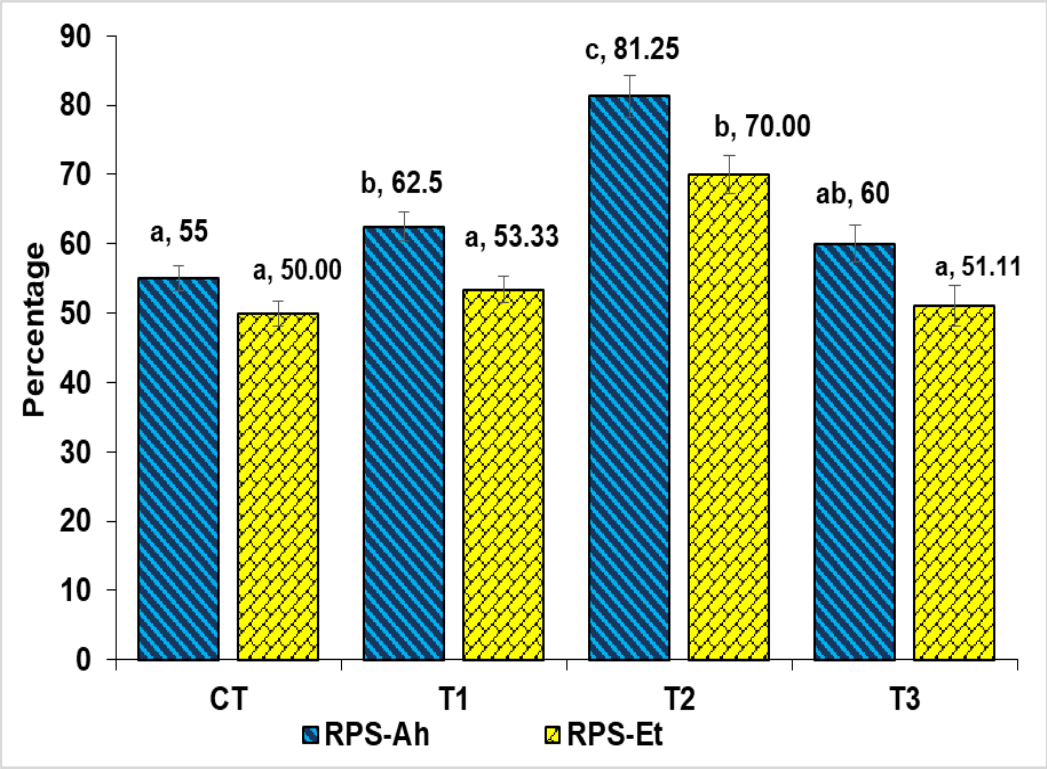


Fig. 20. Relative percentage survival of indoor feed trial followed by challenge study

Table 18. Growth performances, nutrient utilization and body indices of *L. rohita* fingerlings fed with different level of *Terminalia arjuna* bark powder (TABP) under indoor feed trial

Treatment	CT	T1	T2	T3	p-value
IW(g)	20.53± 0.36	20.63± 0.28	20.7± 0.32	20.43± 0.44	**0.283**
FW(g)	40.73± 0.86^{c}	42. 86±1.02^{c}	51.5±1.29^{b}	38.93±0.64^{a}	**0.005**
WG (g/day)	0.22 ± 0.05^{a}	0.25 ± 0.07^{c}	0.34 ± 0.09^{b}	0.20± 0.05^{a}	**0.005**
AWG (g)	20.53±0.141^{b}	22.57±0.94^{b}	30.83±0.334^{c}	18.57±0.475^{a}	**0.003**
FI (g/fish)	5.69±0.037^{ab}	5.70±0.044^{ab}	6.12±0.221^{b}	5.58±0.022^{a}	**0.008**
WG (%)	98.79±0.964^{b}	109.45±0.798^{b}	148.41±0.854^{c}	84.55±0.981^{a}	**0.001**
SGR (% day^{-1})	1.14±0.016^{b}	1.20±0.025^{b}	1.51±0.024^{c}	1.07±0.028^{a}	**0.008**
FCR	2.16±0.197^{ab}	1.94±0.171^{b}	1.63±0.081^{bc}	2.40±0.101^{a}	**0.005**
FER	0.45±0.024^{bc}	0.50±0.023^{ab}	0.52±0.027^{a}	0.41±0.023^{b}	**0.005**
PER	1.26±0.07	1.29±0.08	1.31±0.02	1.07±0.24	**0.234**
PI (g/fish)	1.18±0.04	1.21±0.03	1.25±0.01	1.10±0.02	**0.064**
ANPU (%)	22.05±0.29	22.29±0.43	24.26±0.42	21.19±0.22	**0.212**
FTI (g/fish)	2.63±0.05	2.38±0.02	2.87±0.14	2.61±0.15	**0.108**
FR (%)	31.14±1.02^{b}	30.35±1.33^{b}	35.14±2.14^{a}	28.73±1.64^{c}	**0.005**
EI (g/fish)	3.34±0.02^{a}	3.35±0.01^{ab}	3.43±0.03^{b}	3.28±0.03^{a}	**0.006**
ER (%)	10.83±0.25^{ab}	10.94±0.16^{a,}	13.23±0.07^{b}	10.12±0.19^{a}	**0.008**
SR (%)	87.78±0.86^{b}	88.11±0.73^{b}	97.44±0.86^{c}	82.68±0.67^{a}	**0.005**
GaSI (%)	3.67±0.114^{b}	3.70±0.109^{b}	4.61±0.140^{c}	3.07±0.164^{a}	**0.005**
CSI (%)	0.04±0.003^{a}	0.05±0.002^{a}	0.06±0.001^{b}	0.04±0.001^{a}	**0.006**
HSI (%)	1.88±0.22^{ab}	1.85±0.13^{ab}	2.21±0.09^{c}	1.48±0.08^{a}	**0.008**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. Here, WG (%)- Weight gain percentage; AWG (g)- Average weight gain; FCR- Feed conversion ratio; FER- Feed efficiency ratio; SGR (% day^{-1})-Specific growth rate; PER- Protein efficiency ratio; PI (g/fish)- Protein intake; ANPU- Apparent net protein utilization; FI (g/fish)- Feed intake; FTI(g/fish)- Fat intake; FR (%)- Fat retention; EI- Energy Intake (g/fish); ER (%)- Energy retention.

SR (%)- Survival percentage; GaSI (%)- gastrosomatic index; CSI (%)- Craniosomatic index; HSI (%)- Hepatosomatic index.

4.6.1.4. Nucleic acid analyses

Nucleic acid analyses of indoor feed trial have been provided in Table 19a. DNA content vary insignificantly ($p>0.05$) between the groups exhibiting maximum and minimum values in liver of T2 group (1.12±0.07) and intestine of CT (0.4±0.07), respectively, across the organs used in the study. Upon challenged with *A. hydrophila* and *E. tarda* DNA exhibited no significance ($p>0.05$).

The RNA-M showed insignificant ($p>0.05$) difference between CT (0.25±0.01) and T3 (0.25±0.03) and considerably ($p<0.05$) differ with T1 (0.36±0.02) and T2 (0.55±0.03). The RNA-L showed remarkable variations among treatments ($p<0.05$) and the trends was as follow: CT (1.17±0.03) > T1 (1.20±0.05) > T2 (1.34±0.05) < T3 (0.93±0.04). The RNA-K also showed remarkable changes ($p<0.05$) among groups being highest value for T3 (1.23).

Amongst RNA analyses the greatest values were reported in brain of T2 (1.39±0.01). Irrespective of treatments and across the vital organs the RNA values showed a trend as follows: RNA-B > RNA-L > RNA-K > RNA-I > RNA-M. DNA values of vital organs assumed to be constant; however, tissue specific values were reported. In challenge study, nucleic acid analyses showed decreasing trend for both the bacteria (Table 19b). The trend of RNA in challenge study was as followed, RNA-B > RNA-L > RNA-I >RNA-K > RNA-M.

The value for RNA in both the bacteria has declined as compared to feed experiment but considerably greater as compared with CT ($p<0.05$). Comparatively, the values were diminished in *A. hydrophila* infection, however, values were considerably ($p<0.05$) higher in T3 group as compared with other treatments and same trend was followed by *E. tarda* but values were higher for *E. tarda* as compared to *A. hydrophila*.

Table 19a. Nucleic acid analyses (mg mL^{-1}) of vital organs of *L. rohita* under indoor feed trial

Var/trt	CT	T1	T2	T3	p-value
RNA-M	0.23 ± 0.01^{a}	0.38 ± 0.01^{b}	0.58 ± 0.02^{c}	0.26 ± 0.01^{a}	**0.006**
RNA-L	1.15 ± 0.01^{b}	1.22 ± 0.01^{c}	1.37 ± 0.02^{d}	0.94 ± 0.01^{a}	**0.003**
RNA-K	1.11 ± 0.01^{b}	1.17 ± 0.01^{c}	1.26 ± 0.02^{d}	0.9 ± 0.01^{a}	**0.00**
RNA-I	1.1 ± 0.01^{b}	1.16 ± 0.01^{c}	1.19 ± 0.02^{c}	0.79 ± 0.01^{a}	**0.007**
RNA-B	1.21 ± 0.01^{b}	1.23 ± 0.01^{b}	1.42 ± 0.02^{c}	0.99 ± 0.01^{a}	**0.008**
DNA-M	0.18±0.05	0.23±0.03	0.24±0.07	0.19±0.09	**0.158**
DNA-L	0.94±0.06	1.06±0.04	1.12±0.07	0.91±0.03	**0.345**
DNA-K	0.87±0.08	0.99±0.05	1.02±0.08	0.89±0.07	**0.267**
DNA-I	0.4±0.07	0.49±0.05	0.52±0.07	0.43±0.09	**0.227**
DNA-B	0.51±0.05	0.56±0.05	0.58±0.07	0.53±0.08	**0.167**
RDM	0.78 ± 0.01^{d}	0.6 ± 0.01^{b}	0.41 ± 0.02^{a}	0.73 ± 0.01^{c}	**0.005**
RDL	0.82 ± 0.01^{a}	0.87 ± 0.01^{b}	0.82 ± 0.01^{a}	0.97 ± 0.01^{c}	**0.007**
RDK	0.78 ± 0.01^{a}	0.85 ± 0.01^{c}	0.81 ± 0.01^{b}	0.99 ± 0.01^{d}	**0.005**
RDI	0.36 ± 0.01^{a}	0.42 ± 0.01^{b}	0.44 ± 0.01^{c}	0.54 ± 0.01^{d}	**0.003**
RDB	0.42 ± 0.01^{a}	0.46 ± 0.02^{b}	0.41 ± 0.01^{a}	0.54 ± 0.03^{c}	**0.008**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other.
Here, RNA-M: RNA of muscle; RNA-L; RNA of liver; RNA-K: RNA of kidney; RNA-I; RNA of intestine; RNA-B: RNA of brain; DNA-M: DNA of muscle; DNA-L: DNA of liver; DNA-K: DNA of kidney; DNA-I: DNA of intestine; DNA-B: DNA of brain; RDM: DNA/RNA of muscle; RDL: DNA/RNA of liver; RDK: DNA/RNA of kidney; RDI-I: DNA/RNA of intestine; RDB: DNA/RNA of brain.

Table 19b. Nucleic acid analyses (mg mL^{-1}) of vital organs of *L. rohita* under indoor feed trial followed by challenge study

Challenge with *A. hydrophila*						Challenge with *E. tarda*				
Var/trt	**CT**	**T1**	**T2**	**T3**	**p-value**	**CT**	**T1**	**T2**	**T3**	**p-value**
RNA-M	0.2 ± 0.01^{a}	0.35 ± 0.01^{b}	0.55 ± 0.02^{c}	0.23 ± 0.01^{a}	**0.007**	0.17 ± 0.01^{a}	0.32 ± 0.01^{b}	0.52 ± 0.02^{c}	0.2 ± 0.01^{a}	**0.006**
RNA-L	1.07 ± 0.01^{b}	1.14 ± 0.01^{c}	1.29 ± 0.02^{d}	0.86 ± 0.01^{a}	**0.003**	1.01 ± 0.01^{b}	1.07 ± 0.01^{c}	1.22 ± 0.02^{d}	0.79 ± 0.01^{a}	**0.003**
RNA-K	1.04 ± 0.01^{b}	1.1 ± 0.01^{c}	1.19 ± 0.02^{d}	0.83 ± 0.01^{a}	**0.004**	0.97 ± 0.01^{b}	1.03 ± 0.01^{c}	1.12 ± 0.02^{d}	0.76 ± 0.01^{a}	**0.005**
RNA-I	1.05 ± 0.01^{b}	1.11 ± 0.01^{c}	1.14 ± 0.02^{c}	0.74 ± 0.01^{a}	**0.007**	0.96 ± 0.01^{b}	1.02 ± 0.01^{c}	1.05 ± 0.02^{c}	0.65 ± 0.01^{a}	**0.008**
RNA-B	1.18 ± 0.01^{b}	1.2 ± 0.01^{b}	1.39 ± 0.02^{c}	0.96 ± 0.01^{a}	**0.006**	1.15 ± 0.01^{b}	1.17 ± 0.01^{b}	1.36 ± 0.02^{c}	0.93 ± 0.01^{a}	**0.007**
DNA-M	0.16 ± 0.08	0.21 ± 0.02	0.22 ± 0.02	0.17 ± 0.06	**0.128**	0.14 ± 0.01	0.19 ± 0.01	0.2 ± 0.02	0.15 ± 0.01	**0.231**
DNA-L	0.91 ± 0.01	1.03 ± 0.01	1.09 ± 0.02	0.88 ± 0.01	**0.147**	0.87 ± 0.01^{ab}	0.99 ± 0.01^{c}	1.05 ± 0.02^{d}	0.84 ± 0.01^{b}	**0.007**
DNA-K	0.83 ± 0.01	0.95 ± 0.01	0.98 ± 0.02	0.85 ± 0.01	**0.241**	0.78 ± 0.01	0.9 ± 0.01	0.93 ± 0.02	0.8 ± 0.01	**0.006**
DNA-I	0.4 ± 0.03^{ab}	0.49 ± 0.01^{c}	0.54 ± 0.02^{d}	0.43 ± 0.02^{a}	**0.006**	0.37 ± 0.09	0.44 ± 0.04	0.49 ± 0.08	0.4 ± 0.06	**0.232**
DNA-B	0.5 ± 0.06	0.55 ± 0.03	0.57 ± 0.05	0.52 ± 0.03	**0.123**	0.47 ± 0.07	0.52 ± 0.06	0.54 ± 0.08	0.49 ± 0.05	**0.432**
RDM	0.8 ± 0.01^{d}	0.6 ± 0.01^{b}	0.4 ± 0.02^{a}	0.74 ± 0.01^{c}	**0.004**	0.82 ± 0.01^{d}	0.59 ± 0.01^{b}	0.39 ± 0.02^{a}	0.75 ± 0.01^{c}	**0.004**
RDL	0.85 ± 0.01^{b}	0.87 ± 0.03^{c}	0.85 ± 0.01^{a}	1.02 ± 0.01^{d}	**0.003**	0.86 ± 0.01^{b}	0.92 ± 0.003^{c}	0.86 ± 0.03^{a}	1.06 ± 0.02^{d}	**0.004**
RDK	0.79 ± 0.03^{a}	0.86 ± 0.03^{c}	0.82 ± 0.003^{b}	1.02 ± 0.07^{d}	**0.003**	0.803 ± 0.03^{a}	0.873 ± 0.03^{c}	0.833 ± 0.03^{b}	1.05 ± 0.03^{d}	**0.003**
RDI	0.38 ± 0.02^{a}	0.437 ± 0.05^{b}	0.457 ± 0.05^{b}	0.577 ± 0.03^{c}	**0.007**	0.38 ± 0.04^{a}	0.44 ± 0.06^{b}	0.46 ± 0.09^{c}	0.61 ± 0.03^{d}	**0.008**
RDB	0.42 ± 0.03^{a}	0.45 ± 0.04^{b}	0.41 ± 0.06^{a}	0.54 ± 0.02^{c}	**0.008**	0.41 ± 0.01^{a}	0.44 ± 0.01^{b}	0.4 ± 0.01^{a}	0.53 ± 0.01^{c}	**0.006**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. Here, RNA-M: RNA of muscle; RNA-L; RNA of liver; RNA-K: RNA of kidney; RNA-I; RNA of intestine; RNA-B: RNA of brain; DNA-M: DNA of muscle; DNA-L: DNA of liver; DNA-K: DNA of kidney; DNA-I: DNA of intestine; DNA-B: DNA of brain; RDM: DNA/RNA of muscle; RDL: DNA/RNA of liver; RDK: DNA/RNA of kidney; RDI-I: DNA/RNA of intestine; RDB: DNA/RNA of brain.

4.6.1.5. Physio-chemical parameters of water

The physio-chemical parameters of water were as follow, DO (6.8-7.7 mg L^{-1}), pH (7.4-8.3), TDS (148.45-159.23 mg L^{-1}) WT (27.31-30.95 ℃) and other parameters are represented in Table 20.

Table 20. Physio-chemical parameters of water under indoor feed trial (90 days) and challenge study

Sl.No.	Parameters	Range
1.	Water temperature (WT) ℃	27.31-30.95
2.	Air temperature (AT) ℃	32.56-34.46
3.	pH	7.4-8.3
4.	Free CO_2 (mg L^{-1})	0-1.2
5.	Total alkalinity (mg L^{-1})	160-175
6.	Total hardness (mg L^{-1})	180-210
7.	Ammonia (mg L^{-1})	0.15-0.21
8.	Total dissolved solids (TDS) (mg L^{-1})	148.45-159.23
9.	Nitrite -N (mg L^{-1})	0.002-0.004
10.	Nitrate-N (mg L^{-1})	0.05-0.07
11.	Specific conductivity (ụ sec/cm)	250-278
12.	Dissolved oxygen (DO) (mg L^{-1})	6.8-7.7

4.6.2. Outdoor pond feed trial (60-days)

4.6.2.1. Carcass composition of *L. rohita* fingerlings

Table 21 shows the carcass composition of *L. rohita* in the outdoor pond feed trial. The CP content grew linearly between groups until T2, insignificantly ($p>0.05$), and subsequently decreased insignificantly ($p>0.05$) in T3 as compared to T2. The T2 (17.57±0.79%) recorded the highest value. The T3 had a non-substantial ($p>0.05$) lower EE content as compared to other treatments. T3 had a non-significant ($p>0.05$) increase in moisture content when compared to the other groups, with T3 having the highest value (73.46±2.37%).

Table 21. Carcass composition of *L. rohita* fingerlings under outdoor pond feed trial (% wet weight basis)

Parameters /Treatment	Initial	CT	T1	T2	T3	p-value
Moisture %	73.18±2.28	71.63±1.78	71.56±2.72	71.36±1.23	73.46±2.37	**0.086**
CP (%)	16.33±0.24	16.88±0.37	17.04±0.98	17.57+0.79	16.93+1.09	**0.145**
Crude fat (%)	6.14±0.32	6.88±0.45	7.00±0.68	6.76±0.73	6.38±0.58	**0.261**
Ash (%)	4.36±0.13	4.61+0.38	4.40±0.32	4.31±0.45	4.23±0.59	**0.324**

Values are expressed as Mean ± SE; n = 3

4.6.2.2. Growth performances, nutrient utilization and body indices of *L. rohita* fingerlings

Growth rates, feed conversion and nutrient retention were influenced considerably ($p<0.05$) by dietary TABP in rohu. Maximum average weight gain was reported in T2 (31.8 ± 0.66 g) followed by T1 (22.17± 0.48 g) > T3 and CT. The final weight gain (FW) was highest in T2 (55.1± 1.17 g) followed by T1> T3 and CT. Maximum and minimum growth rates were noticed in T2 and T3, respectively ($p<0.05$).

Feed conversion ratio was inversely proportional to growth rates ($p<0.05$) while PER and ANPU were directly correlated to growth rates ($p<0.05$). Dietary TABP affected the feed intake in *L. rohita* fingerlings ($p<0.05$) while fat intake and energy intake were similar in all treatment groups ($p>0.05$). HSI and GaSI varied substantially ($p<0.05$) in response to dietary TABP while CSI was insignificant ($p>0.05$). Parameters such as FI, FR, CSI and EI exhibited no significance ($p<0.05$) among the treatments (Table 22).

Table 22. Growth performances, nutrient utilization and body indices of *L. rohita* fingerlings

Treatment	CT	T1	T2	T3	p-value
IW (g)	23.7±1.52	23.53±2.9	23.6±1.35	23.87±2.29	**0.188**
FW (g)	45.07 ± 2.58^{ab}	47.2 ± 1.31^{b}	55.5 ± 1.17^{c}	45.93 ± 2.37^{a}	**0.005**
AWG (g)	21.07 ± 1.05^{b}	22.17 ± 0.48^{b}	31.8 ± 0.66^{c}	22.06 ± 0.73^{b}	**0.005**
WG (g/day)	0.36 ± 0.012^{a}	0.39 ± 0.016^{ab}	0.53 ± 0.018^{c}	0.37 ± 0.023^{a}	**0.004**
WG (%)	90.06 ± 3.75^{ab}	100.63 ± 4.56^{b}	134.51 ± 3.31^{c}	92.44 ± 2.47^{a}	**0.003**
SGR (% day^{-1})	1.06 ± 0.004^{ab}	1.15 ± 0.01^{b}	1.42 ± 0.004^{c}	1.09 ± 0.001^{a}	**0.006**
FI (g/fish)	42.66 ± 1.87^{a}	42.51 ± 2.23^{a}	42.52 ± 2.07^{a}	42.96 ± 2.12^{a}	**0.009**
FCR	2.02 ± 0.02^{bc}	1.92 ± 0.09^{b}	1.59 ± 0.01^{a}	2.01 ± 0.01^{c}	**0.005**
FER	0.49 ± 0.006^{ab}	0.53 ± 0.02^{b}	0.67 ± 0.007^{c}	0.51 ± 0.002^{a}	**0.005**
PER	1.65 ± 0.02^{ab}	1.75 ± 0.08^{b}	2.1 ± 0.02^{c}	1.69 ± 0.008^{a}	**0.004**
PI (g/fish)	1.08±0.03	1.15±0.05	1.18±0.05	1.14±0.04	**0.324**
ANPU (%)	23.14 ± 1.46^{a}	29.23 ± 3.79^{ab}	36.66 ± 3.42^{c}	25.72 ± 2.19^{a}	**0.008**
FR (%)	55.02 ± 2.3^{a}	56.85 ± 2.98^{a}	58.51 ± 5.32^{b}	57.71 ± 0.45^{a}	**0.006**
EI(g/fish)	7.42±0.24	7.49±0.07	7.68±0.1	7.5±0.06	**0.156**
ER (%)	22.71 ± 1.35^{ab}	23.96 ± 2.17^{ab}	27.17 ± 2.52^{b}	22.5 ± 2.39^{a}	**0.007**
FTI (g/fish)	2.96±0.56	2.90±0.86	2.88±0.75	2.76±0.49	**0.119**
SR (%)	88.33 ± 4.49^{a}	91.67 ± 1.69^{ab}	95.00 ± 1.50^{b}	91.18 ± 3.39^{a}	**0.005**
HSI (%)	2.04 ± 0.38^{ab}	2.21 ± 0.25^{ab}	2.56 ± 0.02^{b}	2.60 ± 0.17^{a}	**0.007**
CSI (%)	0.05±0.001	0.05±0.003	0.07±0.003	0.05±0.006	**0.231**
GaSI (%)	4.07 ± 0.32^{ab}	4.18 ± 0.26^{ab}	4.82 ± 0.048^{c}	4.09 ± 0.24^{a}	**0.007**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. Here, WG (%)- Weight gain percentage; AWG (g)- Average weight gain; FCR- Feed conversion ratio; FER- Feed efficiency ratio; SGR (% day^{-1})- Specific growth rate; PER- Protein efficiency ratio; PI (g/fish)- Protein intake; ANPU- Apparent net protein utilization; FI (g/fish)- Feed intake; FTI (g/fish)- Fat intake; FR (%)- Fat retention; EI- Energy Intake; ER (%)- Energy retention; SR (%)- Survival percentage; GaSI (%)- gastrosomatic index; CSI (%)- Craniosomatic index; HSI (%)- Hepatosomatic index.

4.6.2.3. Relative percentage survival

In the outdoor pond feed trial, the RPS in T2 increased considerably ($p<0.05$) as compared to the other groups. Between CT and T3, no considerable change ($p>0.05$) was reported. In both *A. hydrophila* and *E. tarda* infection studies, the RPS can be depicted as T2>T1>T3>CT. However, as compared to *A. hydrophila* treatments, *E. tarda* had a lower value (Fig. 21).

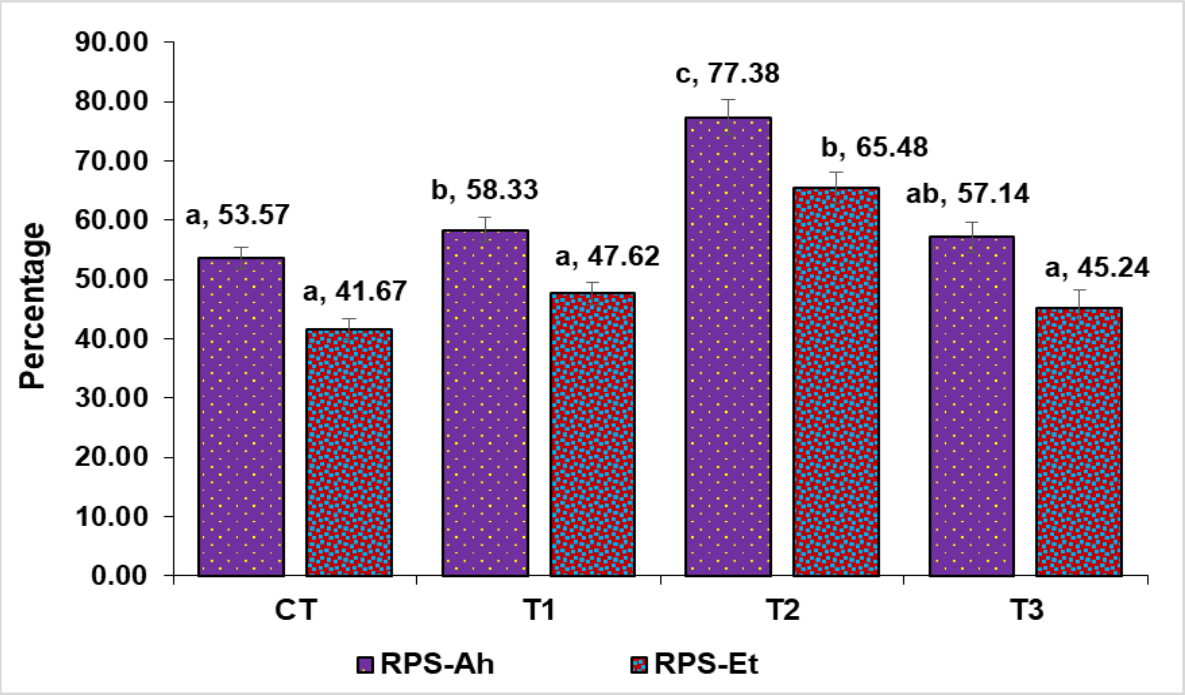


Fig. 21. Relative percentage survival of outdoor pond feed trial followed by challenge study

4.6.2.4. Nucleic acid analyses of vital organs of *L. rohita*

The trend of RNA content depicted as follows; RNA-L > RNA-B > RNA-K > RNA-I > RNA-M (Table 23a). When comparing the treatment and control groups, the results were substantially different ($p<0.05$). T2 had the highest RNA-L value (1.56 ±0.03), followed by T1> T3 and CT. Between the groups, there was no substantial variation in DNA concentration ($p>0.05$). For all the organs, the DNA/RNA ratio exhibited a considerable variation ($p<0.05$). During the challenge study with *A. hyrophila* and E. *tarda*, a substantial difference ($p<0.05$) was found (Table 23b). Nucleic acid values dropped in the challenged study when compared to the pond

feed trial, regardless of organs. The RNA concentration pattern in the challenge study was similar to the pond feed trial, but it was declining in comparison to the pond feed trial. In the challenge study, the DNA/RNA ratio was higher.

Table 23a. Nucleic acid analyses (mg L^{-1}) of vital organs of *L. rohita* under outdoor pond feed trial

Var/trt	CT	T1	T2	T3	P-value
RNA-M	0.3±0.01^{a}	0.42±0.02^{b}	0.61±0.01^{c}	0.32±0.02^{a}	**0.006**
RNA-L	1.44±0.02^{a}	1.49±0.02^{a}	1.56±0.03^{b}	1.46±0.06^{a}	**0.009**
RNA-K	1.21±0.01^{a}	1.32±0.05^{b}	1.49±0.05^{c}	1.27±0.01^{a}	**0.004**
RNA-I	1.15±0.01^{a}	1.27±0.02^{a,b}	1.39±0.02^{c}	1.24±0.02^{a}	**0.008**
RNA-B	1.25±0.01^{a}	1.38±0.02^{b}	1.47±0.03^{c}	1.31±0.02^{a}	**0.007**
DNA-M	0.28±0.03	0.32±0.04	0.3±0.06	0.29±0.04	**0.126**
DNA-L	1.07±0.07	1.11±0.04	1.12±0.05	1.08±0.05	**0.234**
DNA-K	0.99±0.03	1.02±0.03	1.02±0.04	1.01±0.03	**0.321**
DNA-I	0.53±0.08	0.58±0.04	0.57±0.04	0.56±0.03	**0.242**
DNA-B	0.69±0.08	0.72±0.06	0.75±0.08	0.69±0.06	**0.083**
RDM	0.93±0.003^{a}	0.76±0.02^{c}	0.49±0.02^{d}	0.90±0.04^{a}	**0.005**
RDL	0.74±0.08^{a}	0.73±0.01^{a}	0.71±0.01^{b}	0.73±0.06^{a}	**0.006**
RDK	0.81±0.01^{a}	0.77±0.08^{a,b}	0.68±0.02^{c}	0.79±0.03^{a}	**0.004**
RDI	0.46±0.02^{a}	0.45±0.04^{a,b}	0.41±0.01^{c}	0.45±0.3^{a}	**0.007**
RDB	0.55±0.02^{a,b}	0.52±0.01^{a}	0.51±0.01^{c}	0.52±0.02^{a}	**0.006**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. Here, RNA-M: RNA of muscle; RNA-L; RNA of liver; RNA-K: RNA of kidney; RNA-I; RNA of intestine; RNA-B: RNA of brain; DNA-M: DNA of muscle; DNA-L: DNA of liver; DNA-K: DNA of kidney; DNA-I: DNA of intestine; DNA-B: DNA of brain; RDM: DNA/RNA of muscle; RDL: DNA/RNA of liver; RDK: DNA/RNA of kidney; RDI-I: DNA/RNA of intestine; RDB: DNA/RNA of brain.

Table 23b. Nucleic acid analyses (mg L^{-1}) of vital organs of *L. rohita* under outdoor pond feed trial followed by challenge study

Challenge with *A. hydrophila*						Challenge with *E. tarda*				
Var/trt	**CT**	**T1**	**T2**	**T3**	**p-value**	**CT**	**T1**	**T2**	**T3**	**p-value**
RNA-M	0.28±0.01^{b}	0.4±0.02^{c}	0.59±0.01^{d}	0.34±0.02^{a}	**0.003**	0.26±0.01^{a}	0.38±0.02^{b}	0.57±0.01^{c}	0.29±0.02^{a}	**0.007**
RNA-L	1.24±0.02^{a}	1.32±0.02^{b}	1.38±0.03^{c}	1.26±0.06^{a}	**0.005**	1.18±0.03^{a}	1.25±0.02^{b}	1.33±0.03^{c}	1.22±0.07^{a}	**0.008**
RNA-K	1.07±0.07^{a}	1.12±0.005^{b}	1.29±0.05^{c}	1.09±0.01^{a}	**0.006**	1.03±0.07^{b}	1.08±0.01^{b}	1.25±0.05^{c}	1.07±0.01^{a}	**0.007**
RNA-I	1.11±0.01^{a}	1.24±0.02^{a,b}	1.35±0.02^{c}	1.17±0.029^{a}	**0.007**	1.1±0.02b	1.18±0.02b	1.29±0.03^{c}	1.14±0.03^{a}	**0.007**
RNA-B	1.24±0.01^{a}	1.37±0.02^{b}	1.45±0.03^{c}	1.26±0.02^{a}	**0.004**	1.21±0.02^{a}	1.34±0.02^{b}	1.42±0.03^{c}	1.23±0.02^{a}	**0.005**
DNA-M	0.26±0.04	0.3±0.06	0.29±0.04	0.28±0.07	**0.412**	0.24±0.04	0.28±0.04	0.26±0.03	0.23±0.04	**0.167**
DNA-L	1.08±0.07	1.11±0.06	1.14±0.07	1.09±0.08	**0.098**	1.01±0.09	1.04±0.04	1.06±0.07	1.03±0.08	**0.087**
DNA-K	0.95±0.04	0.98±0.06	0.98±0.05	0.97±0.05	**0.276**	0.93±0.03	0.96±0.01	0.96±0.01	0.93±0.01	**0.256**
DNA-I	0.59±0.03	0.64±0.03	0.67±0.07	0.59±0.03	**0.167**	0.49±0.08	0.52±0.05	0.56±0.07	0.50±0.07	**0.184**
DNA-B	0.62±0.04	0.67±0.03	0.69±0.03	0.66±0.03	**0.273**	0.62±0.04	0.64±0.03	0.66±0.04	0.62±0.03	**0.123**
RDM	0.93±0.003^{c}	0.75±0.02^{d}	0.49±0.02^{a}	0.82±0.05^{b}	**0.005**	0.92±0.03^{c}	0.73±0.03^{b,c}	0.45±0.03^{a}	0.79±0.06^{b}	**0.006**
RDL	0.87±0.01^{a}	0.84±0.01^{a}	0.82±0.01^{a}	0.86±0.09^{b}	**0.004**	0.86±0.01^{a}	0.83±0.01^{a}	0.79±0.01^{a}	0.84±0.11^{b}	**0.007**
RDK	0.89±0.06^{b}	0.87±0.008^{b}	0.76±0.03^{a}	0.88±0.008^{b}	**0.009**	0.90±0.07^{b,c}	0.89±0.01^{b}	0.77±0.04^{a}	0.87±0.01^{b}	**0.008**
RDI	0.53±0.02^{c}	0.51±0.02^{b,c}	0.49±0.01^{a}	0.50±0.03^{b}	**0.006**	0.44±0.02^{a}	0.43±0.02^{a}	0.42±0.01^{a}	0.43±0.04^{a}	**0.123**
RDB	0.51±0.02^{b}	0.48±0.01^{a}	0.47±0.01^{a}	0.52±0.02^{b}	**0.009**	0.51±0.03^{b}	0.47±0.02^{a}	0.46±0.01^{a}	0.50±0.02^{b}	**0.008**

Data expressed as Mean ± SE, n = 3; Mean values in the same column with different superscript differ significantly ($p < 0.05$).

4.6.2.5. Physio-chemical parameters of water

The physio-chemical parameters of water were as follow, DO (6.2-8.9 mg L^{-1}), pH (7.6-8.7), TDS (157.92-168.53 mg L^{-1}) WT (28.31-30.69 ℃) and other parameters are represented in table 24.

Table 24. Physio-chemical parameters of water under outdoor pond feed trial of 60 days and challenge study

Sl.No.	Parameters	Range
1.	Water temperature (WT) ℃	28.31-30.69
2.	Air temperature (AT) ℃	31.56-33.46
3.	pH	7.6-8.7
4.	Free CO_2 (mg L^{-1})	0-1.1
5.	Total alkalinity (mg L^{-1})	173-192
6.	Total hardness (mg L^{-1})	212-225
7.	Ammonia (mg L^{-1})	0.13-0.18
8.	Total dissolved solids (TDS) (mg L^{-1})	157.92-168.53
9.	Nitrite -N (mg L^{-1})	0.003-0.005
10.	Nitrate-N (mg L^{-1})	0.04-0.08
11.	Specific conductivity (ụ sec/cm)	270-287
12.	Dissolved oxygen (DO) (mg L^{-1})	6.2-8.9

4.6.3. Intraperitoneal inoculation experiment with *T. arjuna* bark extract

4.6.3.1. Carcass composition of *L. rohita* fingerlings

In intraperitoneal inoculation experiment, during first 15 days no significance ($p>0.05$) was reported in CP and ash content. The maximum values of CP and ash content were reported in T3-15 (16.21+0.92%) and (4.62+0.85%), respectively and minimum values for both, CP and ash content was reported in CT15. An inverse relationship was witnessed between moisture and crude fat content ($p<0.05$). The highest value for moisture content was reported in CT15 (72.85+0.29%) while minimum in T3-15 (70.13+0.22%). Similar trends were followed for subsequent 15 days but slightly lower values were observed during 15-30 days. The values of moisture content increased with statistical variation ($p<0.05$) in T3 and ash content decreased considerably ($p<0.05$) in T3 (Table 25).

Table 25. Carcass composition of *L. rohita* fingerlings under intraperitoneal inoculation experiment (% wet weight basis)

Parameters /Treatment	Moisture (%)	CP (%)	Crude fat (%)	Ash (%)
Initial	74.90+0.53	14.93+0.87	6.94+0.93	3.23+0.82
CT15	72.85+0.29^{b}	15.34+0.75	8.14+0.28^{a}	3.67+0.91
T1-15	71.90+0.39^{b}	15.62+0.84	8.53+0.33^{a}	3.95+0.83
T2-15	71. 15+0.12^{a,b}	15.84+0.86	8.76+0.35^{a}	4.25+0.72
T3-15	70.13+0.22^{a}	16.21+0.92	9.04+0.26^{b}	4.62+0.85
p-value	**0.007**	**0.312**	**0.007**	**0.198**
CT30	72.44+0.38^{a,b}	15.52+0.72	8.45+0.44^{b}	3.59+0.86
T1-30	71.94+0.28^{a}	15.76+0.39	8.62+0.31^{b}	3.68+0.52
T2-30	71.24+0.32^{a}	15.95+0.83	8.95+0.26^{b}	3.86+0.47
T3-30	74.11+0.14^{b}	14.93+0.82	7.84+0.12^{a}	3.12+0.85
p-value	**0.008**	**0.234**	**0.008**	**0.323**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. CT15, T1-15, T2-15, T3-15 indicates value at 15 days time interval and CT30, T1-30, T2-30, T3-30 indicates value at 30 days time interval.

4.6.3.2. Growth performance, nutrient utilization and body indices of *L. rohita* fingerlings

The intraperitoneal inoculation experiment, the parameters showed higher values in first 15 days as compared to 30 days except CT which showed higher value at 30 days as compared to 15 days. A substantial ($p<0.05$) rise in parameters such as different pattern of growth performances indices, nutrient utilization and retention was observed. In first 15 days, WG (%), AWG, SGR, WG (g/day), PER, ANPU, PI, FI, FER, HSI and GaSI enhanced considerably ($p<0.05$) with graded dose of *T. arjuna* ethanolic bark extract and an improvement in FCR was also observed (Table 26a).

Table 26a. Growth performance, nutrient utilization and body indices of *L. rohita* fingerlings with different level of *Terminalia arjuna* bark extract under intraperitoneal inoculation experiment at 15 days

Var./trt	CT15	T1-15	T2-15	T3-15	p-value
IW(g)	23.7±0.87	23.87±0.79	23.66+0.85	23.52±0.096	**0.345**
FW(g)	25.76±0.32^{c}	27.53+0.47^{b}	29.33+0.45^{a}	29.6+0.38^{a}	**0.005**
WG (%)	9.82±0.67^{c}	15.21±0.57^{b}	23.85±0.85^{a}	25.83±0.98^{a}	**0.005**
WG(g/day)	0.14±0.02^{a}	0.24+0.011^{b}	0.39±0.02^{c}	0.40±0.012^{c}	**0.004**
AWG(g)	2.03±0.36^{a}	3.67±0.74^{b}	5.67±0.36^{b}	6.07±0.47^{b,c}	**0.006**
FCR	2.12±0.27^{a}	1.94±0.46^{a}	1.87±0.36^{a,b}	1.78±0.19^{b,c}	**0.006**
FER	0.34±0.05^{a}	0.47±0.08^{b}	0.50±0.03^{b,c}	0.52±0.06^{c}	**0.007**
SGR (% day^{-1})	1.04±0.01^{a}	1.08±0.02^{a}	1.18±0.07^{a,b}	1.23±0.09^{b}	**0.009**
PER	1.02±0.03^{a}	1.09±0.06^{a}	1.14±0.09^{a}	1.18±0.05^{a,b}	**0.009**
PI(g/fish)	0.98±0.03^{a}	1.11±0.04^{a}	1.15±0.02^{a,b}	1.19±0.05^{b,c}	**0.008**
ANPU (%)	14.05±0.46^{a}	17.29±0.33^{a}	18.26±0.53^{a,b}	19.19±0.35^{b}	**0.009**
FI (g/fish)	3.68±0.23^{a,b}	4.70±0.79^{a,b}	5.11±0.28^{a}	5.72±0.43^{c}	**0.008**
FTI(g/fish)	0.17±0.04^{a}	0.23±0.07^{a}	0.27±0.02^{a,b}	0.32±0.05^{c}	**0.007**
FR (%)	7.17±0.48	7.28±0.38	7.42±0.26	7.68±0.55	**0.08**
EI (%)	2.93±0.59	3.17±0.63	3.37±0.68	3.45±0.73	**0.208**
ER (%)	7.33±0.36	7.48±0.50	7.82±0.48	7.98±0.47	**0.197**
SR (%)	86.32±0.69^{a}	89.48±0.88^{a}	95.76±0.73^{b,c}	97.98±0.59^{c}	**0.008**
GaSI (%)	2.30±0.24^{a}	2.43±0.15^{a}	2.66±0.27^{a,b}	2.92±0.32^{c}	**0.007**
CSI (%)	0.02±0.04	0.03±0.03	0.04±0.005	0.06±0.007	**0.206**
HSI (%)	1.13±0.35^{a}	1.34±0.47^{b}	1.86±0.22^{b}	2.03±0.52^{c}	**0.008**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly (p <0.05) when compared to each other.

The parameters such as FTI, ER, FR, CSI, and EI exhibited no significance (p>0.05) from one another. With a graded level of extract dose, the SR (percent) increased remarkably (p<0.05). The WG (%), AWG, SR (percent), GaSI and elevated considerably (p<0.05) after 30 days, although other parameters exhibited no significance (p>0.05) (Table 26b). When compared to the control group, the WG, and AWG increased considerably (p<0.05) until T3-15. T2-15 and T3-15 did not differ substantially (p>0.05), but CT15 and T1-15 differed considerably, and CT15 and T1-15 also differed substantially as well (p<0.05). At 30 days, all parameters decrease in value, and the majority of them did not differ substantially (p>0.05) between CT30, T1-30, T2-30, and T3-30 groups.

Table 26b. Growth performance, nutrient utilization and body indices of *L. rohita* fingerlings with different level of *Terminalia arjuna* bark extract under intraperitoneal inoculation experiment at 30 days

Var./trt	CT30	T1-30	T2-30	T3-30	p-value
IW(g)	25.76±0.32	27.53±0.47	29.33+0.63	29.6±0.48	**0.423**
FW(g)	$28+0.0.57^{c}$	$30.6+0.62^{c}$	$34.8+0.68^{b}$	$35.2+0.73^{a}$	**0.006**
WG (%)	$10.51±0.67^{c}$	$12.32±0.65^{b}$	$18.78±0.85^{a}$	$19.18±0.98^{a}$	**0.006**
WG(g/day)	$0.18±0.03^{a}$	$0.22±0.03^{a}$	$0.37±0.05^{b}$	$0.38±0.04^{b}$	**0.007**
AWG(g)	$2.71±0.36^{c}$	$3.31±0.74^{b}$	$5.51±0.35^{a}$	$5.63±0.47^{a}$	**0.008**
FCR	2.18±0.36	2.14±0.46	2.06±0.36	1.98±0.19	**0.163**
FER	0.32±0.06	0.36±0.07	0.37±0.09	0.43±0.09	**0.234**
SGR (% day^{-1})	$0.85±0.01^{a}$	$0.89±0.02^{a}$	$0.97±0.03^{a,b}$	$1.03±0.06^{b,c}$	**0.007**
PER	1.08±0.09	0.96±0.07	0.98±0.03	1.05±0.07	**0.212**
PI(g/fish)	1.01±0.06	0.99±0.07	1.05±0.08	1.09±0.07	**0.308**
ANPU (%)	15.05±0.45	14.79±0.98	15.98±0.34	17.19±0.85	**0.076**
FI (g/fish)	4.57±0.56	3.73±0.57	3.89±0.75	4.07±0.68	**0.321**
FTI(g/fish)	0.19±0.05	0.21±0.03	0.24±0.07	0.26±0.08	**0.092**
FR (%)	6.56±0.26	6.79±0.38	6.85±0.57	6.97±0.73	**0.431**
EI (%)	3.08±0.25	3.06±0.56	3.16±0.72	3.24±0.57	**0.189**
ER (%)	9.73±0.42	9.69±0.73	9.97±0.79	10.24±0.84	**0.273**
SR (%)	$85.87±0.56^{a}$	$87.99±0.83^{a}$	$94.66±0.47^{b,c}$	$96.69±0.92^{c,d}$	**0.007**
GaSI (%)	$2.68±0.57^{a}$	$2.63±0.38^{a}$	$2.85±0.73^{b}$	$3.03±0.65^{c}$	**0.008**
CSI (%)	0.01±0.005	0.02±0.08	0.03±0.003	0.05±0.006	**0.342**
HSI (%)	$1.09±0.47^{a}$	$1.14±0.23^{a}$	$1.18±0.35^{b,c}$	$1.24±0.56^{c}$	**0.009**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. CT30, T1-30, T2-30, T3-30 indicates value at 30 days time interval.

4.6.3.3. Relative percentage survival

The RPS in intraperitoneal inoculation experiment showed a significant increment ($p<0.05$) among the treatment being maximum in T3 but it has no significance ($p>0.05$) with T2 (Fig. 22). The RPS can be represented as T3>T2>T1>CT in both *A. hydrophila* and *E. tarda*. The highest value was reported in CT (56.25%) when fish infected with *A. hydrophila* and lowest value also in CT (51.25%) due to infection of *E. tarda.*

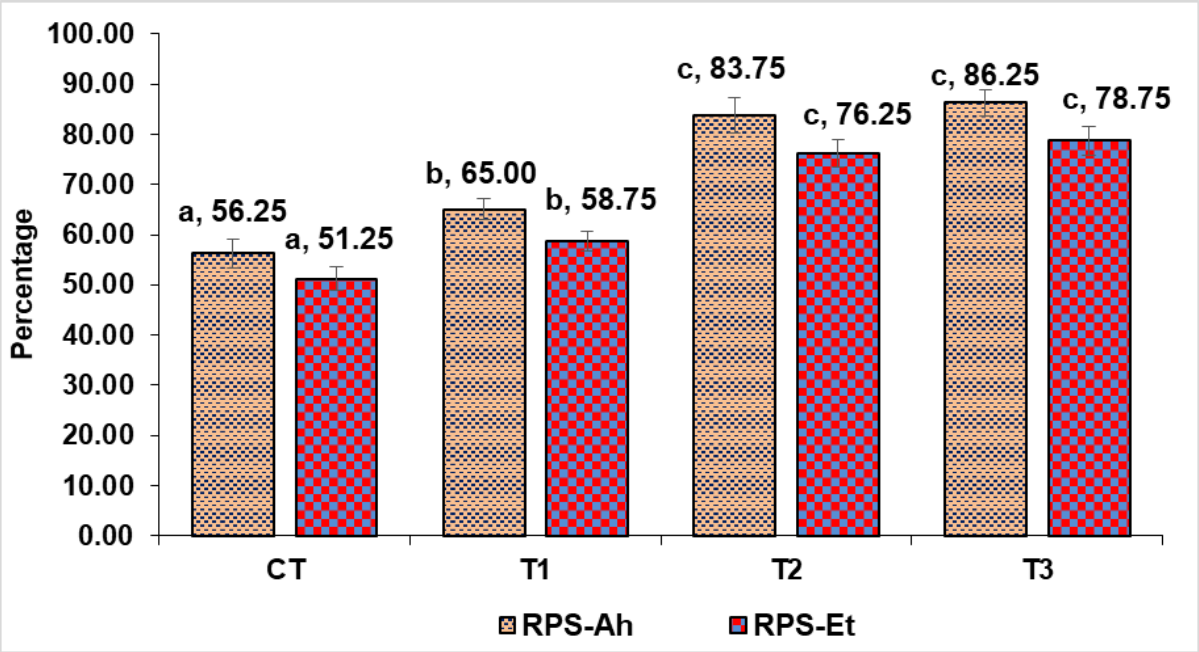


Fig. 22. Relative percentage survival of intraperitoneal inoculation experiment followed by challenge study

4.6.3.4. Nucleic acid analyses of vital organs of *L. rohita*

In intraperitoneal inoculation experiment, the RNA during first 15 days increased considerably ($p<0.05$) between T2 and T3 but did not differ considerably ($p>0.05$) between CT and T1. Next 15 days the trend was same but declining side as compared with first 15 days. Maximum RNA content was recorded in brain in T3 across the experimental duration. The linear regression equation for RNA-B was as followed, $Y=0.174x+0.88$, $R^2=0.89$. The 4 order Polynomial equation for RNA content of different organs of T2 treatment was $Y=0.0588x^4 +0.79x^3-3.79x^2 +7.46x-3.79$, $R^2=1$. DNA was recorded highest in Liver in T3 treatment during both the duration. DNA/ RNA was also differed considerably ($p>0.05$) between T2 and T3 groups and trend was as followed, RDL>RDK>RDB>RDI>RDM. The trend of RNA was as follows: RNA-B> RNA-L >RNA-I> RNA-K and RNA-M. The DNA content followed the trend as follows, DNA-L followed by DNA-K>DNA-B>DNA-I and DNA-M (Table 27a).

Intraperitoneal inoculation experiment, followed by challenge study with *A. hydrophila* and *E. tarda* showed a linear increase in RNA content value that is considerably ($p<0.05$) differ among the treatments (Table 27b). The maximum value

was highest in brain of treatment T3 of Ah and linear regression equation was derived as follows, Y=0.174x+0.85, R^2=0.90. Upon infection with *E. tarda* the values of nucleic acids severely decreased within the treatment but there was increasing trend for the values among the treatments. The linear regression equation for *E. tarda* brain was as follows, Y=0.179x+0.96, R^2=0.95. The DNA also showed same trend but not much significance ($p>0.05$) among the treatments. The four-order polynomial equation for organ specific RNA content was as recorded as, $Y=-0.0537x^4 +1.82x^3-22.79x^2 +125.64x-255.64$, R^2=1.

Table 27a. Nucleic acid analyses (mg $^{L-1}$) of vital organs of *L. rohita* under intraperitoneal inoculation experiment

Trt.	CT15	T1-15	T2-15	T3-15	p-value	CT30	T1-30	T2-30	T3-30	p-value
RNA-M	0.22±0.02^{a}	0.32±0.03^{a}	0.53±0.06^{b}	0.65±0.09^{c}	0.006	0.24±0.01^{a}	0.27±0.03^{a,b}	0.34±0.05^{b}	0.55±0.09^{c}	**0.01**
RNA-L	1.07±0.26^{a}	1.14±0.19^{a,b}	1.27±0.11^{b}	1.43±0.35^{c}	0.005	0.96±0.25^{a}	1.03±0.18^{a,b}	1.14±0.10^{b,c}	1.37±0.37^{c}	**0.01**
RNA-K	0.95±0.12^{b}	1.05±0.23^{c}	1.16±0.36^{d}	1.25±0.41^{a}	0.007	0.91±0.12^{b}	0.95±0.23^{c}	1.08±0.36^{d}	1.23±0.41^{a}	**0.01**
RNA-I	1.05±0.11^{b}	1.11±0.24^{c}	1.16±0.15^{c}	1.37±0.34^{a}	0.008	0.95±0.11^{b}	1.01±0.24^{c}	1.09±0.15^{c}	1.28±0.34^{a}	**0.01**
RNA-B	1.12±0.23^{a}	1.16±0.14^{b}	1.34±0.28^{c}	1.64±0.17^{d}	0.006	1.04±0.23^{a}	1.14±0.14^{b}	1.31±0.28^{c}	1.58±0.17^{d}	**0.01**
DNA-M	0.18±0.03^{a}	0.23±0.06^{a,b}	0.27±0.05^{b}	0.43±0.04^{c}	0.007	0.16±0.03^{a}	0.22±0.03^{a,b}	0.25±0.05^{b}	0.42±0.04^{c}	**0.01**
DNA-L	1.04±0.14	1.08±0.08	1.19±0.82	1.24±0. 15	0.087	1.02±0.14	1.06±0.13	1.17±0.32	1.22±0.12	**0.07**
DNA-K	0.92±0.05	0.95±0.07	0.99±0.04	1.04±0.08	0.077	0.92±0.05	0.94±0.07	0.97±0.02	1.03±0.05	**0.06**
DNA-I	0.42±0.03	0.45±0.05	0.49±0.04	0.53±0.03	0.143	0.42±0.03	0.44±0.05	0.47±0.04	0.51±0.03	**0.24**
DNA-B	0.51±0.06	0.55±0.07	0.58±0.06	0.63±0.08	0.146	0.51±0.04	0.53±0.07	0.56±0.04	0.62±0.06	**0.23**
RDM	0.82±0.06^{c}	0.72±0.04^{b}	0.51±0.07^{a}	0.66±0.03^{d}	0.005	0.89±0.06^{a}	0.92±0.04^{a}	0.74±0.07^{b,c}	0.79±0.03^{d}	**0**
RDL	0.97±0.03^{c}	0.95±0.02^{b}	0.94±0.04^{a}	0.87±0.03^{c}	0.006	1.06±0.03^{a}	1.03±0.02^{a}	1.03±0.04^{a}	0.89±0.03^{b}	**0.01**
RDK	0.97±0.04^{a}	0.90±0.05^{b}	0.85±0.07^{a}	0.83±0.08^{a}	0.006	1.01±0.04^{a}	0.99±0.05^{a,b}	0.89±0.07^{b}	0.84±0.08^{c}	**0.01**
RDI	0.40±0.04^{b}	0.41±0.02^{a}	0.42±0.01^{a}	0.39±0.02^{c}	0.007	0.44±0.04^{b}	0.44±0.02^{a}	0.43±0.01^{a}	0.39±0.02^{c}	**0.01**
RDB	0.46±0.05^{c}	0.47±0.04^{b}	0.43±0.03^{a}	0.38±0.04^{b}	0.007	0.48±0.05^{b}	0.46±0.04^{a,b}	0.42±0.03^{a,c}	0.39±0.04^{c}	**0.01**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. CT15, T1-15, T2-15, T3-15 indicates value at 15 days time interval and CT30, T1-30, T2-30, T3-30 indicates value at 30 days time interval.

Table 27b. Nucleic acid analyses (mg L^{-1}) of vital organs of *L. rohita* under intraperitoneal inoculation experiment followed by challenge study

Challenge with *A. hydrophila*						Challenge with *E. tarda*				
Vrl/Trt	**CT25**	**T1-25**	**T2-25**	**T3-25**	**p-value**	**CT25**	**T1-25**	**T2-25**	**T3-25**	**p-value**
RNA-M	0.19 ± 0.03^{a}	0.29 ± 0.02^{a}	0.5 ± 0.05^{b}	0.62 ± 0.09^{c}	0.004	0.12 ± 0^{a}	0.18 ± 0.02^{ab}	0.28 ± 0.04^{b}	0.47 ± 0.08^{c}	**0.005**
RNA-L	1.04 ± 0.05^{a}	1.11 ± 0.01^{ab}	1.24 ± 0.02^{b}	1.4 ± 0.05^{c}	0.006	0.9 ± 0.04^{a}	0.97 ± 0.07^{ab}	1.08 ± 0.06^{bc}	1.31 ± 0.06^{c}	**0.007**
RNA-K	0.92 ± 0.11^{b}	1.02 ± 0.13^{c}	1.13 ± 0.15^{d}	1.22 ± 0.1^{a}	0.007	0.85 ± 0.11^{b}	0.89 ± 0.14^{c}	1.02 ± 0.11^{d}	1.17 ± 0.13^{a}	**0.008**
RNA-I	1.02 ± 0.1^{b}	1.08 ± 0.09^{c}	1.13 ± 0.12^{c}	1.34 ± 0.11^{a}	0.005	0.89 ± 0.1^{b}	0.95 ± 0.13^{c}	1.03 ± 0.15^{c}	1.22 ± 0.12^{a}	**0.007**
RNA-B	1.09 ± 0.07^{a}	1.13 ± 0.06^{b}	1.31 ± 0.03^{c}	1.61 ± 0.06^{d}	0.004	0.98 ± 0.12^{a}	1.08 ± 0.11^{b}	1.25 ± 0.1^{c}	1.52 ± 0.11^{d}	**0.006**
DNA-M	0.15±0.08	0.2±0.06	0.24±0.08	0.33±0.14	0.068	0.1±0.02	0.16±0.02	0.19±0.04	0.26±0.13	**0.079**
DNA-L	1.01±0.13	1.05±0.22	1.16±0.11	1.18±0.12	0.089	0.96±0.08	1.00±0.11	1.11±0.15	1.16±0.12	**0.098**
DNA-K	0.89±0.02	0.92±0.06	0.96±0.08	1.01±0.04	0.078	0.86±0.04	0.88±0.06	0.91±0.06	0.97±0.04	**0.196**
DNA-I	0.39±0.06	0.42±0.07	0.46±0.03	0.5±0.08	0.256	0.36±0.02	0.38±0.04	0.41±0.03	0.45±0.02	**0.345**
DNA-B	0.48±0.06	0.52±0.08	0.55±0.06	0.5±0.05	0.346	0.44±0.06	0.47±0.08	0.5±0.07	0.56±0.07	**0.423**
RDM	0.79 ± 0.05^{d}	0.69 ± 0.03^{c}	0.48 ± 0.06^{a}	0.63 ± 0.02^{b}	0.003	0.83 ± 0.05^{a}	0.89 ± 0.03^{b}	0.68 ± 0.06^{c}	0.77 ± 0.02^{d}	**0.003**
RDL	0.94 ± 0.02^{c}	0.92 ± 0.01^{b}	0.91 ± 0.06^{a}	0.82 ± 0.02^{c}	0.004	1.07 ± 0.02^{a}	1.03 ± 0.01^{a}	1.03 ± 0.03^{a}	0.89 ± 0.02^{b}	**0.006**
RDK	0.94 ± 0.03^{a}	0.87 ± 0.04^{b}	0.82 ± 0.06^{a}	0.8 ± 0.07^{a}	0.005	1.01 ± 0.03^{a}	0.99 ± 0.04^{ab}	0.89 ± 0.06^{b}	0.83 ± 0.07^{c}	**0.007**
RDI	0.37 ± 0.03^{b}	0.38 ± 0.08^{a}	0.39 ± 0.05^{a}	0.36 ± 0.01^{c}	0.008	0.4 ± 0.02^{b}	0.4 ± 0.04^{a}	0.4 ± 0.07^{a}	0.37 ± 0.01^{b}	**0.009**
RDB	0.43 ± 0.04^{c}	0.44 ± 0.03^{b}	0.4 ± 0.02^{a}	0.35 ± 0.03^{b}	0.007	0.45 ± 0.04^{b}	0.44 ± 0.03^{ab}	$0.4\pm0.02a^{c}$	0.37 ± 0.03^{c}	**0.008**

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p < 0.05$) when compared to each other. CT25, T1-25, T2-25, T3-25 indicates value at 25 days time interval.

4.6.3.5. Physio-chemical parameters of water

The Physio-chemical parameters of water were as follow, DO (6.7-7.3 mg L^{-1}), pH (7.3-8.2), TDS (154.83-158.92 mg L^{-1}) WT (29.02-30.88 ℃) and other parameters are represented in table 28.

Table 28. Physio-chemical parameters of water under intraperitoneal inoculation experiment (30 days) and challenge study

Sl.No.	Parameters	Range
1.	Water temperature (WT) ℃	29.02-30.88
2.	Air temperature (AT) ℃	31.32-32.40
3.	pH	7.3-8.2
4.	Free CO_2 (mg L^{-1})	0-0.7
5.	Total alkalinity (mg L^{-1})	173-182
6.	Total hardness (mg L^{-1})	220-226
7.	Ammonia (mg L^{-1})	0.18-0.21
8.	Total dissolved solids (TDS) (mg L^{-1})	154.83-158.92
9.	Nitrite -N (mg L^{-1})	0.004-0.006
10.	Nitrate-N (mg L^{-1})	0.03-0.05
11.	Specific conductivity (ụ sec/cm)	259-278
12	Dissolved oxygen (DO) (mg L^{-1})	6.7-7.3

4.7. Hematological parameters

4.7.1. Indoor feed trial

4.7.1.1. Humoral responses

In feed experiment Lysozyme at 90days (Lys90), Bactericidal activity at 90days (Bact90) and NBTat 90 days (NBT90) increased in T2 as compared to CT and T1 and decreased considerably ($p<0.05$) in T3. Challenge study showed a substantially ($p<0.05$) decreasing trend at 90 days to 100 days in CT and T3 but there is no difference in values of T2 treatment. The trend is also followed in challenged study with *A. hydrophila* (Ah) and *E. tarda* (Et) (Fig. 23).

The linear regression equation for NBT of T2 treatment and challenged study as follows, $Y= -0.12x+1.26$, $R^2=0.92$. The equations for lys90 and bact90 are, $Y=-1.87x+162.09$, $R^2=0.99$ and $Y= -0.96x+ 42.09$, $R^2=1$, respectively. The value at 90

days followed by infection trial to 100 days indicated a significant ($p<0.05$) decreasing trend within the group, although there was insignificant ($p>0.05$) difference was reported between CT and T3 for any parameters.

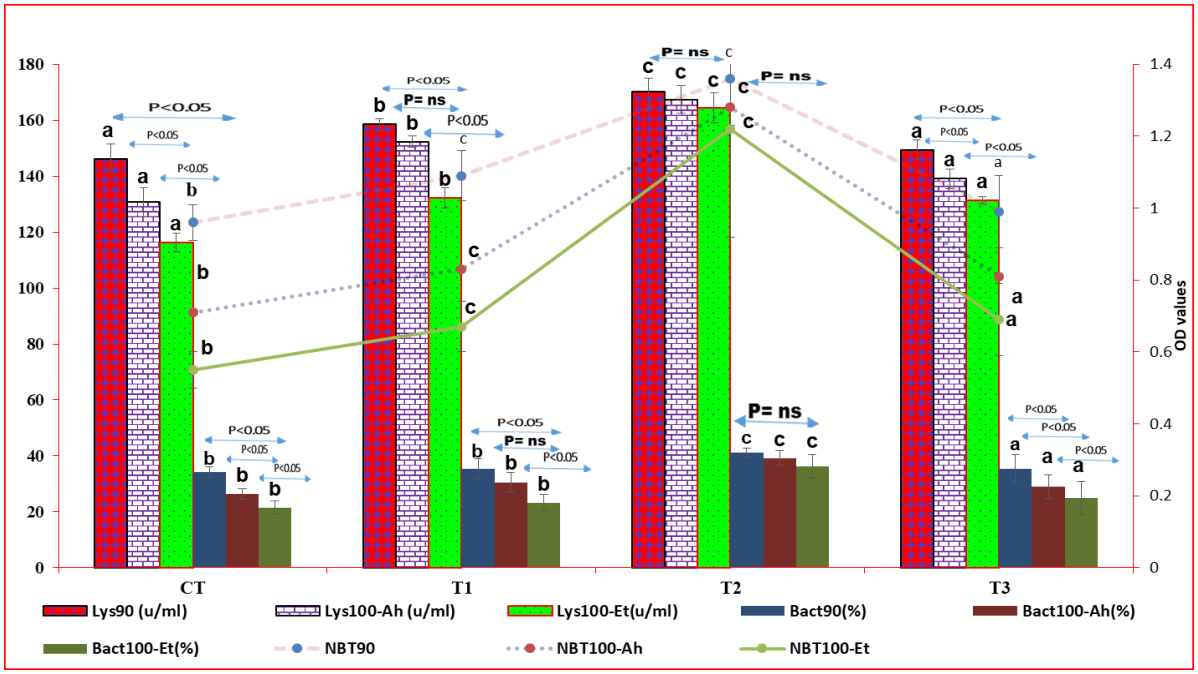


Fig. 23. Humoral responses in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.7.1.2. Complete blood count (CBC)

4.7.1.2.1. Haematological parameters of *L. rohita* under indoor pond feed trial and challenge study

Table (29) demonstrated that when comparing feed treatments to challenge studies with *A. hydrophila* (Ah) and *E. tarda* (Et), metrics including haematocrit (%) at 90 days (HCT90), mean corpuscular volume at 90 days (MCV90), mean corpuscular haemoglobin at 90 days (MCH90), and platelets at 90 days (PLT90) exhibited a substantially ($p<0.05$) increasing trend in T2. While upon infection the trend was same but it was observed to be decreasing side. Among the treatments, a feed trial followed by infection investigation revealed that practically all of the parameters in CT and T1 after 90 days and challenge with Et (100-Et) exhibited a substantial ($p<0.05$)

decrease in value. The values in T3 were also decreasing side but exhibited no significance (p>0.05). The values in T2 did not vary substantially (p>0.05).

Table 29. Showing hematological parameters of *L. rohita* under indoor feed trial and challenge study

Trt/parameters	CT	T1	T2	T3	p-value
HCT90 (%)	34.2±0.76^{a}	37.67±6.23^{ab}	43.74±4.53^{c}	34.83±0.26^{a}	**0.003**
HCT100-Ah (%)	31.66±0.37^{a}	35.15±5.27^{ab}	41.81±2.7^{c}	33.25±0.15^{a}	**0.003**
HCT100-Et (%)	29.91±0.34^{a}	30.41±0.38^{a}	40.86±2.71^{b}	32.54±0.15^{c}	**0.004**
p-value	**0.003**	**0.005**	**0.08**	**0.007**	
MCV90(fL)	123.66±5.65^{a}	126.88±7.91^{ab}	136.08±3.06^{b}	117.45±6.11^{a}	**0.006**
MCV100-Ah(fL)	120.32±5.65^{a}	123.1±6.26^{a}	134.97±3.06^{b}	115.67±6.11^{a}	**0.005**
MCV100-Et(fL)	116.69±5.65^{a}	1118.25±5.51^{ab}	132.78±2.26^{c}	115.48±6.11^{a}	**0.005**
p-value	**0.004**	**0.005**	**0.08**	**0.007**	
MCH90(pg)	37.92±0.58^{a}	38.96±0.58^{a}	44.3±2.15^{b}	36.67±2.31^{a}	**0.005**
MCH100-Ah(pg)	33.37±1.15^{a}	35.26±1.81^{a}	43.27±1.03^{b}	34.64±2^{a}	**0.005**
MCH100-Et(pg)	26.34±5.57^{a}	30.88±3.04^{a}	41.81±1.8^{b}	33.72±0.63^{a}	**0.005**
p-value	**0.003**	**0.003**	**0.06**	**0.006**	
MCHC90(g/dl)	36.21±1.47^{a}	37.97±1.51^{a}	42.4±2.52^{b}	34.67±1.15^{a}	**0.005**
MCHC100-Ah(g/dl)	33.62±1.47^{a}	35.38±1.51^{a}	41.14±2.65^{b}	32.08±1.15^{a}	**0.005**
MCHC100-Et(g/dl)	28.54±1.47^{ab}	31.3±1.51^{bc}	40.73±0.59^{c}	31±1.15^{a}	**0.005**
p-value	**0.003**	**0.003**	**0.06**	**0.006**	
PLT90(x10^{3} nos./ µL)	37.69±1.44^{ab}	40.73±1.88^{b}	51.19±2.73^{c}	35.2±2.72^{a}	**0.005**
PLT100-Ah x10^{3} nos./ µL)	32.66±1.9^{a}	38.6±2.19^{b}	49.92±0.71^{c}	30.19±1.24^{a}	**0.005**
PLT100-Et x10^{3} nos./ µL)	25.72±2.26^{a}	34.19±5.39^{b}	47.89±3.47^{c}	27.41±3.84^{a}	**0.005**
p-value	**0.003**	**0.003**	**0.06**	**0.006**	

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly (p <0.05) when compared to each other. *Different superscripts in same row showed significance (p<0.05) between the treatments at 0.05 level,

** Different superscript in column showed significance (p<0.05) of interaction within the treatment at 0.05 level and indicated by p-values. The suffix 90 indicates time interval and 100-Ah and 100-Et challenge study at 100 days with *A. hydrophila* and *E. tarda,* respectively.

4.7.1.2.2. RBC, WBC and Hg content in indoor feed trial and challenge study

When comparing T2 treatment to the others, RBC90 increased substantially ($p<0.05$). There was an insignificant ($p>0.05$) change in RBC90 for CT, T1, or T3, however there was a considerable ($p<0.05$) decline in values after infection with *A. hydrophila*, with the greatest value in T2. The treatment, T2 has a considerably higher WBC90 than the other groups ($p<0.05$), and there is no significant difference ($p>0.05$) between T1 and T3, although CT and T1 differ substantially ($p<0.05$). When compared to a 90-day feed trial, infection followed by a feed trial exhibited a declining pattern. The Hg levels followed the same pattern as RBC and WBC levels. The feed experiment (90 days) was followed by a challenge study, which revealed that after infection, it fell considerably ($p<0.05$) as compared to 90 days. Between 90 days following infection with Ah, there were non-substantial ($p>0.05$) variations in CT and T3, and insignificant ($p>0.05$) variations in T2 and T1 (Fig. 24).

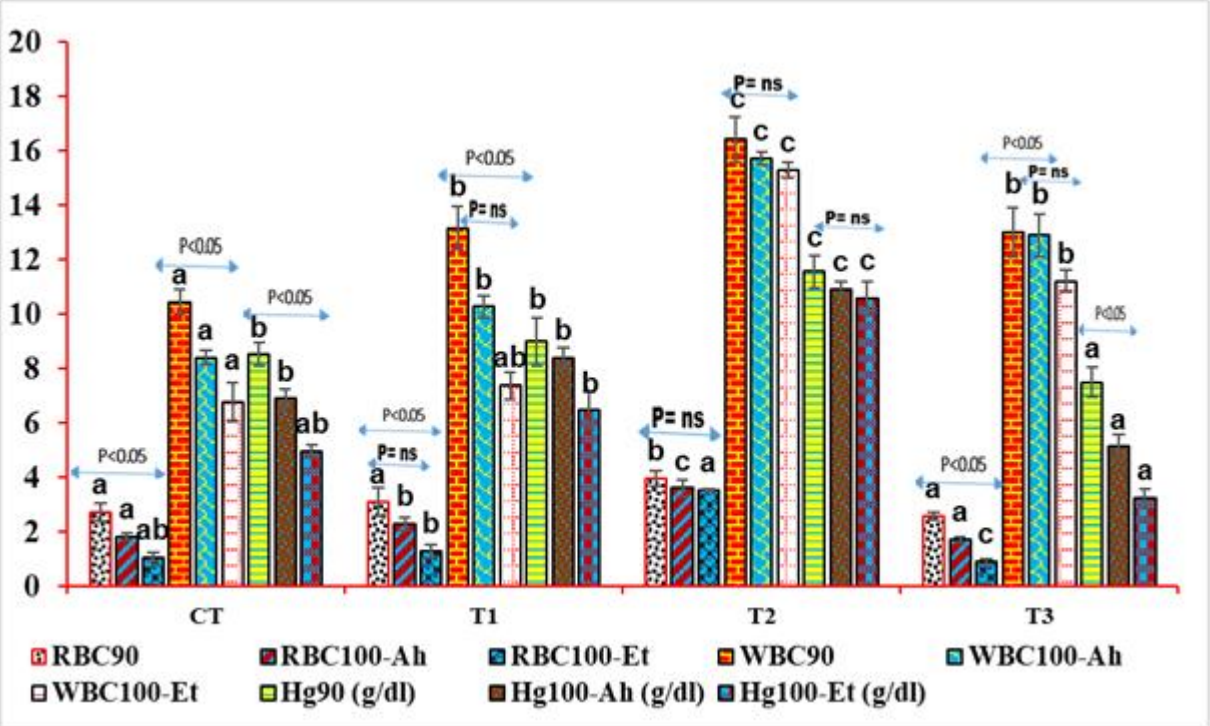


Fig. 24. Showing, RBC, WBC and Hg content in indoor feed trial and challenge study

*Here, RBC is expressed as ($x10^3$ nos./µl); WBC as ($x10^6$ nos./µl). The graph showed variation between and within the treatments.
**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.7.1.3 Protein, albumin, globulin and albumin: globulin in indoor feed trial and challenge study

In the indoor feed trial, albumin at 90 days (ALB90) substantially improved in T2 ($p<0.05$). The CT and T1 are not substantially different ($p>0.05$), however T2 and T3 are reported a substantial variation ($p<0.05$). When compared to the other groups, PRO90 increased considerably ($p<0.05$) in T2, then decreased ($p<0.05$) in T3. Protein levels in all groups differ greatly ($p<0.05$). The glogulin at 90 days (Glob90) is considerably different ($p<0.05$) between CT and T1, but not between T2 and T3. T2 had the highest value, followed by T1>T3 and CT.

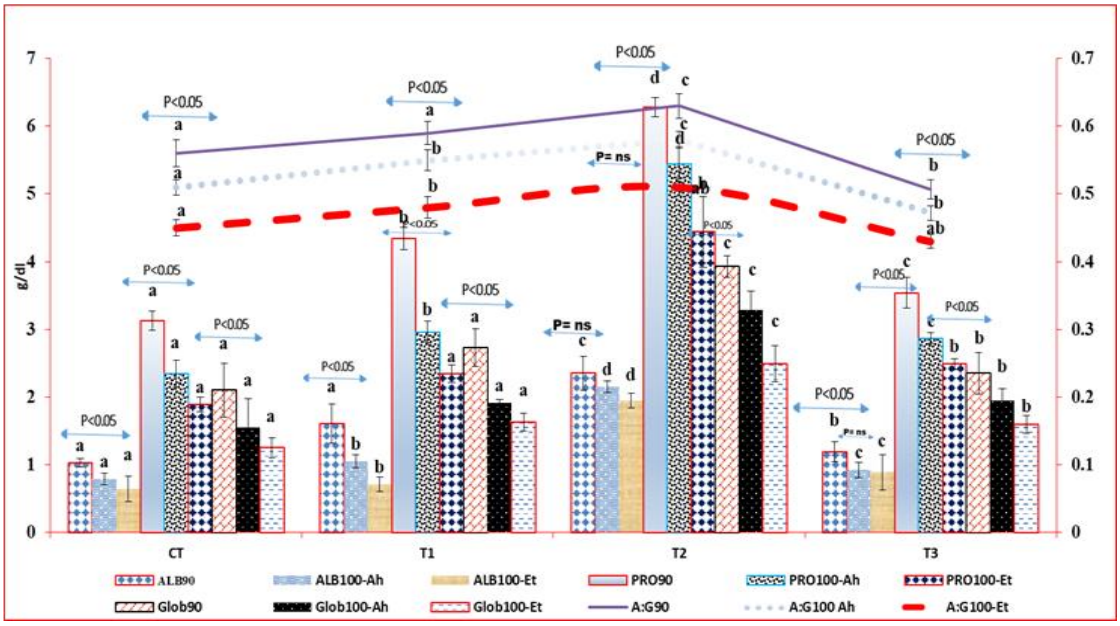


Fig. 25. Protein, albumin, globulin and albumin globulin ratio in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

When T2 was compared to the other groups, the albumib: globulin ratio at 90 days (A:G90) was substantially ($p<0.05$) different. In CT and T1 A:G90 values exhibited no significance ($p>0.05$), and T2 and T3 did not differ substantially ($p>0.05$). The same pattern was seen for albumib: globulin ratio at 100 days with infection of *A. hydrophila* (A: G100-Ah) and albumib: globulin ratio at 100 days with infection of *E. tarda* (A: G100-Et).

Following the feeding trial and infection with Ah and Et, all parameter values decreased in all groups. The value of CT decreased drastically ($p<0.05$) with Ah and Et, but T1 exhibited a significance ($p<0.05$) with Et but not with Ah ($p>0.05$). After being infected with Ah and Et, the values in the groups were declined. All measures showed a substantial ($p<0.05$) decline in CT and T1, but not in T2. The value in T3 decreased substantially ($p<0.05$) with Et but not considerably ($p>0.05$) with Ah levels (Fig. 25).

4.7.2. Outdoor pond feed trial

4.7.2.1. Humoral responses

The NBT at 60 days (NBT60), lysozyme at 60 days (Lys60), and bactericidal activity (%) at 60 days (Bact60) levels increased considerably ($p<0.05$) in T3 and T2 during an outdoor pond feed trial. There was no substantial variation ($p>0.05$) between CT and T1, or T2 and T3. The same tendency was seen in the pond trial that followed the challenge study (Fig. 26).

The linear regression equation for T3 of NBT60, Lyso60, and Bact60 was: $Y=-0.07x+1.0886$, $R^2=0.98$; $Y=-2.035x+144.31$, $R^2=0.99$; and $Y=-0.125x+31.05$, $R^2=0.65$.

When we compare the values of different parameters within the groups, we see a different tendency. Lyso60, Bact60, and NBT60 levels fell considerably ($p<0.05$) after infection with Ah and Et in CT, but there was no considerable ($p>0.05$) difference in the values of these parameters between 60 days and Ah in T1.

At 60 days, there was no significant ($p>0.05$) difference between T2 and T3, which was followed by infection with Ah and Et (at 70 days).

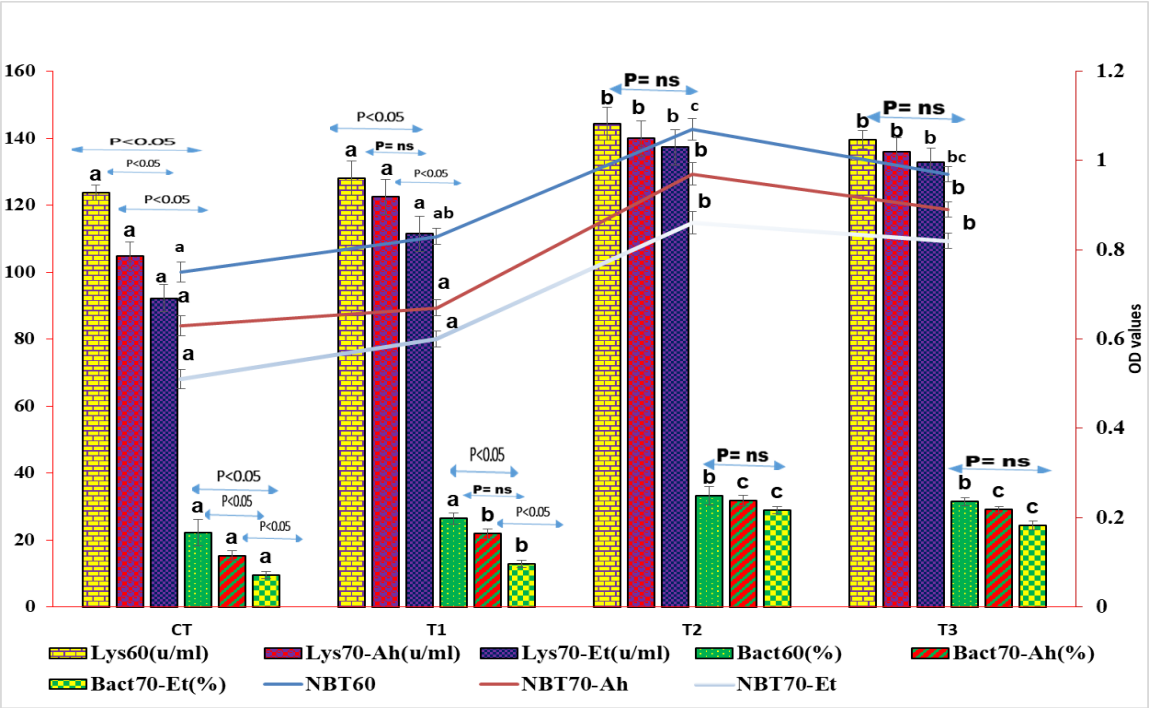


Fig. 26. Humoral responses in outdoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.7.2.2. Complete blood count (CBC)

4.7.2.2.1. Hematological parameters of *L. rohita* under outdoor pond feed trial and challenge study

The hematocrit (%) at 60 days (HCT60), mean corpuscular volume (fL) at 60 days (MCV60), mean corpuscular haemoglobin concentration(pg) at 60 days (MCHC60), and platelets (x10^3nos./µL) at 60 days (PLT60) levels were considerably ($p<0.05$) higher in T2 and T3 than in the other groups, but there was no considerable change ($p>0.05$) between CT and T1. The PLT60 did not differ substantially across CT, T1, and T3 ($p>0.05$). The 60-day feed trial in pond circumstances, followed by an infection investigation with Ah and Et, revealed that, with the exception of T2, all parameters reduced considerably ($p<0.05$) in challenge study. When comparing T2 to 60 days, no substantial ($p>0.05$) change was detected.

The HCT60 and MCH60 in T1 did not demonstrate a significant ($p>0.05$) decline with Ah. MCV60 levels in T2 were substantially greater ($p<0.05$) as compared

with other groups (Table 30). The values of the feed trial followed by the infection study demonstrated the same pattern between treatments, with the T2 group having a considerably ($p<0.05$) higher value. Significant (p0.05) decreases were detected in CT for both Ah and Et, in T1 and T3 with Et, but not in T2. When compared to the other groups, T3 had a considerably lower PLT ($p<0.05$).

Table 30. Showing hematological parameters of *L. rohita* under outdoor pond feed trial and challenge study

Trt/parameters	CT	T1	T2	T3	p-value
HCT60 (%)	35.2 ± 0.76^{ab}	37.34 ± 0.95^{bc}	39.41 ± 1.9^{c}	38.83 ± 0.26^{c}	**0.005**
HCT70-Ah (%)	31.66 ± 0.37^{a}	35.15 ± 0.25^{bc}	37.81 ± 1.11^{c}	36.25 ± 0.15^{c}	**0.005**
HCT70-Et (%)	26.91 ± 0.34^{a}	31.41 ± 0.38^{ab}	35.2 ± 0.62^{c}	32.54 ± 0.15^{c}	**0.003**
P-value	**0.003**	**0.005**	**0.07**	**0.005**	
MCV60 (fL)	$123.66\pm5.65a^{b}$	126.88 ± 7.91^{ab}	134.42 ± 4.02^{c}	117.45 ± 6.11^{a}	**0.005**
MCV70-Ah(fL)	119.32 ± 4.73^{a}	121.1 ± 7.91^{ab}	132.64 ± 4.02^{c}	115.67 ± 6.11^{a}	**0.005**
MCV70-Et(fL)	111.36 ± 5.11^{a}	114.91 ± 7.91^{ab}	130.45 ± 4.82^{c}	111.48 ± 6.11^{a}	**0.003**
P-value	**0.003**	**0.005**	**0.06**	**0.005**	
MCH60(pg)	37.92 ± 0.58^{a}	38.96 ± 0.58^{a}	44.3 ± 5.07^{b}	36 ± 2^{a}	**0.005**
MCH70-Ah(pg)	32.04 ± 0.57^{a}	35.26 ± 0.87^{a}	42.94 ± 5.11^{b}	34.64 ± 2^{a}	**0.005**
MCH70-Et(pg)	27.67 ± 0.58^{a}	27.88 ± 0.87^{a}	40.31 ± 2.73^{b}	32.39 ± 2.26^{a}	**0.005**
P-value	**0.003**	**0.005**	**0.08**	**0.005**	
MCHC60(g/dl)	36.21 ± 1.47^{ab}	37.97 ± 1.51^{bc}	39.4 ± 0.59^{c}	34.67 ± 1.15^{a}	**0.005**
MCHC70-Ah (g/dl)	32.62 ± 1.47^{ab}	35.38 ± 1.51^{bc}	37.81 ± 0.59^{c}	32.08±1.15a	**0.005**
MCHC70-Et(g/dl)	26.54 ± 1.47^{ab}	31.3 ± 1.51^{bc}	35.73 ± 0.59^{c}	29 ± 1.15^{a}	**0.005**
P-value	**0.003**	**0.005**	**0.07**	**0.005**	
PLT60 $x10^3$nos./µL	37.02 ± 0.46^{b}	37.06 ± 0.35^{b}	41.86 ± 0.43^{c}	38.87 ± 1.24^{b}	**0.003**
PLT70-Ah $x10^3$nos./µL	32.33 ± 0.46^{b}	31.27 ± 0.35^{b}	39.92 ± 0.43^{c}	35.19 ± 1.24^{b}	**0.002**
PLT70-Et $x10^3$nos./µL	22.05 ± 0.46^{b}	24.19 ± 0.35^{b}	36.89 ± 0.43^{c}	32.08 ± 1.24^{a}	**0.004**
P-value	**0.003**	**0.003**	**0.005**	**0.005**	

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly ($p<0.05$) when compared to each other. *Different superscripts in same row showed significance ($p<0.05$) between the treatments at 0.05 level, ** Different superscript in column showed significance ($p<0.05$) of interaction within the treatment at 0.05 level and indicated by p-values. The suffix 60 indicates time interval and 70-Ah and 70-Et challenge study at 70 days with *A. hydrophila* and *E. tarda*, respectively.

4.7.2.2.2. RBC, WBC and Hg content in outdoor pond feed trial and challenge study

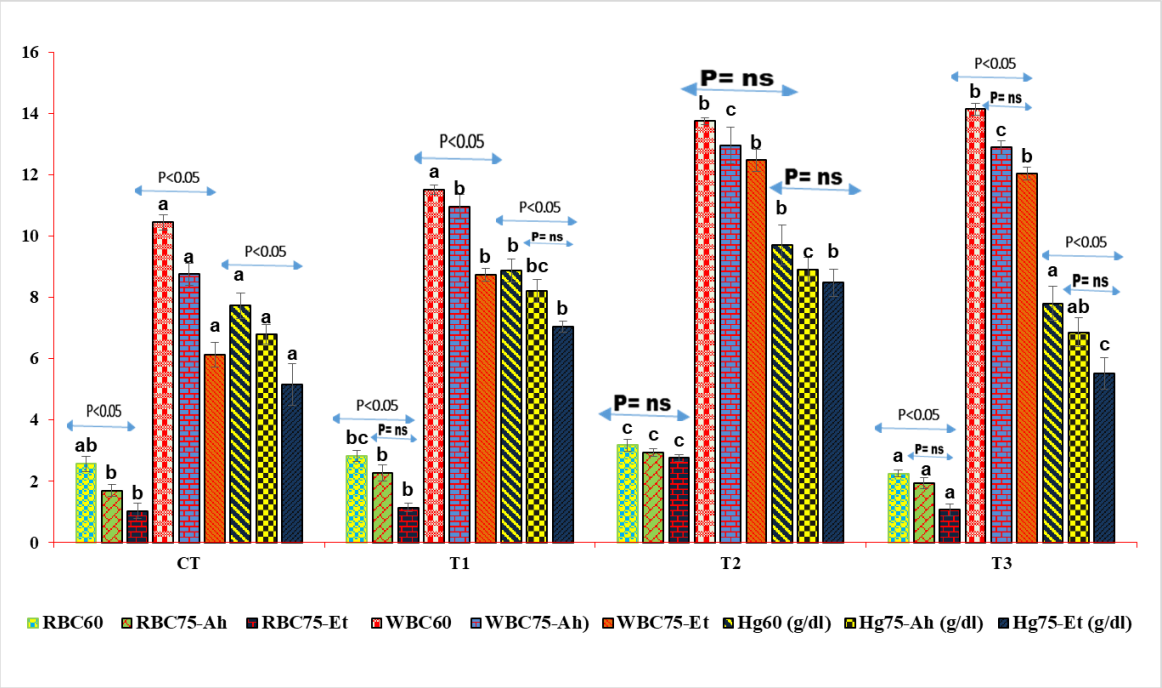


Fig. 27. Showing, RBC, WBC and Hg content in outdoor pond feed trial and challenge study

*Here, RBC is expressed as (x10^6 nos./µl); WBC as (x10^3 nos./µl). The graph showed variation between and within the treatments.

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The red blood cells at 60 days (RBC60), haemoglobin at 60 days (Hg60), and mean corpuscular haemoglobin at 60 days (MCH60) levels were considerably ($p<0.05$) higher in T2 as compared with other groups, and there was a considerable ($p<0.05$) difference between T2, CT, and T3, but no considerable difference was reported between CT and T1 ($p>0.05$), and T3 and CT treatments. The same tendency was seen in the challenge study, with most of the parameters in the Et experiment falling in value when compared to the Ah values. When compared to the other groups, the WBC showed a distinct pattern, with considerably ($p<0.05$) higher values in the T3 treatment. WBC levels did not differ substantially ($p>0.05$) between CT and T1, nor between T2 and T3. In T2 and T3, the Hg60 levels increased considerably ($p<0.05$). There is no significant ($p>0.05$) difference between Hg60 and Hg70-Et values before and after challenge in T2, while there was a substantial ($p<0.05$) change was reported between Hg60 and Hg70-Et values in T3 (Fig. 27).

4.7.2.2. Protein, Albumin, Globulin and Albumin globulin ratio in outdoor pond feed trial and challenge study

In the pond experiment, albumin at 60 days (ALB60), protein at 60 days (PRO60), globulin at 60 days (Glob60), and the albumin:globulin ratio at 60 days(A:G60) showed a substantial ($p<0.05$) variations for T2 and T3 treatments when compared to the other groups. When T2 and T3 groups were compared to the other groups, the ALB60 value increased remarkably ($p<0.05$). For most of the metrics, except the A:G ratio, there was no substantial ($p>0.05$) change was reported between T2 and T3, and CT and T1. The feed trial under pond conditions, followed by the infection, revealed the similar pattern between treatments as the 60-day value. Within the treatment, after infection with Ah and Et, the values reduced considerably ($p<0.05$) in CT and T1, but not in T2 and T3. At 60 days, the A:G ratio revealed a linear ($p>0.05$) rise in T2 and T3 when compared to CT and T1 (Fig. 28). Upon infection the ratio showed a diverse zigzag pattern. The linear equation of ALB60, ALB70-Ah, ALB70-Et, PRO60, PRO70-Ah, PRO70-Et, Glob60, Glob70-Ah, Glob70-Et and A:G 60, A:G70-Ah, A:G70-Et are as follows: $Y=-0.605x+3.16$, $R^2=0.99$, $Y=-1.19x+5.98$, $R^2=0.96$, $Y= 0.58x+2.82$, $R^2=0.91$ and $Y= 0.0342x+1.139$, $R^2=0.083$, respectively.

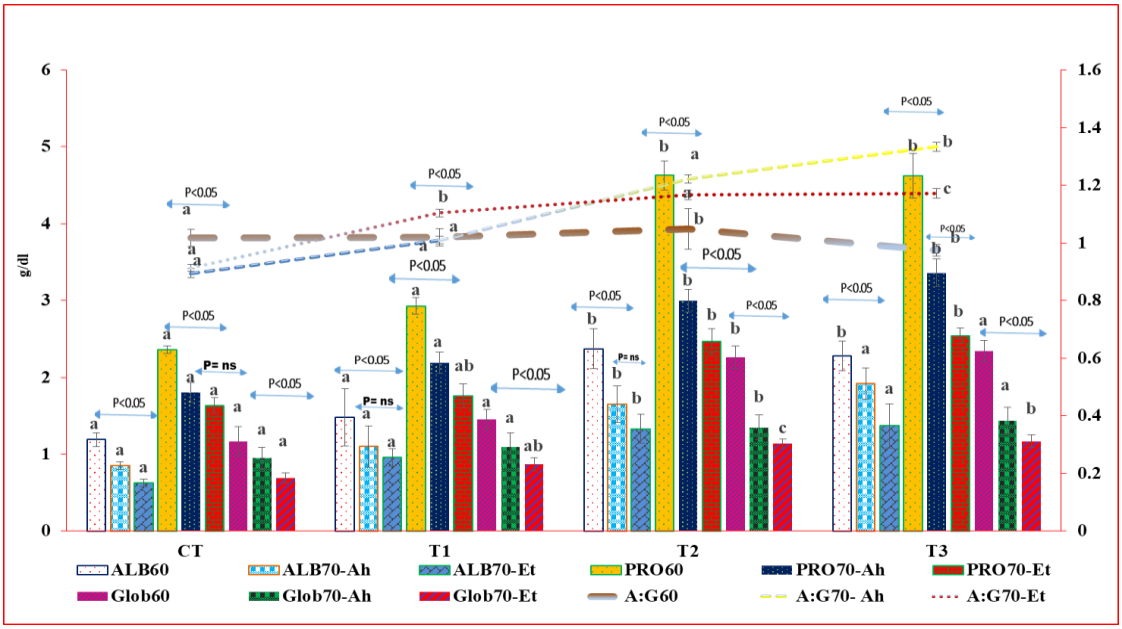


Fig. 28. Protein, Albumin, Globulin and Albumin globulin ratio in outdoor pond feed trial and challenge study

4.7.3. Intraperitoneal inoculation experiment

4.7.3.1. Humoral responses

At 15 days, humoral immune responses such as NBT at 15 days (NBT15), lysozyme activity (u/ml) at 15 days (Lys15), and bactericidal activity (%) at 15 days (Bact15) were at their highest in each group, but at 30 days, the values in treatments decreased considerably ($p<0.05$) except CT that showed insignificant ($p>0.05$) decrease. When compared to the other groups, the value in T2 and T3 increased considerably ($p<0.05$). T2 and T3 showed no considerable ($p>0.05$) changes after intraperitoneal inoculation experiment, and challenge study, whereas T1 and CT showed considerable ($p>0.05$) alterations.

T1 exhibited no significance ($p>0.05$) between 15 days and Ah, although CT values decreased considerably ($p<0.05$). No substantial ($p>0.05$) changes were seen in T2 and T3 at 25 days, followed by a challenge study with Ah and Et (Fig. 29). The linear equation of NBT15, NBT25-Ah NBT25-Et, NBT30 is as follows, $Y=-0.019x+0.645$, $R^2=0.96$. For Lyso15, lyso25, lyso 25-Ah, lyso25-Et, lyso30, and bact15, bact25-Ah, bact25-Et, bact30 are; $Y=-1.981x + 144.91$, $R^2=0.75$ and $Y=-1.605x+38.37$, $R^2=0.93$, respectively.

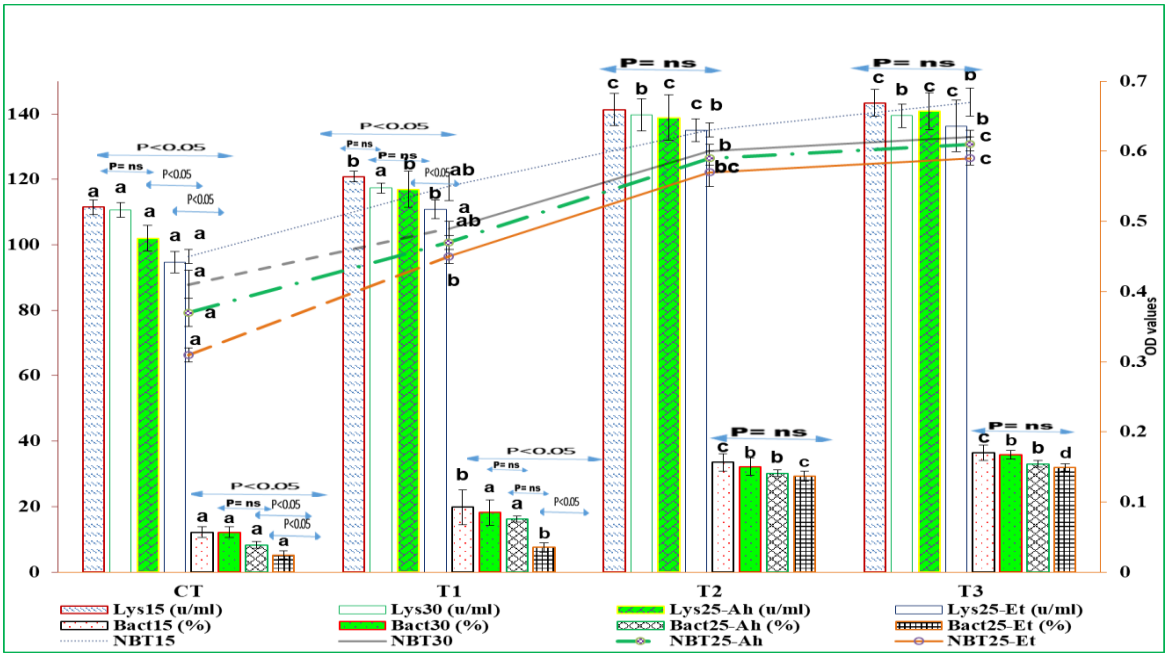


Fig. 29. Showing humoral responses in intraperitoneal inoculation experiment and challenge study

4.7.3.2. Complete blood count (CBC)

4.7.3.2.1. Hematological parameters of *L. rohita* under intraperitoneal inoculation experiment and challenge study

Table (31) shows that at 30 days, the values of haematocrit (%) at 15 days (HCT15), mean corpuscular volume (fL) at 15 days (MCV15), Mean corpuscular haemoglobin concentration (g/dl) at 15 days (MCHC15), and platelets ($x10^3$nos./µL) at 15 days (PLT15) declined modestly in all groups except CT. There was a substantial ($p<0.05$) drop in CT after extract inoculation followed by challenge studies with Ah and Et, and there was no considerable ($p>0.05$) changes were reported between the values of the parameters of T1 at 15 days and Ah15 value, but there was a substantial ($p<0.05$) variation with Et15.

The difference between T2 and T3 was insignificant ($p>0.05$). Between the treatments, T2 and T3 values increased considerably ($p<0.05$) after 15 days of injection, whereas in CT and T1 values did not change considerably ($p>0.05$). After infection (at 25 days), the values for ET in CT and T1 fell considerably ($p<0.05$), but not in T2 or T3. Except for T2 and T3, where the value did not differ substantially ($p>0.05$) within the treatment, there was a considerable ($p<0.05$) difference between and among the treatments for PLT15, PLT25-Ah, and PLT25-Et.

Table 31. Showing hematological parameters of *L. rohita* under intraperitoneal inoculation experiment and challenge study

Trt/parameters	CT	T1	T2	T3	p-value
HCT15 (%)	28.26±3.47^{a}	30.67±1.3^{ab}	33.74±0.79^{b}	33.83±0.98^{b}	**0.005**
HCT25-Ah (%)	23.99±2.8^{a}	25.15±1.19^{b}	31.15±0.82^{bc}	32.59±1.09^{a}	**0.005**
HCT25-Et (%)	22.25±1.18^{a}	28.41±1.32^{b}	30.2±0.39^{c}	30.87±0.44^{c}	**0.003**
HCT30	28.56±3.47^{a}	28.87±1.29^{a}	29.64±0.79^{b}	30.03±0.98^{b}	**0.005**
p-value	**0.004**	**0.005**	**0.08**	**0.09**	
MCV15 (fL)	118.33±1.4^{a}	122.88±0.96^{b}	130.42±3.48^{c}	130.12±2.5^{c}	**0.004**
MCV25-Ah (fL)	109.99±5.11^{a}	118.44±0.59^{b}	125.64±3.48^{c}	127±2.32^{c}	**0.0004**
MCV25-Et (fL)	108.36±5.11^{a}	115.58±0.5^{b}	123.11±2.25^{c}	124.81±1.8^{7c}	**0.004**
MCV30	119.03±1.40^{a}	121.88±0.95^{a}	128.62±3.47^{b}	129.32±2.50^{b}	**0.005**
p-value	**0.003**	**0.005**	**0.03**	**0.07**	
MCH15 (pg)	31.26±0.58^{a}	34.96±1.53^{b}	38.96±1.34^{c}	39.33±1.53^{c}	**0.004**
MCH25-Ah(pg)	26.71±1.01^{a}	31.93±0.29^{b}	35.6±0.7^{c}	36.64±1.73^{c}	**0.003**
MCH25-Et(pg)	23.01±0.58^{a}	29.22±0.77^{b}	33.98±1.12^{c}	35.06±2.08^{c}	**0.004**
MCH30(pg)	32.45±0.58^{a}	34.16±1.53^{a}	38.36±1.3^{b}	39.51±1.01^{b}	**0.005**
p-value	**0.003**	**0.005**	**0.07**	**0.08**	
MCHC15 (g/dl)	32.21±2.1^{a}	34.3±2.07^{ab}	38.06±0.57^{bc}	40.67±3.79^{c}	**0.005**
MCHC25-Ah (g/dl)	26.96±1.7^{a}	33.05±1.98^{b}	34.14±1.99^{b}	35.41±1^{b}	**0.005**
MCHC25-Et (g/dl)	30.21±3.98^{a}	33.97±0.98^{b}	33.39±2.5^{b}	33±0.58^{b}	**0.005**
MCHC30(g/dl)	32.94±1.99^{a}	33.66±1.53^{ab}	37.46±0.56^{bc}	40.2±3.78^{c}	**0.005**
p-value	**0.003**	**0.005**	**0.06**	**0.06**	
PLT15(x10^{3}nos./µL)	28.69±1.64^{a}	34.73±0.79^{b}	38.86±0.43^{c}	39.2±0.84^{c}	**0.003**
PLT25-Ah(x10^{3}nos./µL)	23.33±1.55^{a}	31.6±0.92^{b}	36.25±2.29^{c}	37.86±1.13^{c}	**0.004**
PLT25-Et(x10^{3}nos./µL)	19.38±1.11^{a}	29.19±2.86^{b}	34.89±2.86^{c}	35.08±0.59^{c}	**0.004**
PLT30(x10^{3}nos./µL)	30.08±1.64^{a}	31.72±0.56^{a}	36.46±0.42^{b}	38.0±0.84^{b}	**0.005**
p-value	**0.003**	**0.005**	**0.06**	**0.07**	

Values are expressed as Mean ± SE, n = 3; mean values in the same column with different superscripts differ significantly (p <0.05) when compared to each other. *Different superscripts in same row showed significance (p<0.05) between the treatments at 0.05 level, ** Different superscript in column showed significance (p<0.05) of interaction within the treatment at 0.05 level and indicated by p-values. The suffix 15 indicates time interval and 25-Ah and 25-Et challenge study at 25 days with *A. hydrophila* and *E. tarda,* and suffix 30 shows the time interval of 30 days.

4.7.3.2.2. RBC, WBC and Hg content in intraperitoneal inoculation experiment and challenge study

After 30 days, there is a small drop in red blood cell (x10^6nos./µL) at 15 days (RBC15), white blood cells ((x10^3/µL) at 15 days (WBC15) WBC15, and haemoglobin content (g/dl) at 15 days (Hg15) parameters between groups compared to the values after 15 days in an intraperitoneal inoculation experiment (Fig. 30).

The T2 and T3 had substantially higher values for all parameters ($p<0.05$) than the other groups. For majority of the metrics, there is no considerable ($p>0.05$) difference between CT and T1, and T2 and T3, and the similar trend was observed in infection with Ah and Et. Within the treatments, the value of all parameters fell considerably ($p<0.05$) in CT, and there was no considerable difference ($p>0.05$) between the value of Ah and 15 days and 30 days in T1, but Et exhibited significance ($p<0.05$). The value did not drop considerably in the T2 and T3 groups ($p>0.05$).

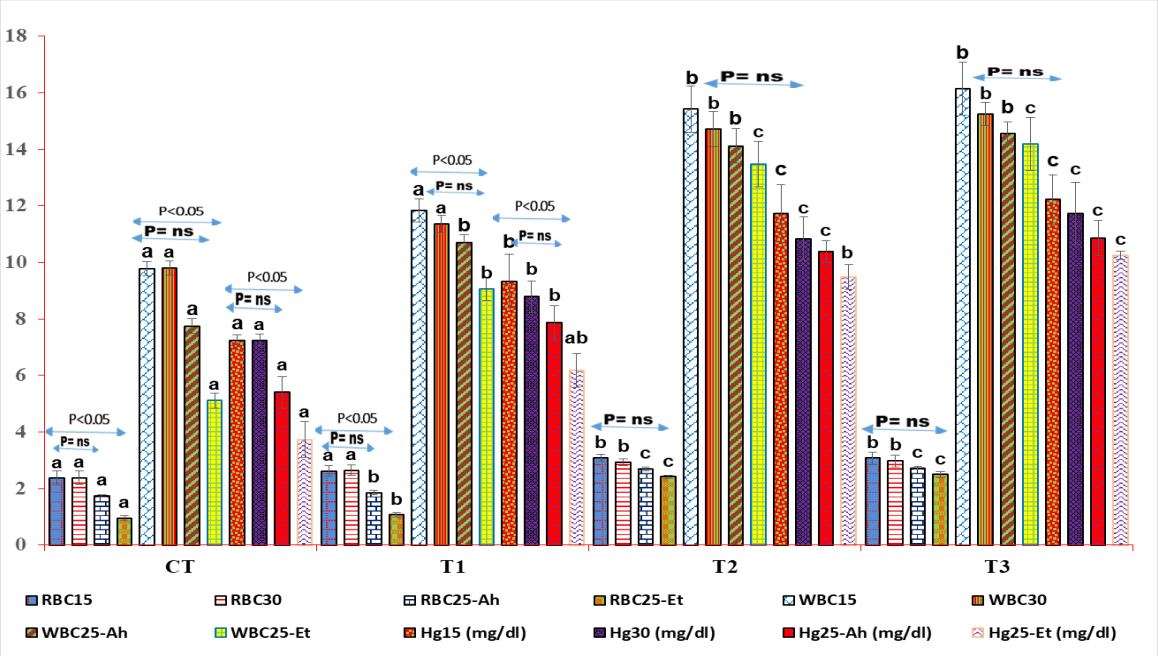


Fig. 30. RBC, WBC and Hg content in intraperitoneal inoculation experiment and challenge study

*Here, RBC is expressed as (x10^6/µl); WBC as (x10^3 /µl). The graph showed variation between and within the treatments.
**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.7.3.3. Protein, albumin, globulin and albumin globulin ratio in intraperitoneal inoculation experiment and challenge study

The values of albumin (g/dl) at 15 days (ALB15), protein (g/dl) at 15 days (PRO15), and globulin (g/dl) at 15 days (Glob15) increased considerably (p0.05) among the parameters in the intraperitoneal inoculation experiment, followed by a minor reduction in all treatments at 30 days. At 25 days after infection, the values for Et fell considerably (p<0.05) in CT and T1, but differed insignificantly (p>0.05) in T2 and T3 for ALB25-Ah, Glob25-Ah, ALB25-Et, and ALB25-Et, but considerably (p<0.05) in PRO25-Ah and PRO25-Et. The values declined considerably (p<0.05) within the groups (Fig. 31).

The A: G15 levels grew until T1, then declined substantially. In all groups, the ratio of A:G30 is decreasing. The A:G for Ah and Et showed an irregular pattern of distribution. The linear equation for A:G of T2 and T3 is as follows, Y=- 0.404x+0.53, R^2=0.77 and Y=0521x+0.56, R^2 =0.79.

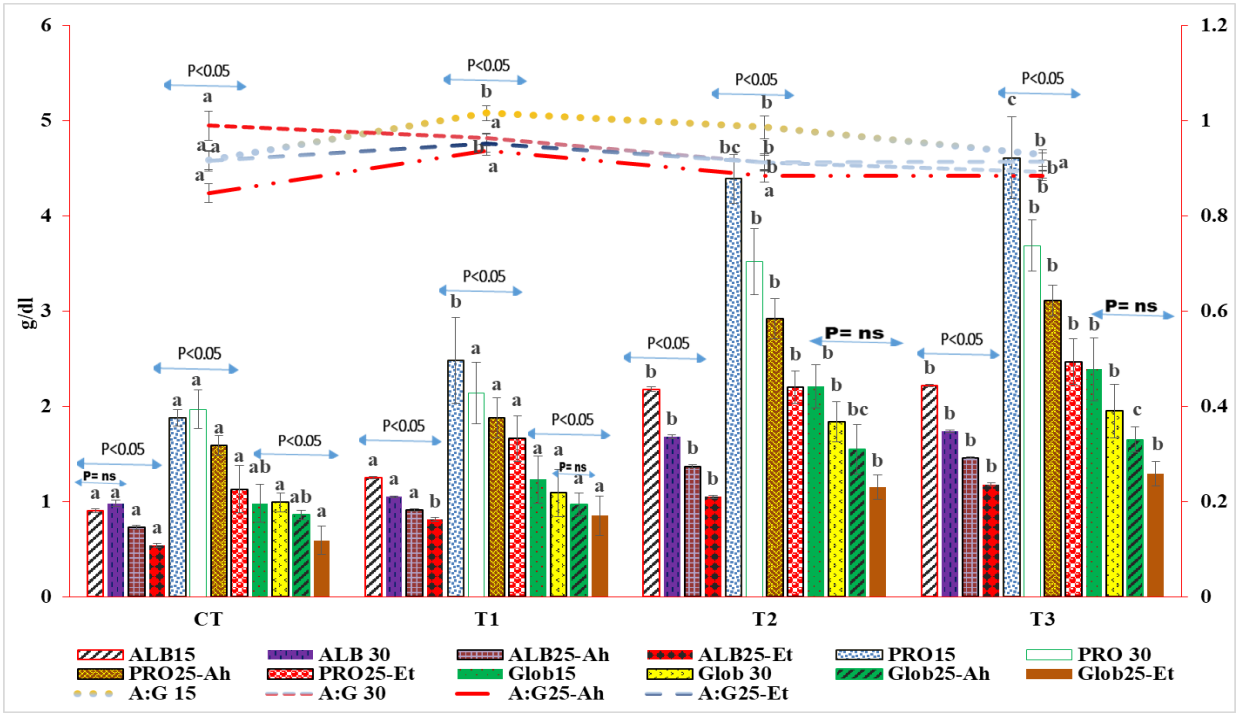


Fig. 31. Showing protein, albumin, globulin and albumin: globulin in intraperitoneal inoculation experiment and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.8. Studies on serum immuno-biochemical parameters, gut-microbiome, histological changes in vital organs and gene expression in *L. rohita* under different trials.

4.8.1. Indoor feed trial

4.8.1.1. Serum enzymes

The values of creatine kinase (U/L) at 90 days, the end of indoor feed trial (CKNac90) exhibited no significance ($p>0.05$) between the groups, however they were considerably lower ($p<0.05$) in T2. There was a considerable difference between T1 and T2 after infection with Ah (*A. hydrophila*) and Et (*E. tarda*) ($p<0.05$). When bacterial pathogens were infected, CKNac90 levels augmented considerably ($p<0.05$) between treatments.

In the CT and T3 groups, there was a substantial ($p>0.05$) rise in CKNac90 and CKNac100-Ah and CKNac100-Et, but T2 had no significance ($p>0.05$) (Fig. 32). In T1, there was insignificant difference between CKNac90 and CKNac100-Ah ($p>0.05$), but there was a considerable difference between CKNac100-Et and CKNac90 ($p<0.05$).

The linear equation of variable of CKNac in T2 group is, $Y=8.67x+227.96$, $R^2=0.99$. The polynomial regression equation of CKNa100-Ah and CKNac100-Et among CT, T1, T2 and T3 are; $Y=26.978x^2-139.07x+432.97$, $R^2=0.89$ and $Y=30.22x^2-157.08x+467.37$ $R^2=0.89$, respectively. The creatinine (mg/dl) ay 90 days, end of idoor feed trial (Creat90) and gamma glutamyl transferase (U/L) at 90 days (GGT90), exhibited a considerably ($p<0.05$) lowering trend in T2, and a substantially ($p<0.05$) increasing trend in T3, however there was insignificant ($p>0.05$) difference between CT and T1.

The creatine at 100 days when infected with *A. hydrophila* (Creat100-Ah) and creatine at 100 days when infected with *E. tarda* (Creat100-Et) did not differ across T1, T2, and T3, or CT and T3 ($p>0.05$). GGT100-Ah and GGT100-Et did not differ considerably ($p>0.05$).

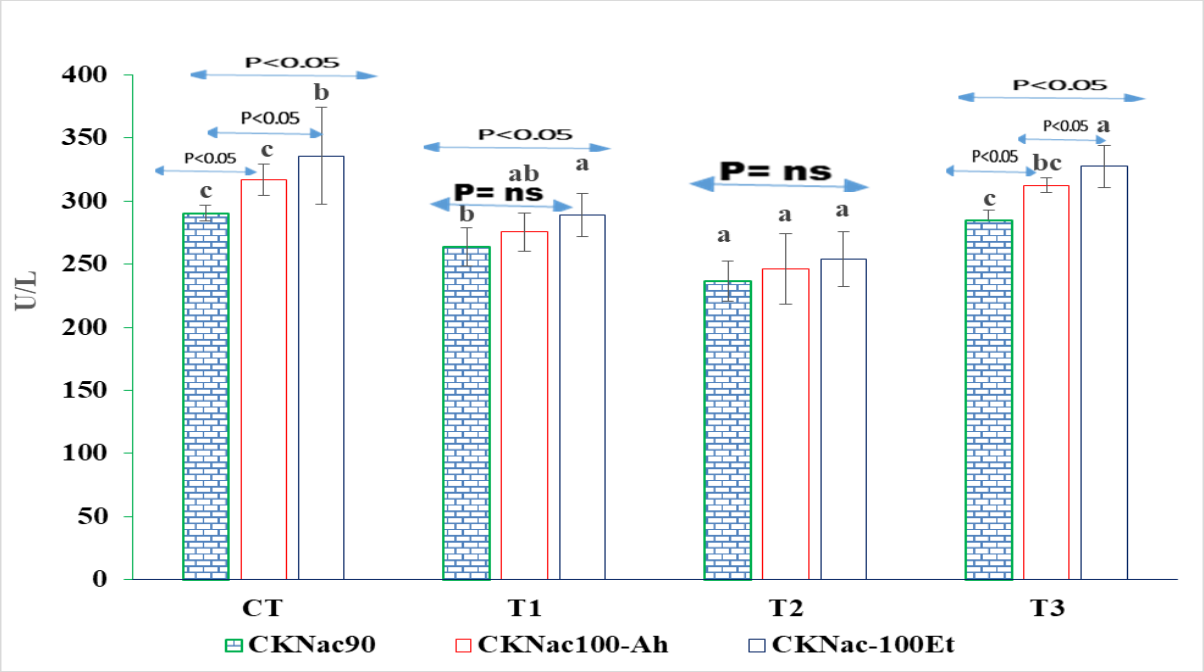


Fig. 32. Showing variation in serum creatine kinase (CKNac) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

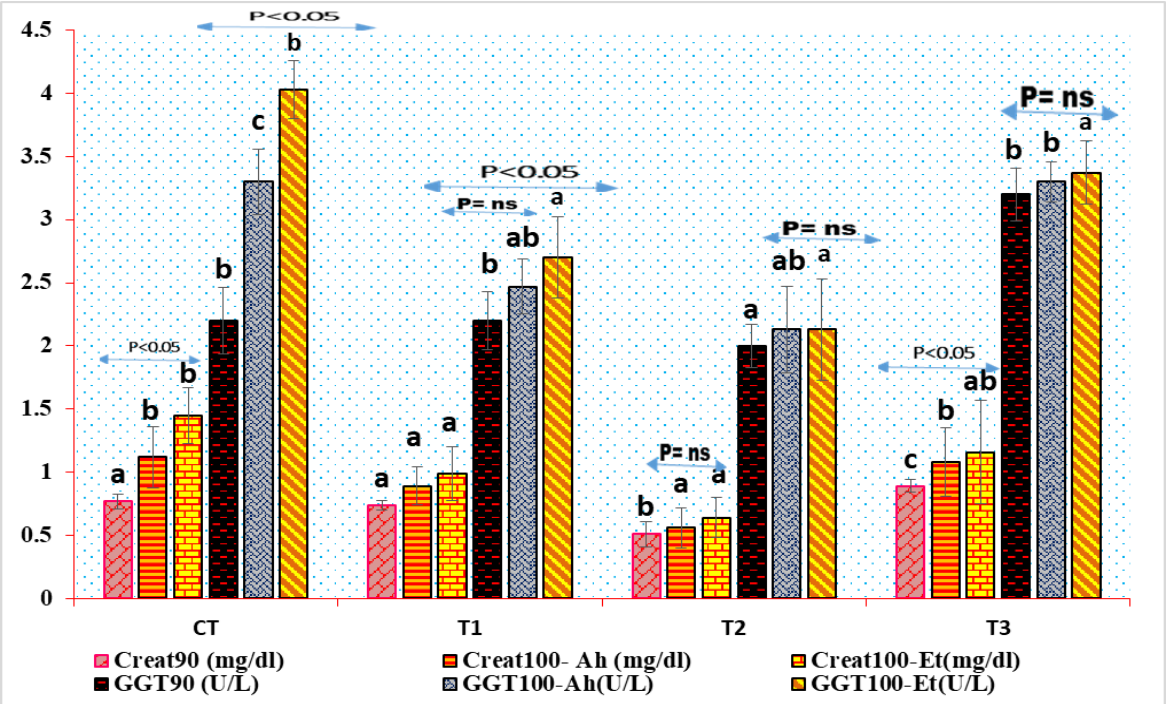


Fig. 33. Showing variation in serum creatine (Creat) and gamma glutamyl transferase (GGT) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The values of Creat90 and GGT90 increased considerably ($p<0.05$) between Creat90 and Creat100-Ah and Creat100-Et and same trend was also followed for GGT90 and GGT100-Ah and GGT100-Et in CT. In T1, GGT90 and Creta90 did not show considerable ($p<0.05$) increase with GGT100-ah and GGT100-Et. In T2 no considerable ($p>0.05$) change was reported between Creat90, Creat100-Ah and Creat100-Et and same trend was also observed for GGT90, GGT100-Ah and GGT100-Et. In T3, insignificance ($p>0.05$) was reported between GGT90, GGT100-Ah and GGT100-Et while it exhibited a considerable ($p<0.05$) change between Creat90, Creat100-Ah and Creat100-Et (Fig. 33).

The linear equation of variable of Creat in T2 group is, $Y=0.0067x+0.44$, $R^2=0.98$. The polynomial regression equation of Creat100-Ah and Creat100-Et among CT, T1, T2 and T3 are; $Y=0.1875x^2-9825x+1.9625$, $R^2=0.77$ and $Y=0.245x^2-1.347x+2.59$ $R^2=0.91$, respectively. The linear equation of variable of GGT in T2 group is, $Y=0.065x+1.9567$, $R^2=0.75$. The polynomial regression equation of GGT100-Ah and GGT100-Et among CT, T1, T2 and T3 are; $Y=0.5x^2-2.534x+5.385$, $R^2=0.95$ and $Y=0.6425x^2-3.4671x+6.9075$ $R^2=0.97$, respectively.

MgXB90 showed no significance ($p>.0.05$) between CT and T1, while, it has decreased considerably ($p<0.05$) in T2 and then again increased statistically ($p<0.05$) in T3. The MgXB100-Ah and MgXB100-Et did not differ ($p<0.05$) between T1, T2 and T3. Within treatments, MgXB90 showed increasing trend with considerable ($p<0.05$) increase in CT. MgXB90, MgXB100-Ah and MgXB100-Et differ considerably ($p<0.05$) in CT. In T1, MgXB90 showed no significance ($p>0.05$) with MgXB100-Ah but it has significant ($p<0.05$) difference with MgXB100-ET.

In T2, MgXB90 did no show significance ($p>0.05$) with MgXB100-Ah and MgXB100-Et while in T3 it has significance ($p<0.05$) with MgXB100-Ah (Fig. 34). The linear equation of variable of MgXB in T2 group is, $Y=0.07x+2.4833$, $R^2=0.99$. The polynomial regression equation of MgXB100-Ah and MgXB100-Et among CT, T1, T2 and T3 are; $Y=0.4175x^2-2.1685x+5.5925$, $R^2=0.87$ and $Y=0.5625x^2-3.0815x+7.0175$ $R^2=0.97$, respectively.

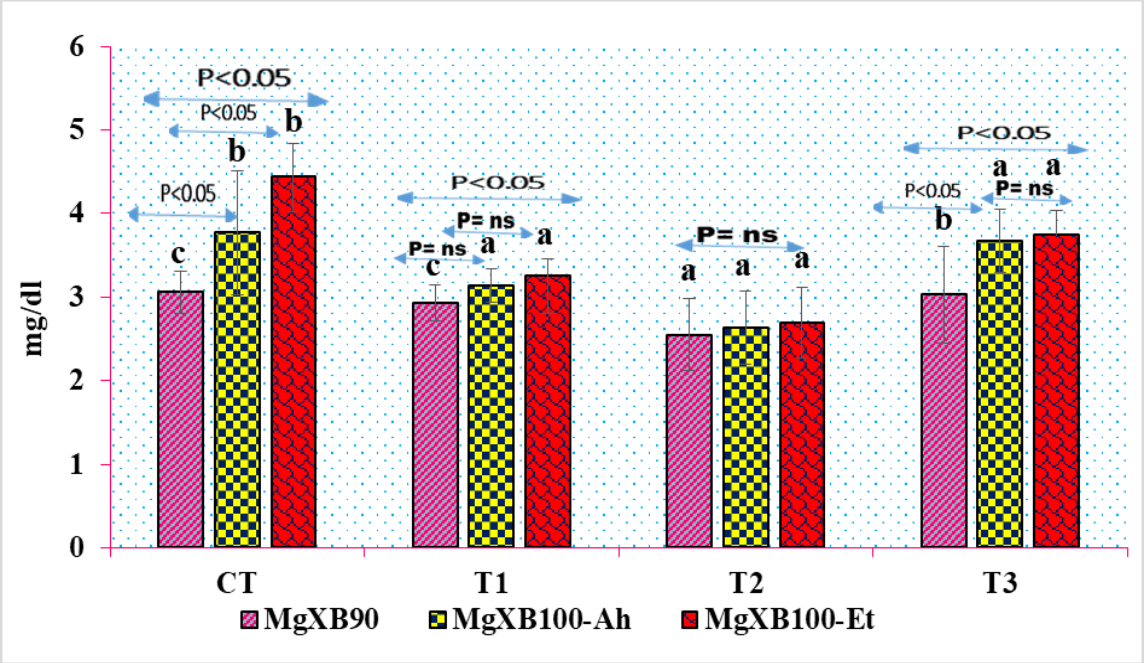


Fig. 34. Showing variation in serum MgXB values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

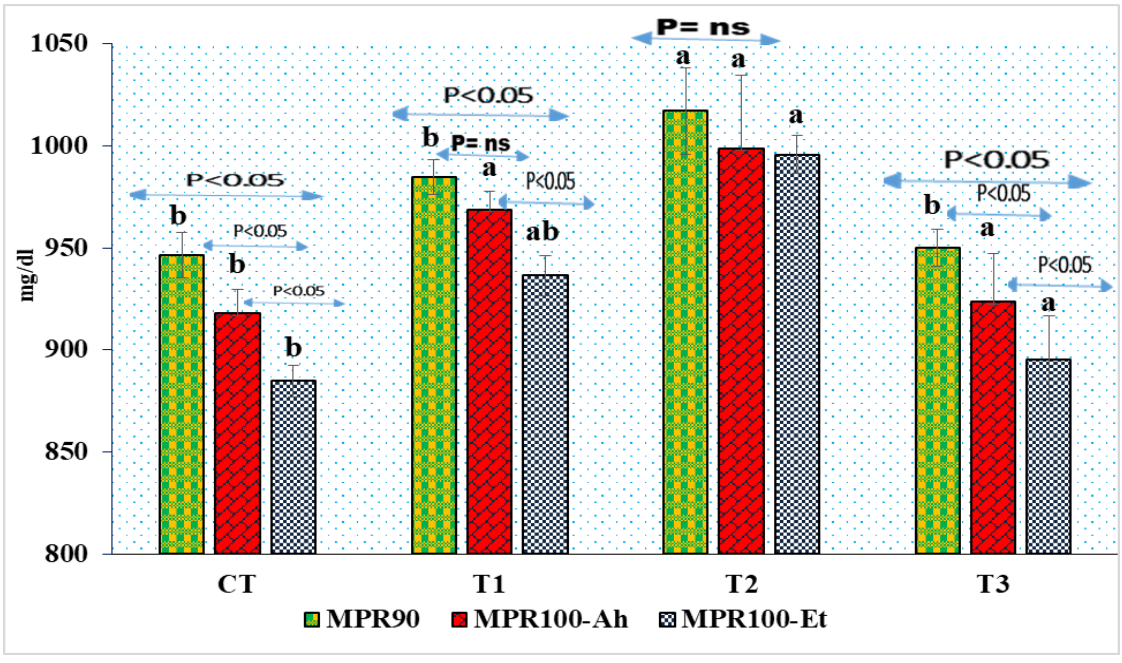


Fig. 35. Showing variation in serum microProtein (MPR) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The microProtein (mg/dl) at 90 days (MPR90) values considerably ($p<0.05$) elevated in T2 and there was no significance was observed between CT, T1 and T3 ($p>0.05$). The microProtein at 100 days when infected with *A. hydrophila* (MPR100-Ah) and the microProtein at 100 days when infected with *E. tarda* (MPR100-Et) had no significance ($p>0.05$) between T1, T2 and T3. Within treatments, the values declined upon infection with bacterial pathogens, Ah and Et. In CT, MPR90 showed significance ($p<0.05$) with MPR100-Ah and MPR100-Et. In T1, MPR90 did not vary substantially ($p>0.05$) with MPR100-Ah but it has significant ($p<0.05$) with MPR100-Et.

In T2, no significance ($p>0.05$) was reported between MPR90, MPR100-Ah and MPR100-ET. In T3, the values were considerably ($p<0.05$) declined between MPR90, MPR100-Ah and MPR100-Et (Fig. 35). The linear equation of variable of MPR in T2 group is, $Y=-10.69x+1025.4$, $R^2=0.85$. The polynomial regression equation of MPR100-Ah and MPR100-Et among CT, T1, T2 and T3 are; $Y=-31.55x^2-162.47x+782.72$, $R^2=0.92$ and $Y=-38.052x^2+199.31x+715.22$, $R^2=0.81$, respectively.

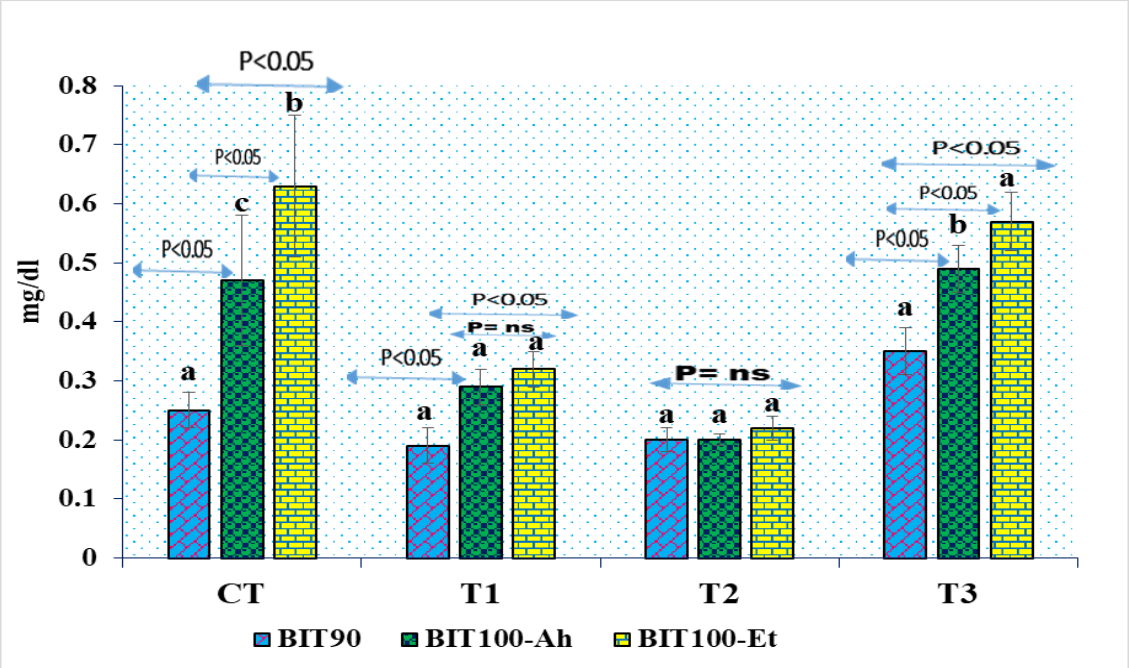


Fig. 36. Showing variation in serum bilirubin total (BIT) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The bilirubin total (mg/dl) at 90days (BIT90) had no significance ($p>0.05$) between the groups and maximum value was observed in T3. The bilirubin total (mg/dl) at 100days when fish infected with *A. hydrophila* (BIT100-Ah) and the bilirubin total (mg/dl) at 100days when fish infected with *E. tarda* (BIT100-Et) follow the same trend as in case of BIT90. The BIT100-Ah and BIT100-Et exhibited no significance ($p<0.05$) between T1 and T2 but differed considerably ($p<0.05$) with CT and T3.

Within groups, the BIT90 increased drastically ($p<0.05$) in CT and T3 groups whereas in T1, BIT90 did not show significance ($p<0.05$) with BIT100-Ah but it has significance ($p<0.05$) with BIT100-Et. In T2, BIT90, BIYT100-Ah and BIT100-Et differed insignificantly ($p>0.05$) (Fig. 36). The linear equation of variable of BIT in T2 group is, $Y=0.01x+0.1867$, $R^2=0.75$. The polynomial regression equation of BIT100-Ah and BIT100-Et among CT, T1, T2 and T3 are; $Y=0.1175x^2-5905x+9575$, $R^2=0.92$ and $Y=0.165x^2-0.853x+1.33$, $R^2=0.97$, respectively.

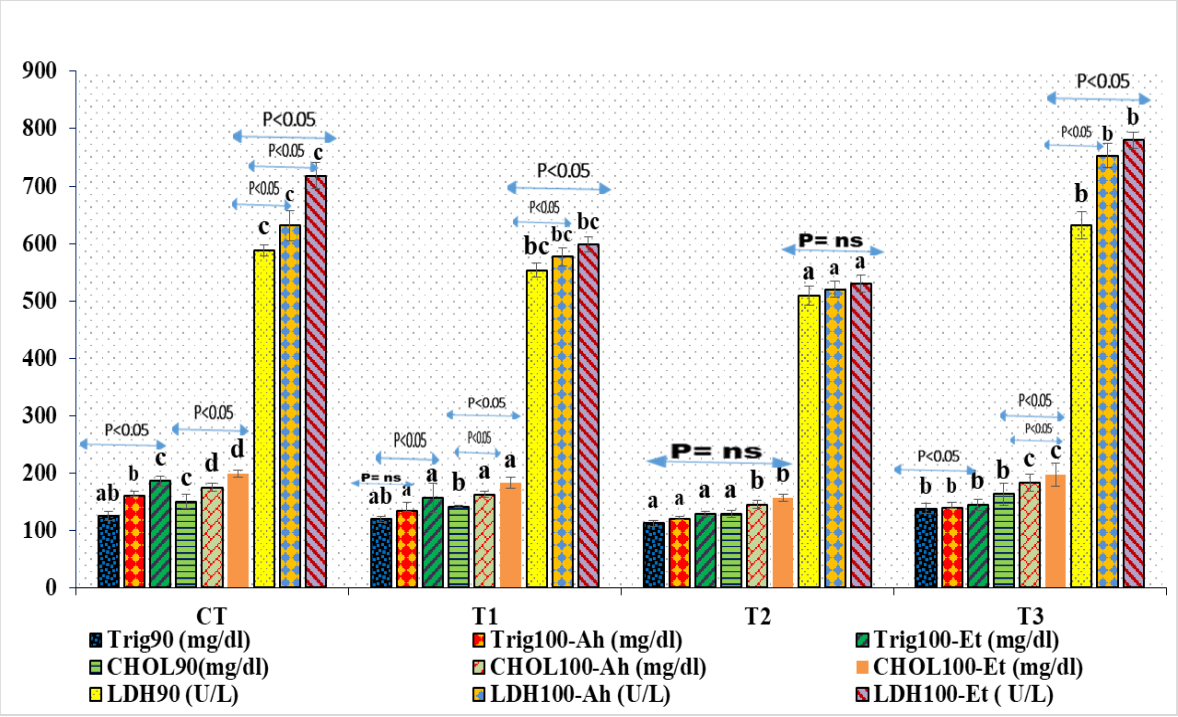


Fig. 37. Showing variation in serum triglycerides (Trig), cholesterol (CHOL) and lactate dehydrogenase (LDH) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The triglycerides (mg/dl) at 90 days (Trig90) exhibited no significance between treatments ($p>0.05$) except T3 and the triglycerides (mg/dl) at 100 days when fish infected with *A. hydrophila* (Trig100-Ah) and the triglycerides (mg/dl) at 100 days when fish infected with *E. tarda* (Trig100-Et) also followed the same trend between the groups. The cholesterol (mg/dl) at 90 days (CHOL90) showed no significance ($p>0.05$) between T1 and T3 and differ considerably ($p<0.05$) with T2. The LDH90 showed no significance ($p>0.05$) between CT and T1 and decreased considerably ($p>0.05$) in T2 and then increased in T3 ($p>0.05$).

The triglycerides (mg/dl) at 100 days when fish infected with *A. hydrophila* (Trig100-Ah) and the triglycerides (mg/dl) at 100 days when fish infected with *E. tarda* (Trig100-Et) had no significance ($p>0.05$) between T1 and T2. The CHOL100-Ah and CHOL100-Et vary considerably between the groups ($p<0.05$). The lacatate dehydrogenase (U/L) at 100 days when fish infected with *A. hydrophila* (LDH100-Ah) and the lacatate dehydrogenase (U/L) at 100 days when fish infected with *E. tarda* (LDH100-Et) showed no significance ($p>0.05$) between CT and T1, and T1 and T3. Within groups, the values of Trig90, CHOL90 and LDH90 increased drastically in CT and T3 ($p<0.05$).

In CT, Trig90 showed considerable ($p<0.05$) difference with Tri100-Ah and Trig 100-ET and same trend was followed for CHOL90, CHOL100-Ah and CHOL100-Et, and LDH90, LDH100-Ah and LDH100-Et also. In TI all parameters showed no considerable ($p>0.05$) trend with ah but showed significance ($p<0.05$) with Et. In T2 no significance ($p>0.05$) was observed within the groups. In T3 considerable ($p<0.05$) change was reported within the group for all variables. The linear equation of variable of Trig in T2 group is, $Y=8.165x+103.77$, $R^2=0.99$. The polynomial regression equation of Trig100-Ah and Trig100-Et among CT, T1, T2 and T3 are; $Y=11.645x^2-65.714x+226.1$, $R^2=0.97$ and $Y=11.645x^2-73.173x+249.53$, $R^2=0.94$, respectively.

The linear equation of variable of CHOL in T2 group is, $Y=14x+1115.34$, $R^2=0.99$. The polynomial regression equation of CHOL100-Ah and CHOL100-Et among CT, T1, T2 and T3 are; $Y=12.585x^2-62.291x+227.43$, $R^2=0.77$ and $Y=14.08x^2-73.591x+261.93$, $R^2=0.75$, respectively (Fig. 37). The linear equation of variable of LDH in T2 group is, $Y=10.5x+498.74$, $R^2=0.99$. The polynomial regression

equation of LDH100-Ah and LDH100-Et among CT, T1, T2 and T3 are; $Y=71.808x^2-328.13x+902.23$, $R^2=0.85$ and $Y=92.222x^2-449.37x+1088.5$, $R^2=0.91$, respectively.

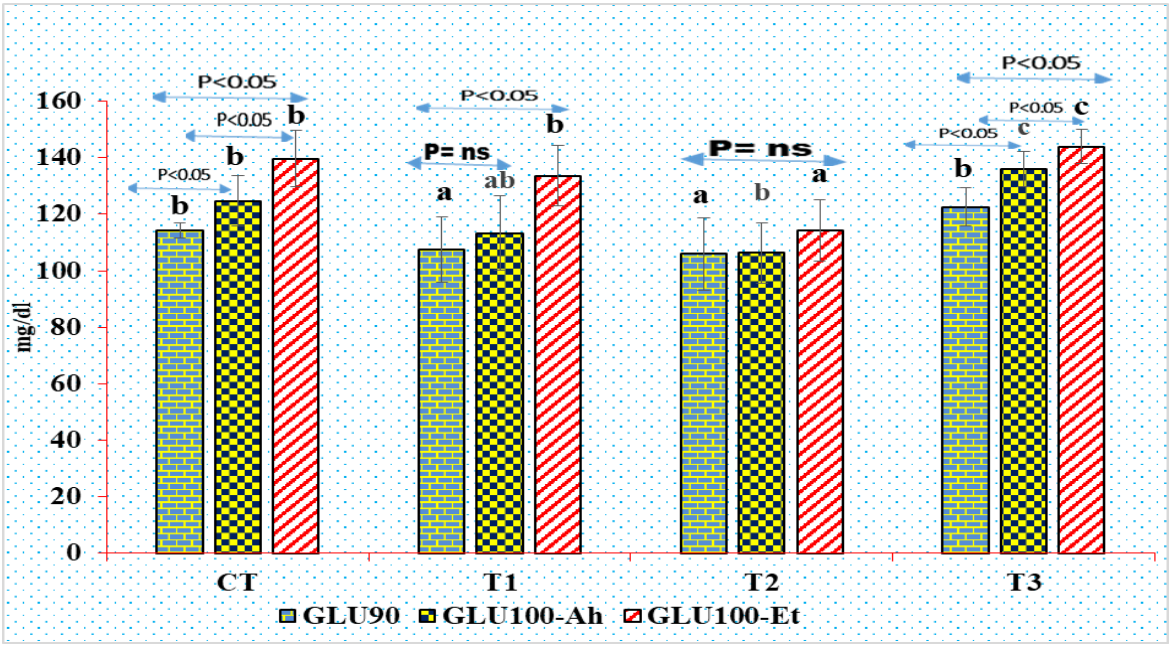


Fig. 38. Showing variation in serum glucose (GLU) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

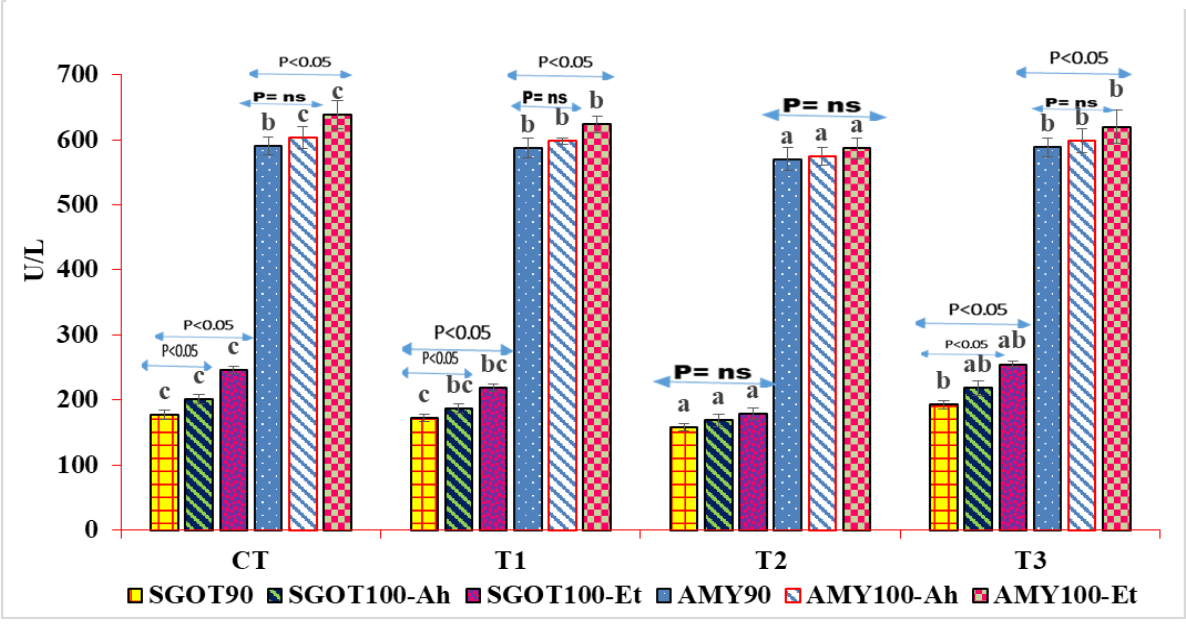


Fig. 39. Showing variation in serum glutamic oxaloacetic transaminase (SGOT) and amylase (AMY) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The CT and T3, as well as T1 and T2, had no considerable differences in glucose at 90 days (GLU90) ($p>0.05$). When compared to the other groups, GLU90 reduced considerably ($p<0.05$) in T3. The glucose at 100 days when fish infected with *A. hydrophila* (GLU100-Ah) and glucose at 100 days when fish infected with *E. tarda* (GLU100-Et) values did not differ considerably between CT, T1, and T2 ($p>0.05$), however they exhibited significance in T3 ($p<0.05$). The values were higher in all groups when compared to GLU90.

In CT GLU90 exhibited a significance ($p<0.05$) with glucose at 100 days when fish infected with *A. hydrophila* (GLU100-Ah) and glucose at 100 days when fish infected with *E. tarda* (GLU100-Et). In T1, GLU90 showed no significance ($p>0.05$) with GLU100-Ah but it has significance ($p<0.05$) with GLU100-Et. In T2 no significance ($p>0.05$) was observed among GLU90, GLU100-Ah and GLU100-Et. In T3, significance ($p<0.05$) was observed among GLU90, GLU100-Ah and GLU100-Et (Fig. 38). The linear equation of variable of GLU in T2 group is, $Y=4.18x+100.49$, $R^2=0.79$. The polynomial regression equation of GLU100-Ah and GLU100-Et among CT, T1, T2 and T3 are; $Y=10.253x^2-48.543x+164.58$, $R^2=0.89$ and $Y=8.92x^2-45.246x+179$, $R^2=0.67$, respectively.

The serum glutamic oxaloacetic transaminase (U/L) at 90 days (SGOT90) showed significance ($p<0.05$) among the groups except CT and T1. The serum glutamic oxaloacetic transaminase at 100 days when fish infected with *A. hydrophila* (SGOT100-Ah) and serum glutamic oxaloacetic transaminase at 100 days when fish infected with *E. tarda* (SGOT-100Et) also differ drastically ($p<0.05$) among the groups. The amylase (U/L) at 90 days (AMY90) considerably decreased in T2 ($p<0.05$) with being maximum value in T3 as compared with other groups. The AMY90 did not vary among CT, T1 and T3. Within groups, the values increased in all groups for both, AMY90 and SGOT90. The AMY90 varied substantially ($p<0.05$) with SGOT100-Ah and SGOT-100Et in CT.

In T1, SGOT90 has significance ($p<0.05$) with SGOT-100Ah and SGOT100-Et. In T2, no significance was reported within the group ($p>0.05$). In T3, SGOT90 showed a significance ($p<0.05$) with SGOT100-Ah and SGOT-100Et but AMY90 did not show significance ($p>0.05$) with AMY100-Ah but it has significance ($p<0.05$) with

AMY100-Et. The linear equation of variable of SGOT in T2 group is, $Y=10.835x+146.76$, $R^2=0.99$.

The polynomial regression equation of SGOT100-Ah and SGOT100-Et among CT, T1, T2 and T3 are; $Y=16.113x^2-77.144x+265.64$, $R^2=0.82$ and $Y=25.86x^2-130.71x+357.71$, $R^2=0.77$, respectively (Fig. 39). The linear equation of variable of AMY in T3 group is, $Y=8.32x+589.18$, $R^2=0.97$. The polynomial regression equation of AMY100-Ah and AMY100-Et among CT, T1, T2 and T3 are; $Y=16.11x^2-77.144x+265.64$, $R^2=0.82$ and $Y=25.867x^2+130.71x+357.71$, $R^2=0.77$, respectively.

The serum glutamic pyruvic transaminase at 90 days (SGPTD90) differed insignificantly ($p>0.05$) among CT, T1 and T3, and decreased substantially ($p<0.05$) in T2 as compared with other groups. The lipase at 90 days (LIP90) and alkaline phosphatase at 90 days (ALPU90) vary considerably ($p<0.05$) among groups being maximum value in T3. Within groups, the values for all three enzymes increased as compared to SGPTD90, LIP90 and ALPU90 values. The linear equation of variable of SGPTD in T2 group is, $Y=0.25x+1.673$, $R^2=0.99$.

The polynomial regression equation of SGPTD90-Ah and SGPTD90-Et among CT, T1, T2 and T3 are; $Y=0.645x^2+3.501+6.628$, $R^2=0.05$ and $Y=0.885x2-4.717x+8.49$, $R^2=0.92$, respectively. The linear regression equation of variable of LIP in T2 group is, $Y=0.685x+17.67$, $R^2=0.97$. The polynomial regression equation of LIP100-Ah and LIP100-Et among CT, T1, T2 and T3 are; $Y=1.764x^2-9.762+34.33$, $R^2=0.69$ and $Y=2.716x^2-15.04x+42.75$, $R^2=0.74$, respectively (Fig. 40).

The linear regression equation of variable of ALPU in T2 group is, $Y=2.165x^2+14.78+$, $R^2=0.95$. The linear equation of ALPU100-Ah and ALPU100-Et among CT, T1, T2 and T3 are; $Y=4.082x^2-21.04x+47.41$, $R^2=0.94$ and $Y=3.835x^2+50.03$, $R^2=0.68$, respectively.

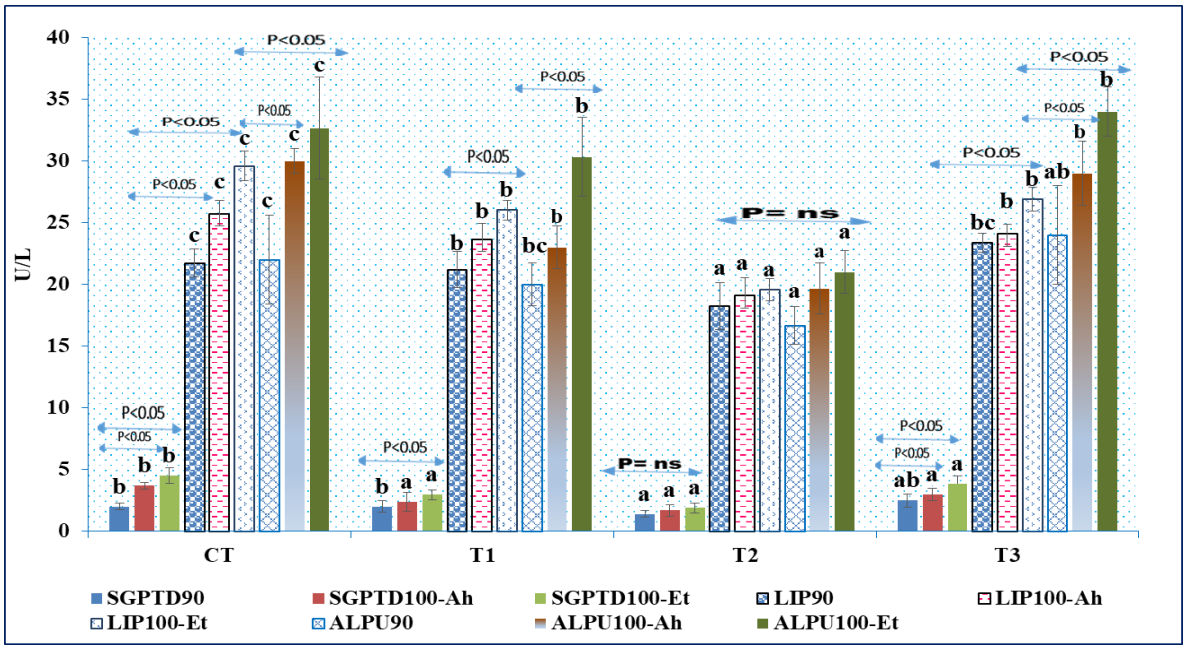


Fig. 40. Showing variation in serum glutamic pyruvic transaminase (SGPTD), lipase (LIP) and alkaline phosphatase (ALPU) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.8.1.2. Digestive enzymes

The indoor feed trial revealed a substantial ($p<0.05$) increase in protease at 90 days (PROT90) in T2 when compared to CT and T3, but no significance ($p>0.05$) in T1 when compared to CT and T3. The pattern continued after infection. When compared to the other groups, T3 had considerably lower AMY90 activity ($p<0.05$). Infection followed the same pattern, except Et was unrelated to T1, T2, or T3. When compared to the other groups, LIP90 activity rose non-significantly ($p>0.05$) up to T2, then dropped considerably ($p<0.05$) in T3. When T1 and T2, as well as CT and T3, were infected, the LIP100-Ah activity did not differ substantially ($p>0.05$).

The lipase at 100 days when fish infected with *E. tarda* (LIP100-Et) also showed same trend as in case of lipase at 100 days when fish infected with *A. hydrophila* (LIP100-Ah). The ALPU100 showed substantial ($p<0.05$) rise in T2 as compare to other groups and the order was as follow, T2>T1>T3>CT. ALPU100 Ah and Et showed almost same trend. Within the treatments, all enzymes showed a substantially ($p<0.05$) declining trend upon infection with Ah and Et in CT group and

over all AMY differed in substantially ($p<0.05$) among CT, T1 and T2. In T1 and T2 a insignificant ($p>0.05$) variation was reported in the value of PROT and LIP for Ah and T2 showed for Et also. In T3 except ALPU other enzyme activity showed non-significant change ($p>0.05$) (Fig. 41).

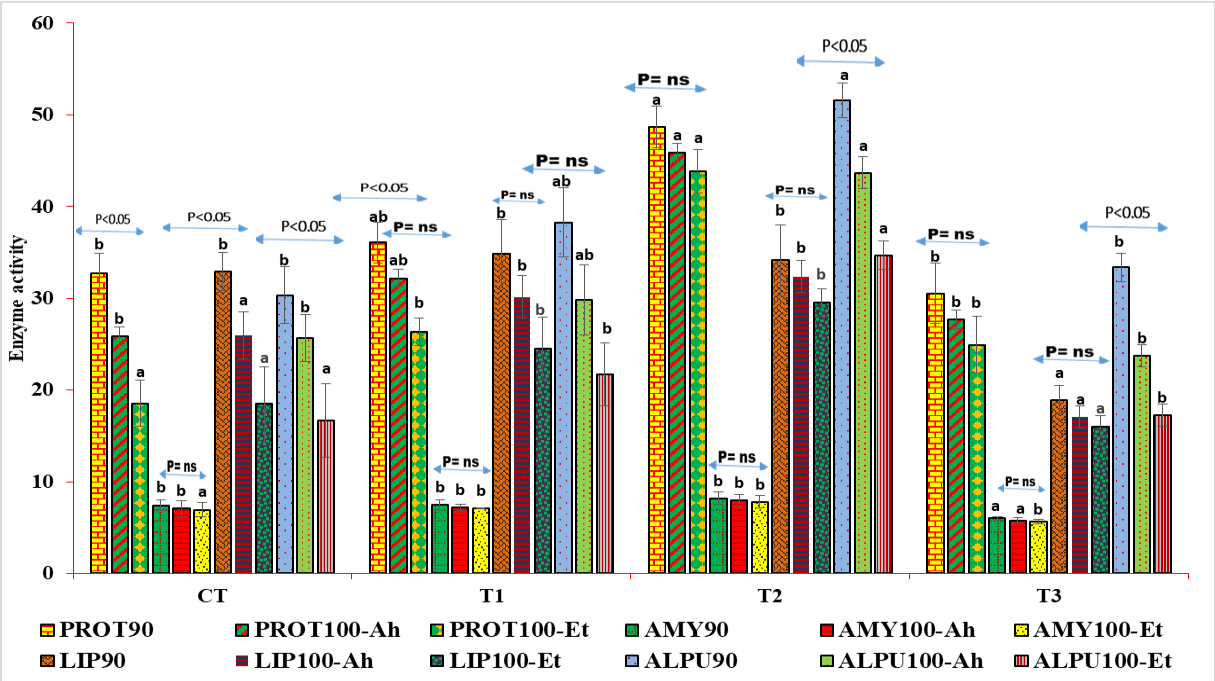


Fig. 41. Showing variation in digestive protease (PROT), lipase (LIP), amylase (AMY) and alkaline phosphatase (ALPU) values in indoor feed trial and challenge study

Here, Protease activity expressed as micromoles of tyrosine released/min/mg protein at 37 ℃; Maltose activity expressed as micromoles of maltose released/min/mg protein at 37 ℃; Lipase activity expressed as units/mg protein at 37 ℃; Alkaline phosphatase activities expressed as nanomoles p-nitrophenol released/min/mg protein at 37 ℃.

4.8.1.3. Enzyme of oxidative damage

The catalase at 90 days (CAT90) exhibited no significance ($p>0.05$) between CT, T1, and T2 in the indoor feed trial, but increased considerably ($p<0.05$) in T3 when compared with other groups. In challenge study, the activity of CAT100-Ah rose in CT, the most compared to the other groups, and there was no significance

was reported (p>0.05) between CT and T3 or T1 and T2. The superoxide dismutase at 90 days (SOD90) dropped considerably (p<0.05) in T2 compared to CT and T1, then increased substantially (p>0.05) in T3. The T2 showed a considerable (p<0.05) variation from the other groups after being infected with Ah. The glutathione-s-transferase at 90 days (GST90) was found to be at its highest level in T3.

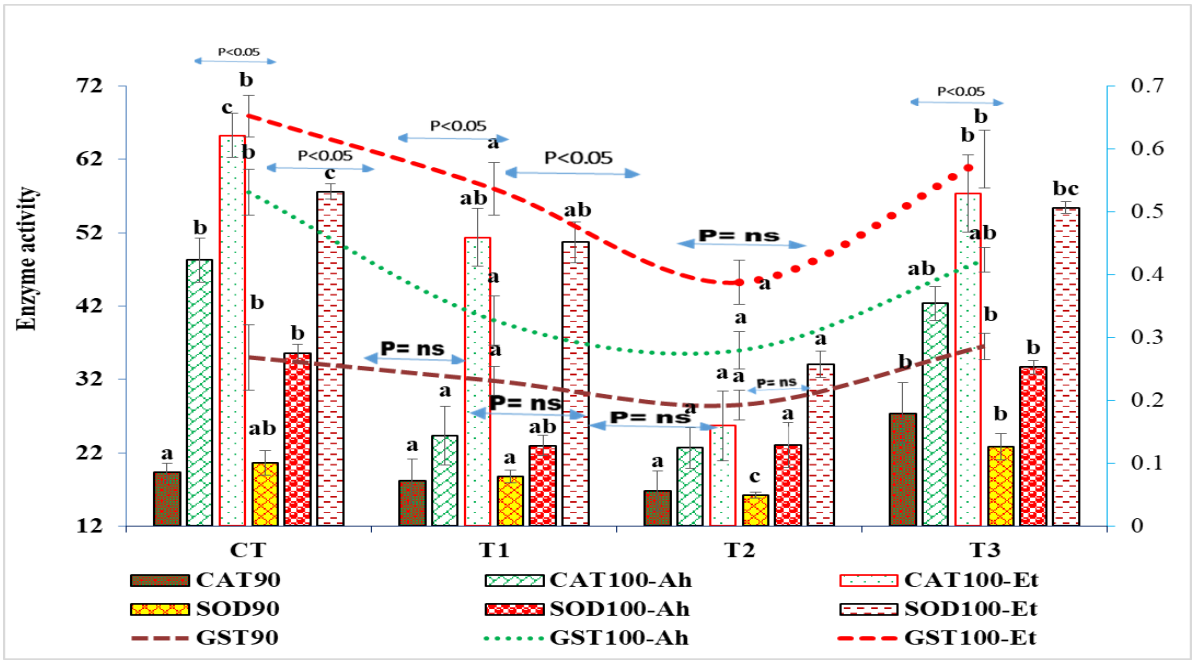


Fig. 42. Showing variation in catalase (CAT), superoxide dismutase (SOD) and glutathione-s-transferase (GST) values in indoor feed trial and challenge study

Here, Catalase: mmol H_2O_2 decomposed /min/ mg protein at 37 ℃; SOD (superoxide dismutase): µmol mg^{-1} protein/min at 37 ℃; GST: Glutathione-S-transferase Units/mg protein.

Trend for Et (*E*. tarda) was almost same as in Ah (*A. hydrophila*). The activity within the treatments exhibited a significance (p<0.05) rise in CT, T1 and T3 as compared to T1 for all enzymes. The GST90 declined drastically (p<0.05) in T2 and increased substantially in T3 (p<0.05) as compared to other groups (Fig. 42).

The polynomial equations for CAT90, SOD90 and GST90 are as follows, $Y= 2.915x^2-12.294x+29.285$; $R^2=0.89$, $Y= 2.1357x^2 – 10.261x+29.243$, $R^2=0.80$; $Y=0.0327x^2-0.1619x+0.4042$, $R^2=0.83$, respectively.

4.8.1.4. Enzyme of neuro-transmission

For acetyl choline esterase at 90 days (AchE90), there was no significance ($p>0.05$) between CT and T3, T1 and T2 in the indoor feed trial. The pattern was nearly identical among all groups after infection with Ah (*A. hydrophila*) and Et (*E. tarda*). Within the treatments, AchE90 activity rose considerably ($p<0.05$) in acetyl choline esterase at 100 days when fish was challenged with *A. hydrophila* (AchE100-Ah) and acetyl choline esterase at 100 days when fish was challenged with *E. tarda* (AchE100-Et) in CT and T3, but no significance was detected in T1 and T2 ($p>0.05$) (Fig. 43). The polynomial equation for AchE at 90 is as follows, $Y= 0.4889x^2-2.1271x+4.1358$, $R^2=0.99$.

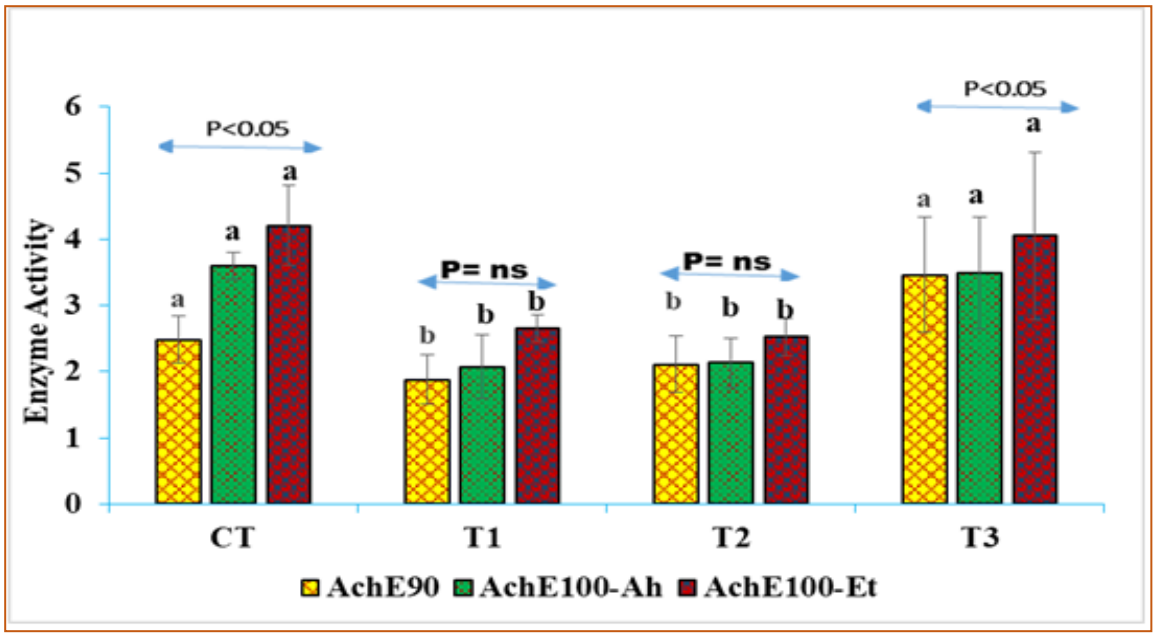


Fig. 43. Showing variation in acetyl choline esterase (AchE) values in indoor feed trial and challenge study

Here, AchE (acetylcholine esterase) activity: µmol of acetylcholine hydrolyzed/min/mg protein at 37 ℃.

4.8.1.5. Triiodothyronine (T3), thyroxine(T4) and cortisol

Between the groups, there was no substantial change in cortisol at 30 days (CORT-30) ($p<0.05$) but in cortisol at 60 days (CORT-60), cortisol at 90 days (CORT-90), cortisol at 100 days when fish infected with *A. hydrophila* (CORT100-Ah), and

cortisol at 100 days when fish infected with *E. tarda* (CORT100-Et) there was no significance was reported (p>0.05). The CORT-60, CORT-90, and CORT100-Ah did not indicate a considerable (p>0.05) change between the groups especially in T1, T2 and T3 when compared among the groups. Following infection, there was a considerable (p<0.05) decline in values of CORT100-Ah and CORT100-Et till T2, thereafter, there was a rising trend in T3 (Fig. 44).

There was a considerable (p<0.05) difference in treatments, but in T4-60, there was no significance (p>0.05) in CT and T1. When T1 and T2 groups were compared, T4-30 showed a substantial (p<0.05) change, while T4100-Et exhibited a sharp deviation (p<0.05) in CT. The T4-30, T4-90, T4100-Ah, and T4100-Et in T3 indicated a substantial (p<0.05) rise in values until T2, after which they showed a decline in T4-30, T4-90, T4100-Ah, and T4100-Et.

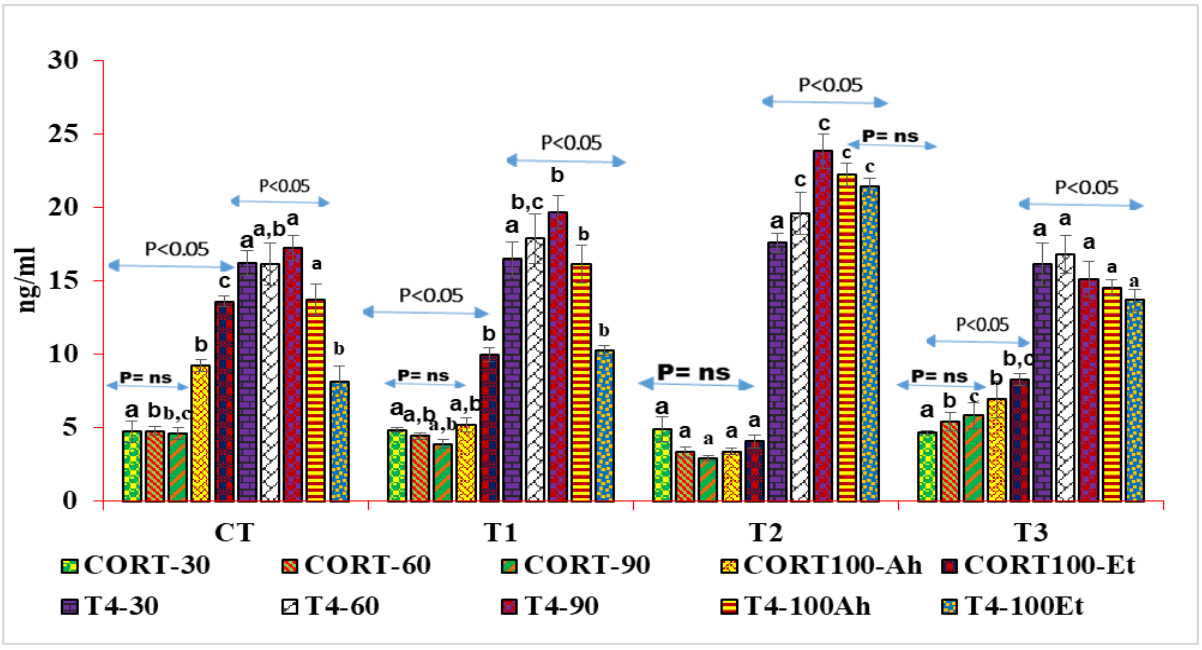


Fig. 44. Showing variation in cortisol (CORT) and thyroxine (T4) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

With the exception of triiodothyronine at 60 days (T3-60) in T1, all of the triiodothyronine at 30 days (T3-30), triiodothyronine at 90 days (T3-90), triiodothyronine at 100 days when fish was challenged with *A. hydrophila* (T3100-Ah),

and triiodothyronine at 100 days when fish was challenged with *E. tarda* (T3100-Et) revealed a substantial change between the groups ($p<0.05$).

There was no considerable variation between CT and T1 ($p>0.05$) in any of the metrics when comparing the treatments, although there was a significance ($p<0.05$) between T2 and T3 for T3-100Et and T3-90. In CT, T1, and T2 values increased considerably ($p<0.05$), followed by a considerable ($p<0.05$) reduction in T3. When comparing T3-30, T3-60, and T3-90 in treatment CT to T3-100Ah and T3-100Et, there was no substantial change ($p>0.05$). Except in T3-100Ah and T3-100Et, there was a considerable ($p<0.05$) change in all parameters in treatment T2 (Fig. 45).

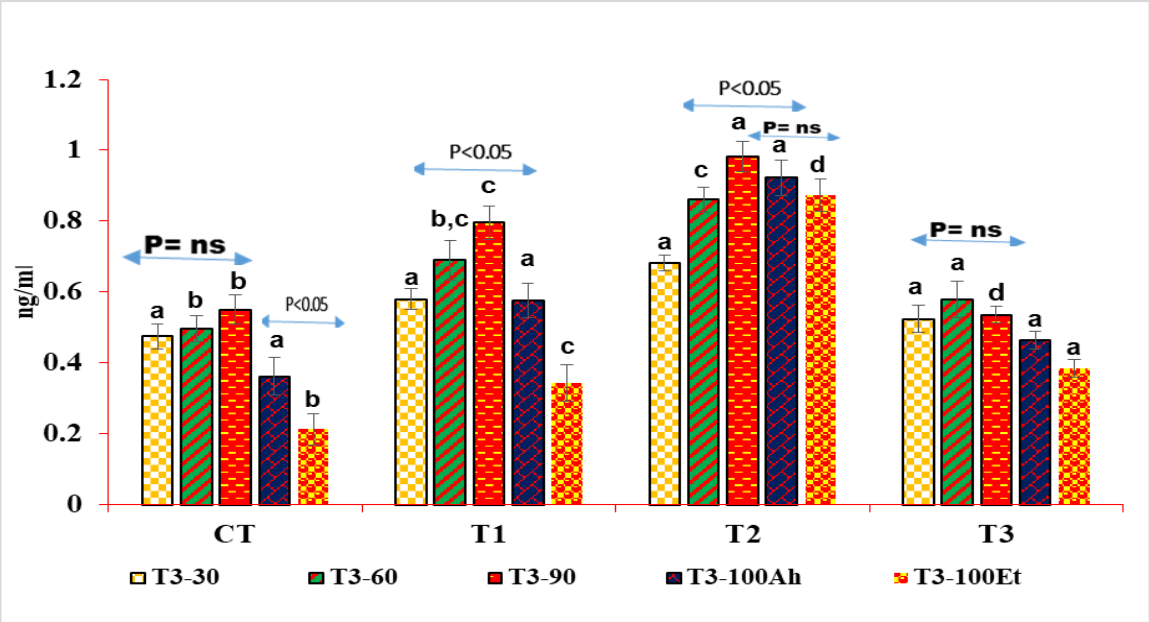


Fig. 45. Showing variation in triiodothyrinine (T3) values in indoor feed trial and challenge study
**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.8.1.6. Antibody titre

At 30 days, there was no substantial difference in IgM levels between the treatments ($p>0.05$), however at 60 days, there was a considerable change ($p<0.05$). After then, the T2 group's values increased drastically ($p<0.05$), while the T3 group's values declined dramatically ($p<0.05$). Except for CT and T3, the IGM levels in 100Ah

and 100Et differ substantially ($p<0.05$) among the treatments. In CT, there was no considerable change in Ah and Et ($p>0.05$). T1 did not exhibit a significance ($p>0.05$) between control at 30 days (CT-30) and control at 60 days (CT-60), but there was a substantial ($p<0.05$) change between T2 group at 60 days (T2-60), T2 group at 90 days (T2-90), T3 group at 60 days (T3-60), and T3 group at 90 days (T3-90) value, and there was a substantial ($p<0.05$) difference between 90 days values and 100 days (Ah), but no substantial ($p>0.05$) difference between Ah and Et (Fig. 46).

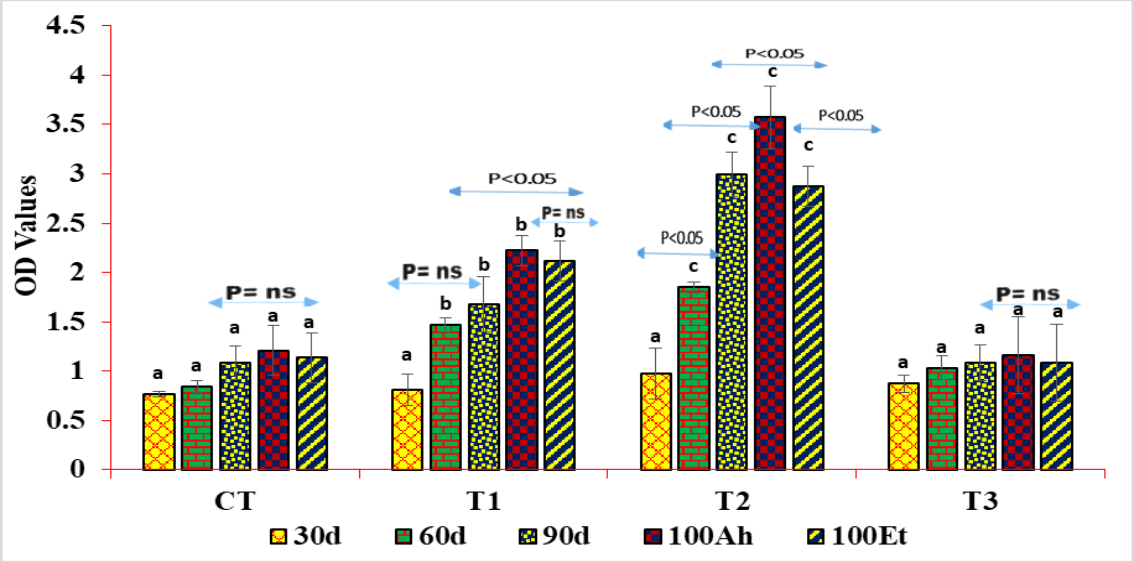


Fig. 46. Showing variation in serum immunoglobulin M (IgM) values in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.8.1.7. Gene expression

4.8.1.7.1. RNA isolation

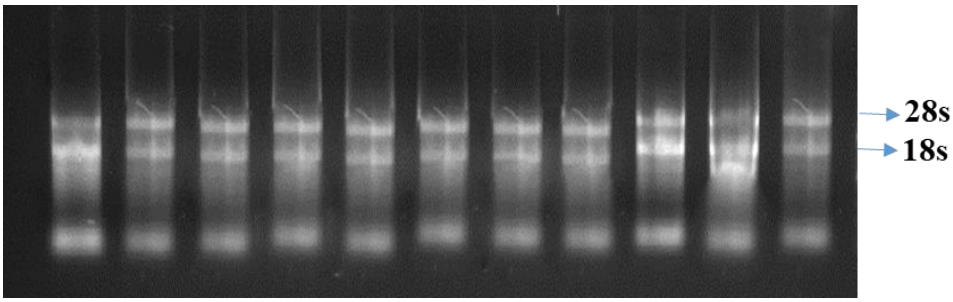


Fig. 47. Showing RNA gel with two distinct bands

For gene expression studies RNA was isolated using Qiagen RNA isolation Kit. Isolated RNA was separated on 1% agarose gel and both 28s and 18s RNA bands were seen (Fig. 47).

4.8.1.7.2. Semi-quantitative PCR

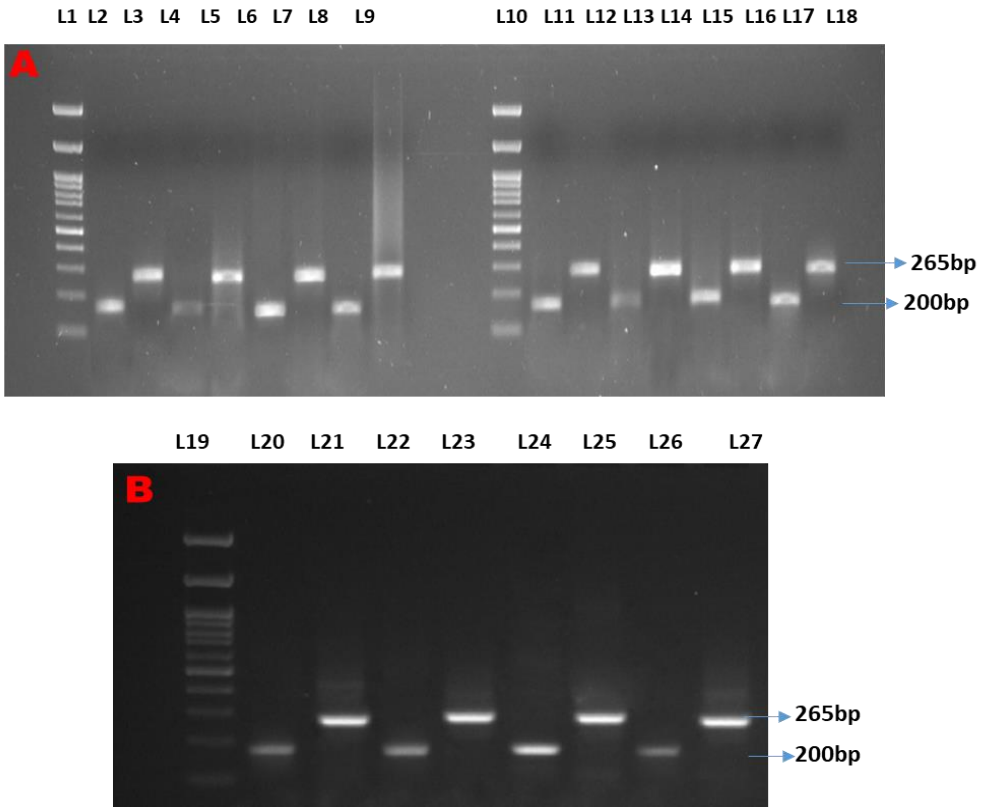


Fig. 48a. Showing semi-quantitative PCR of ISG15

Legends: L-1, 11 and 19: 1Kb molecular marker; L-2,4,6,8,11,13, 15, 17, 20, 22, 24 and 26: β-actin (housekeeping gene); L-3: CT90; L-5: T1-90; L-7: T2-90; L-9: T3-90; L-12: CT100-Ah; L-14: T1-100Ah; L-16: T2-100Ah; L- 18: T3-100Ah; L-21: CT100-Et; L-23: T1-100Et; L-25: T2-100Et; L-27: T3-100Et

actin (as housekeeping gene) and ISG15, respectively (Fig. 48a). Semi- quantitative analysis showed two distinct bands at 200bp and 265bp for, β-actin (as housekeeping gene) and Mx, respectively (Fig. 48b). Two distinct bands were observed at 200bp and 700bp for, β-actin (as housekeeping gene) and STAt1, respectively (Fig. 48c).

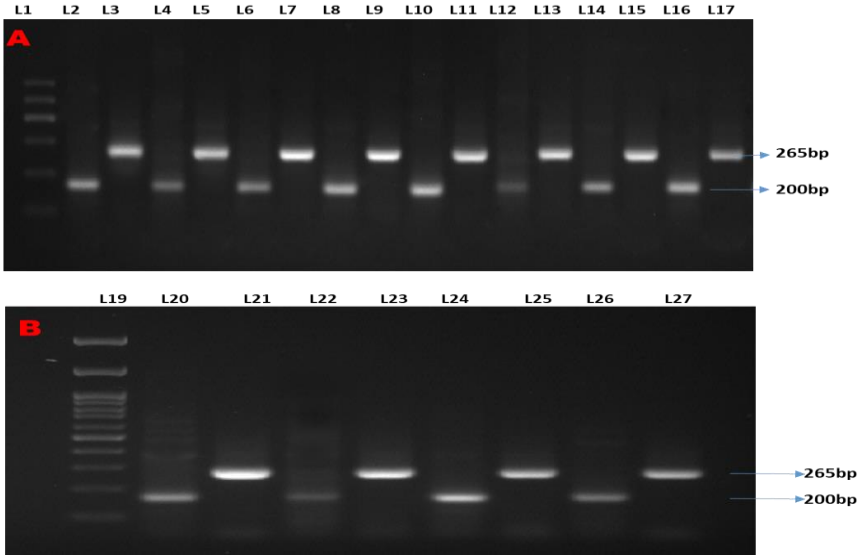


Fig. 48b. Showing semi-quantitative PCR of Mx gene

Legends: L-1, 11 and 19: 1Kb molecular marker; L-2,4,6,8,11,13, 15, 17, 20, 22, 24 and 26: β-actin (housekeeping gene); L-3: CT90; L-5: T1-90; L-7: T2-90; L-9: T3-90; L-12: CT100-Ah; L-14: T1-100Ah; L-16: T2-100Ah; L- 18: T3-100Ah; L-21: CT100-Et; L-23: T1-100Et; L-25: T2-100Et; L-27: T3-100Et

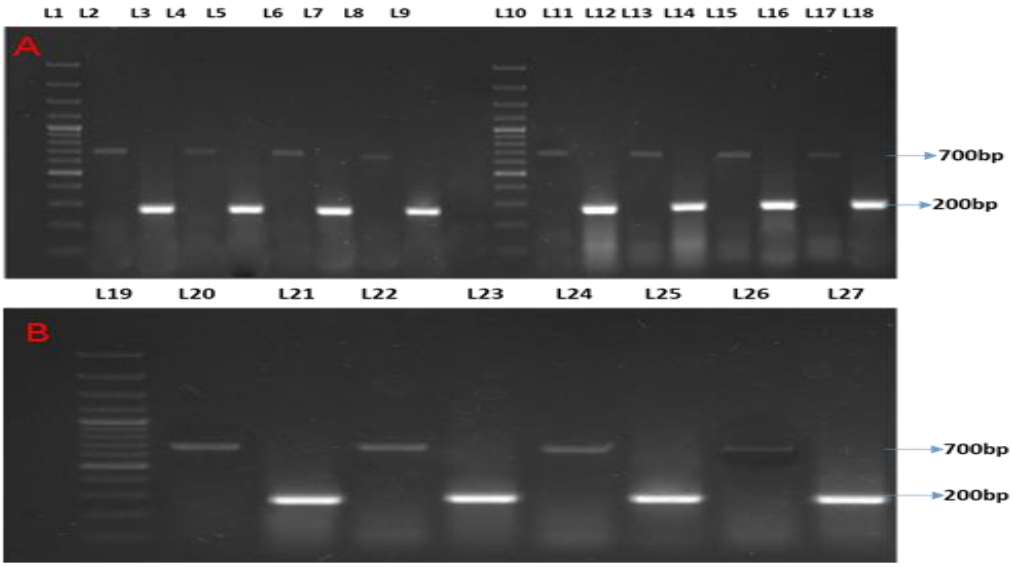


Fig. 48c. Showing semi-quantitative PCR of STAT1 gene

Legends: L-1, 11 and 19: 1Kb molecular marker; L-2,4,6,8,11,13, 15, 17, 20, 22, 24 and 26: β-actin (housekeeping gene); L-3: CT90; L-5: T1-90; L-7: T2-90; L-9: T3-90; L-12: CT100-Ah; L-14: T1-100Ah; L-16: T2-100Ah; L- 18: T3-100Ah; L-21: CT100-Et; L-23: T1-100Et; L-25: T2-100Et; L-27: T3-100Et

The Mx had the highest expression in all groups in the indoor feed trial, followed by ISG15 and STAT1. The ISG15 gene expression differs considerably

(p<0.05) between treatments, with a substantial (p<0.05) increase in T2 and subsequently a substantial (p<0.05) drop in T3. The control at 90 days (CT90) values were slightly lower, with no statistical significance (p>0.05) between CT90, control at 100 days when fish was challenged with *A. hydrophila* (CT100-Ah), and control at 100 days when fish was challenged with *E. tarda* (CT100-Et). It has raised considerably (p<0.05) in T1 and T2 after infection. The ISG15 was expressed more in Ah (*A. hydrophila*) than in Et (*E. tarda*). The Mx expression increased considerably (p<0.05) till T2, then reduced drastically (p>0.05) in T3. The highest Mx value was found in T2, followed by T3>T1 and CT.

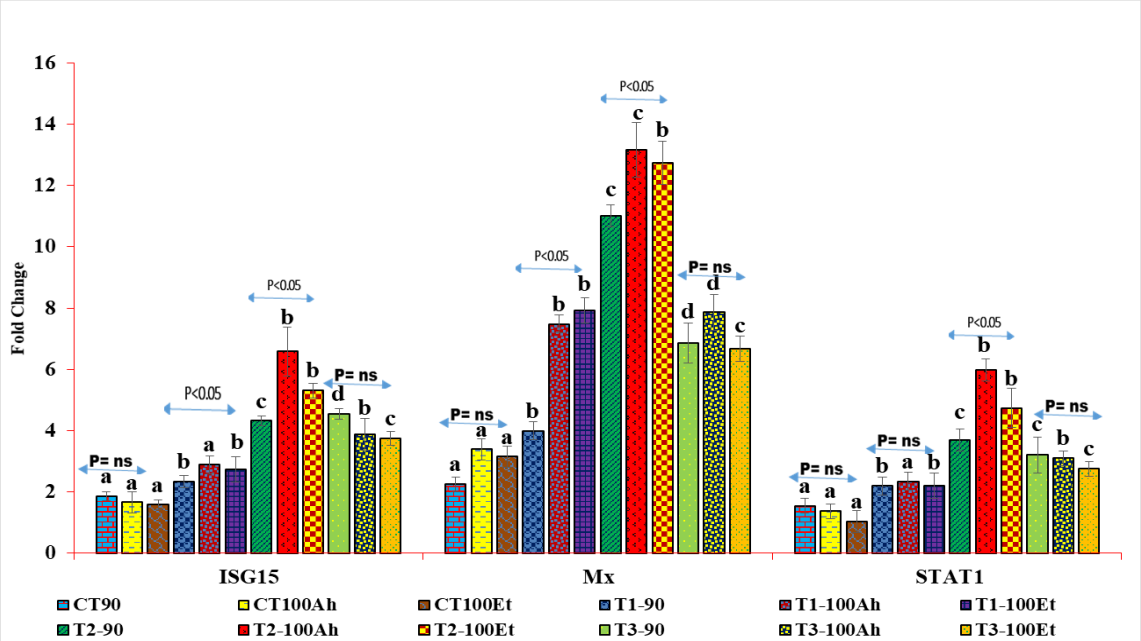


Fig. 49. Expression of three immunogenic genes in indoor feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The Mx levels during infection varied considerably between the treatments (p>0.05). Within the treatments, the Mx value increased considerably (p<0.05) after Et infection, but exhibited no significance after Ah infection (p>0.05). However, between 90 days and Et infection, the Mx value grew considerably (p>0.05) in T1, T2, and T3, but no significance in T3 (p>0.05). The treatment effects on STAT1 expression were staggered. The CT and T1, as well as T2 and T3, exhibited no

statistical significance (p>0.05). The T3, on the other hand, indicated a small reduction in fold change values. When Ah and Et were infected, the fold change value the CT treatment, fell with no significance (p>0.05). Following infection with Ah and Et, the values in T1 increased in an insignificant manner (p>0.05). The T2 exhibited a substantial (p<0.05) rise with Ah, followed by a considerable (p<0.05) drop with Et, and finally an insignificant decline in T3 (p>0.05) (Fig. 49).

4.8.1.8. Gut-microbiome

At the end of indoor feed trial, at 90 days, the gut microbial community structure revealed a distinct difference among the treatments. Family wise stacked chart showing a clear variation in the community composition in T2 groups as compared to other treatments. CT treatments have more species from aeromoadaceae then it's decreasing till T2 and again increasing in T3 (red colour stacked) while others are increasing and *vice-versa* (Fig. 50).

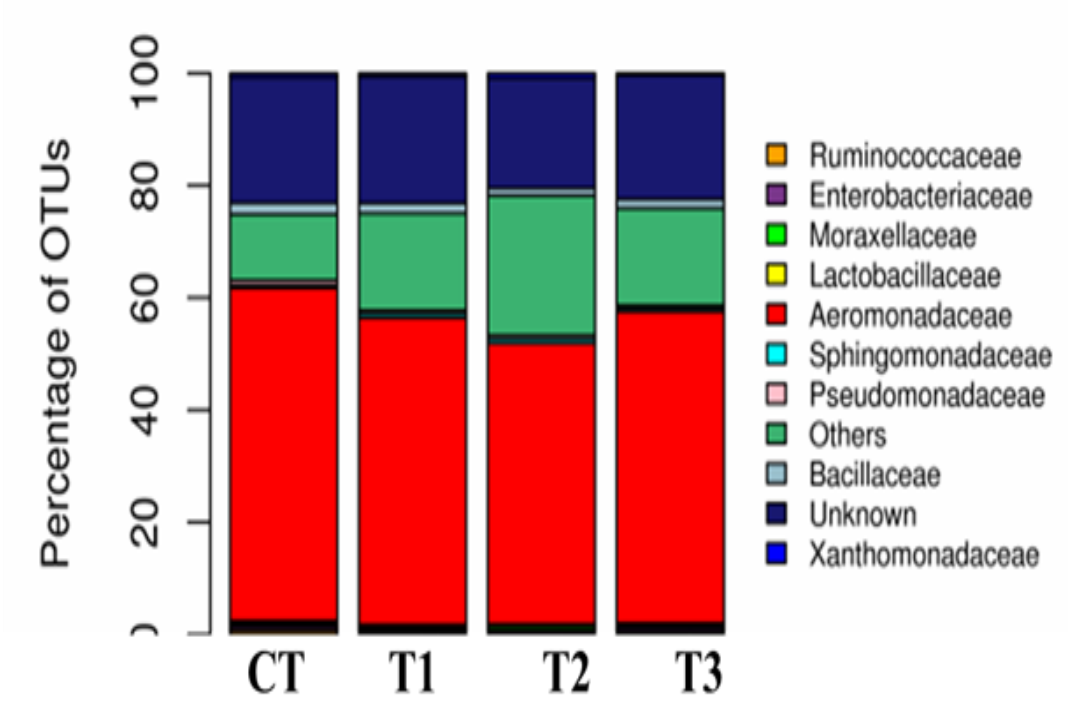


Fig 50. Comparative gut microbiome of *L. rohita* in indoor feeding trial

Dendrogram showed two distinct clusters cluster one has only T3 (0.23) whereas cluster two has two sub-clusters, in one sub-cluster CT and T1 (0.20) fall

and another cluster is made by T2 (0.21) which is showing distinct pattern as compared to CT and T1 (Fig.51).

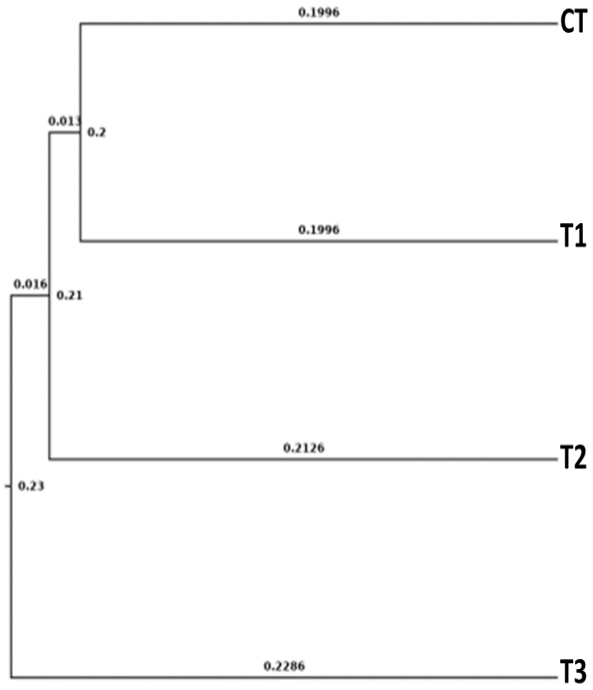


Fig. 51. Dendrogram showing association between gut microbiome of *L. rohita* in indoor feeding trial

4.8.1.9. Histo-architectural changes

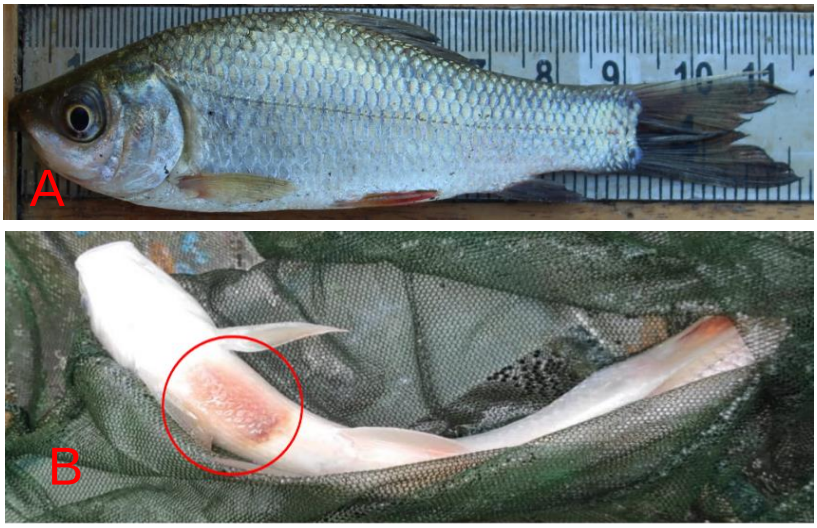
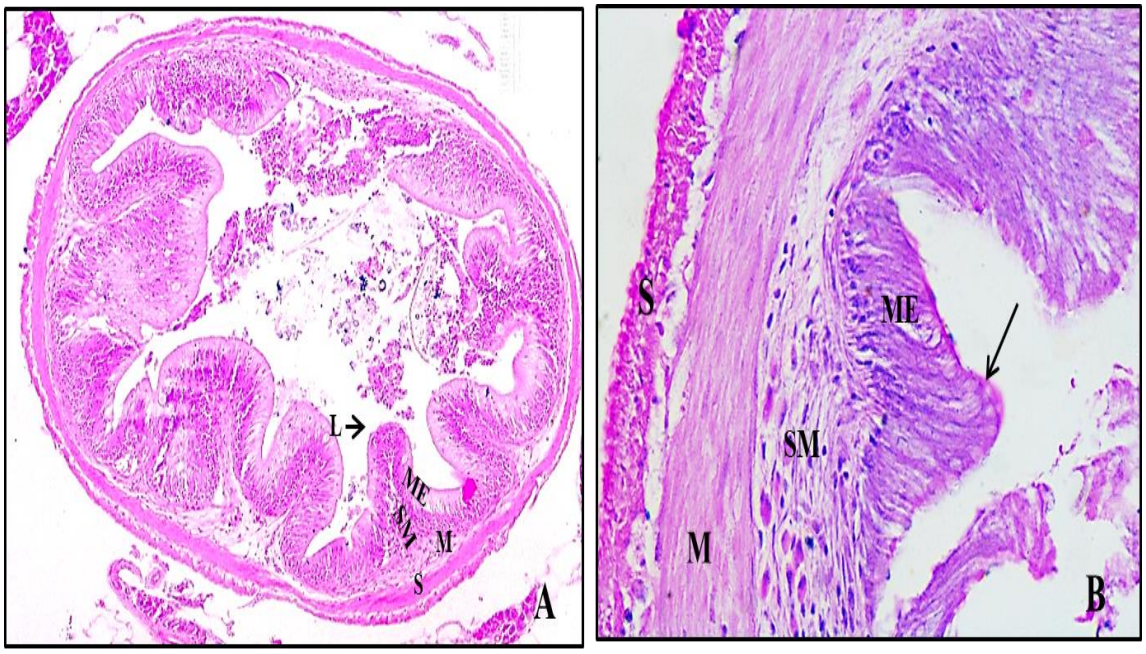
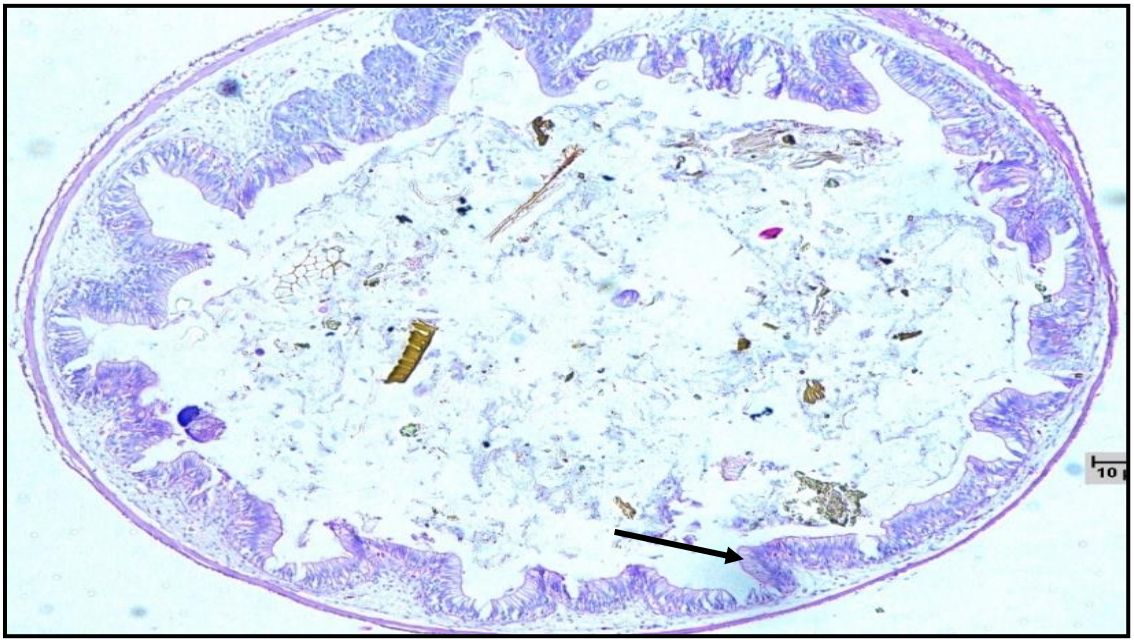


Fig. 52. Showing A) control and B) *A. hydrophila* infected *L. rohita*

PLATE



Photomicrograph of CT intestine (mid gut) showed its characteristic layers (A -10X) & (B-60X): mucosal epithelium (ME), submucosa (SM), muscularis (M), and serosa layers



Photomicrograph of T1 intestine (10X) showing sloughing of the mucosal epithelial layer of microvilli (black arrow)

Plate 8. Showing photomicrograph of intestine of CT and T1

PLATE 9

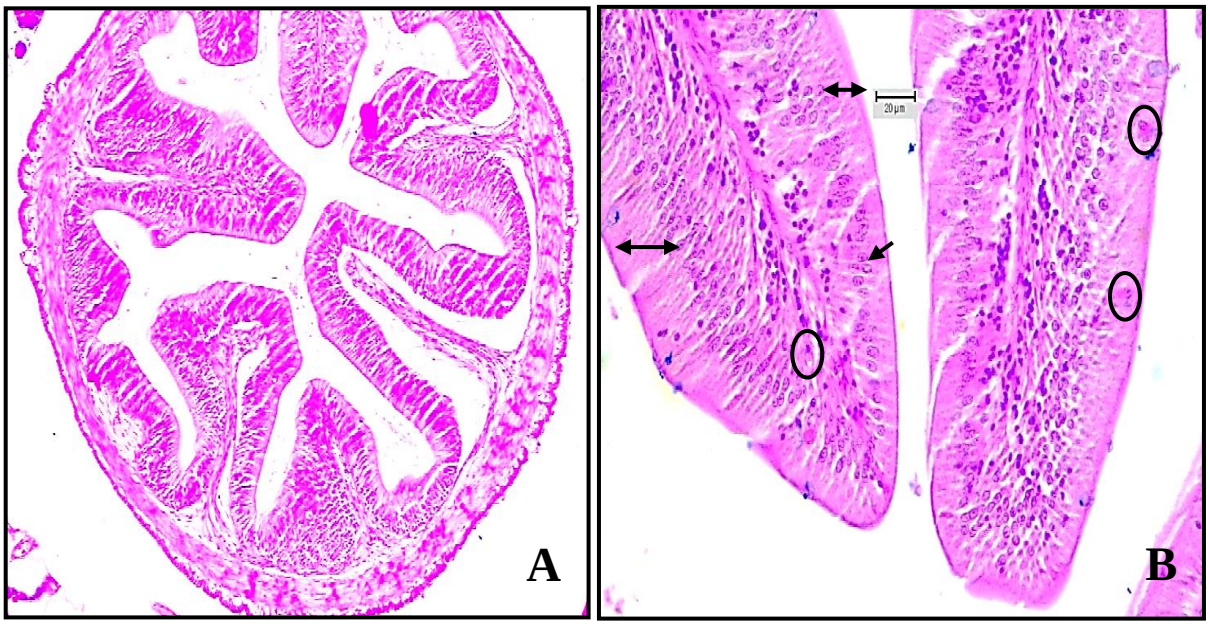
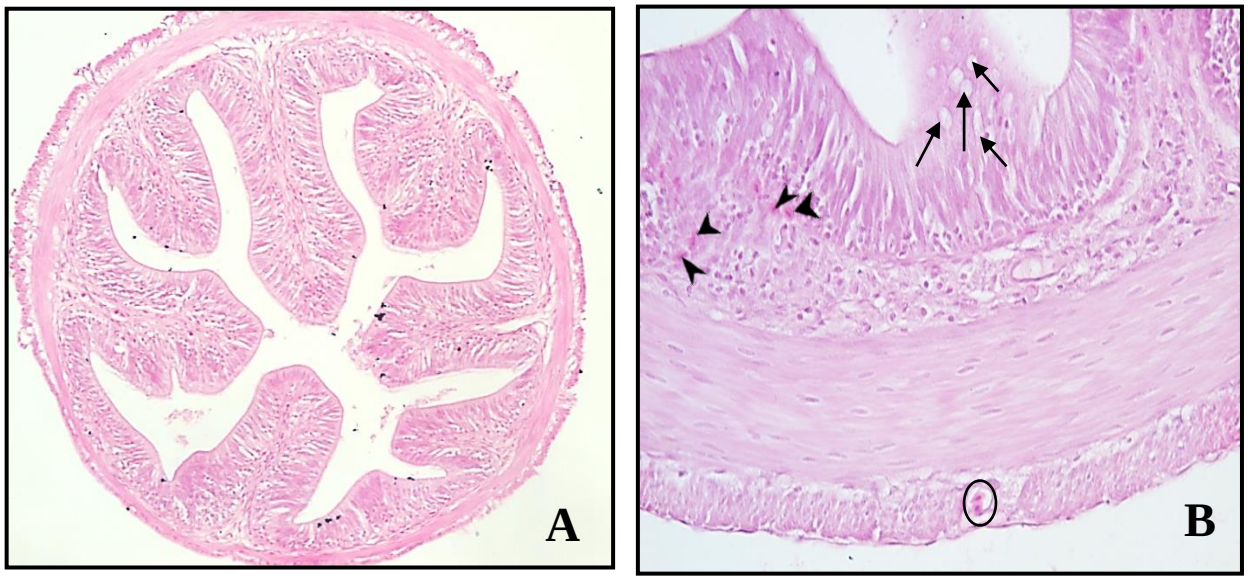


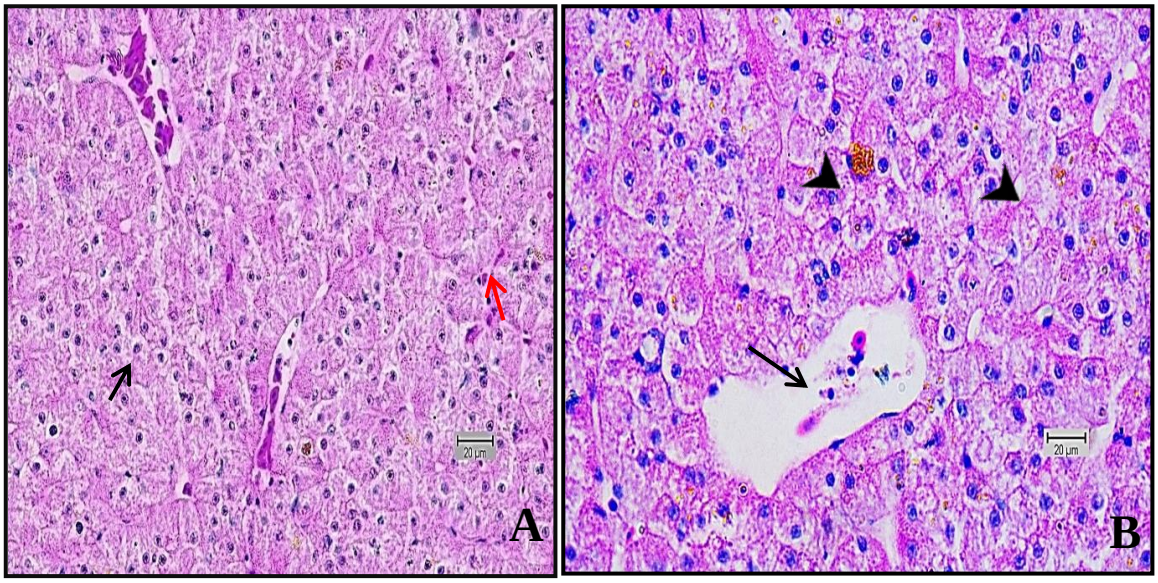
Photo micrograph of histological changes in T2 intestine (mid gut) (A-10X). It shows more mucosal folds and thickening of mucosal epithelial area (double arrow) than control (B-40X). Number of Eosinophilic granular cell is more than Control & treatment-1 and more number of goblet cells and presence of gastric gland (circle).



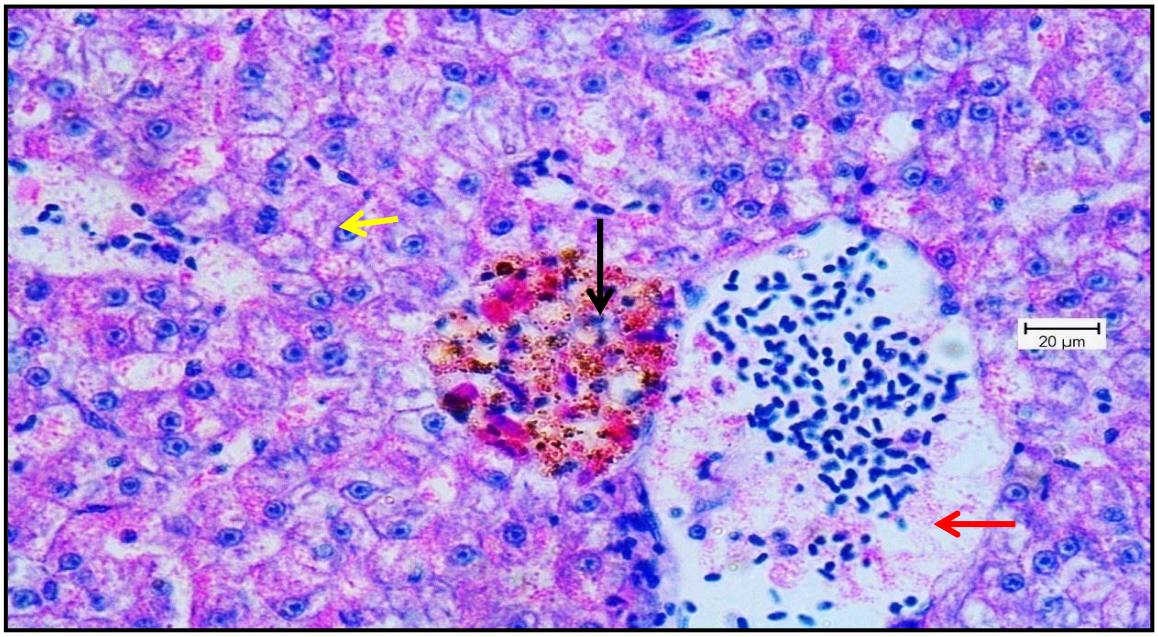
Photomicrograph of histological changes in T3 intestine (mid gut) (A-10X). It shows more mucosal folds & thick mucosal epithelium area (B-40X) Comparatively more eosinophilic granular cells (EGC) accumulation (Arrow head) in the lamia propia, necrosis of lamellar epithelium & more mucous cell proliferation (arrow); height of the mucosal fold is lesser than T2.

Plate 9. Showing photomicrograph of intestine of T2 and T3 groups

PLATE 10



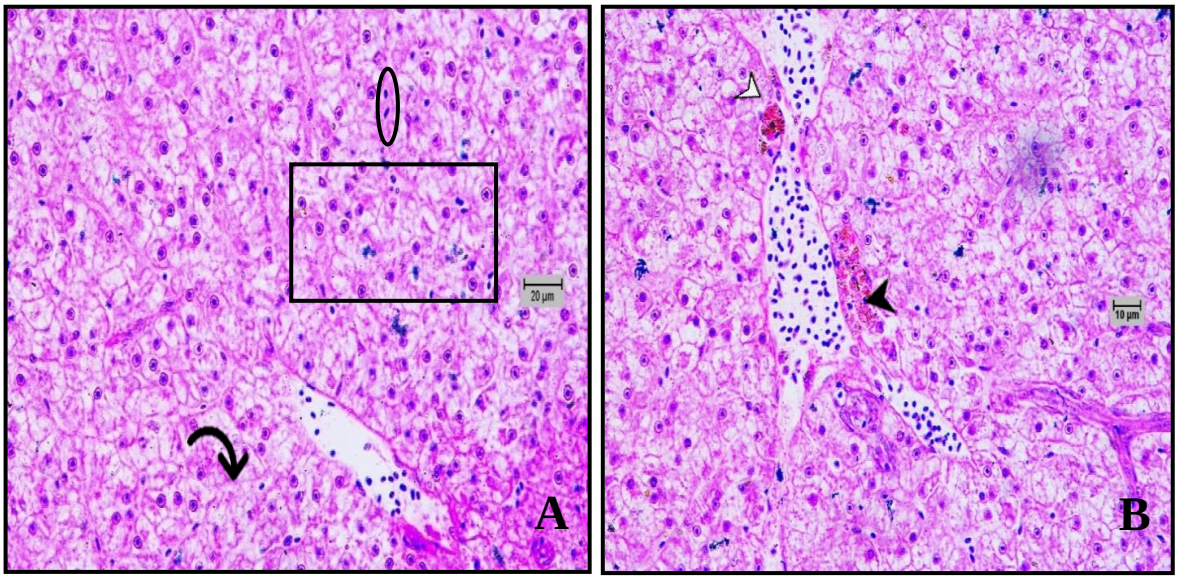
Photomicrograph of CT liver showing (A-10X) Norrmal organization of polygonal hepatocytes (black arrow), sagittal section of sinusoidal capillary (red arrow), (B-40X) Magnified view shows central vein (black arrow), pigmentation (arrow head).



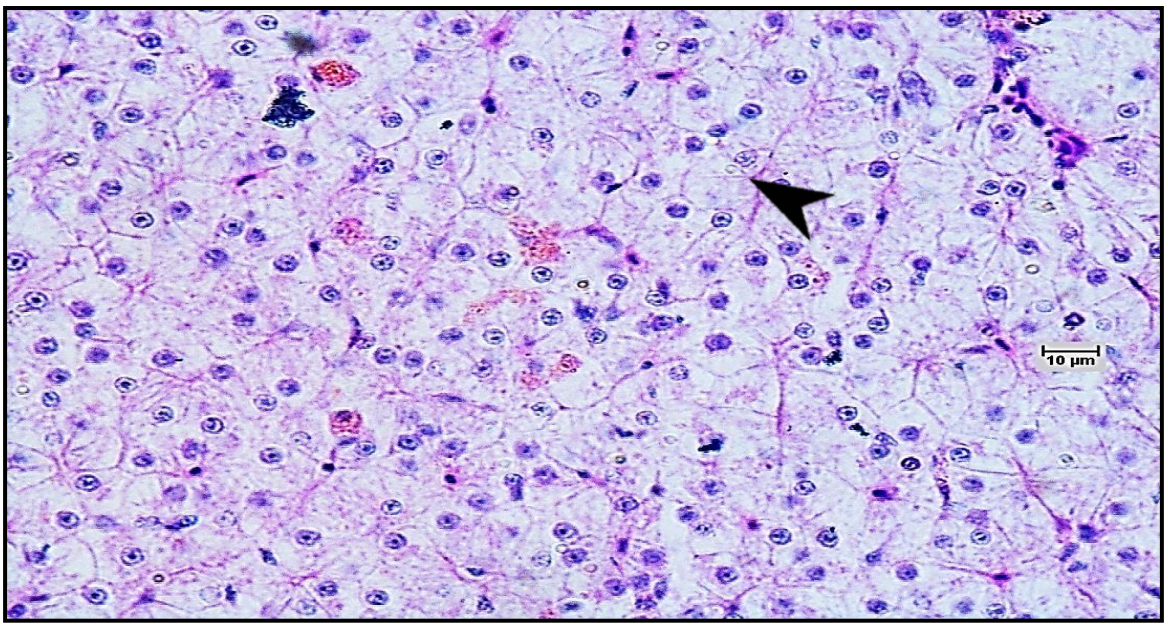
Photomicrograph of T1 liver (60X) showing fully matured melano macrophage (black arrow), Central vein (red arrow) & comparatively less sinusoidal capillary (yellow

Plate 10. Showing photomicrograph of intestine of CT and T1

PLATE 11



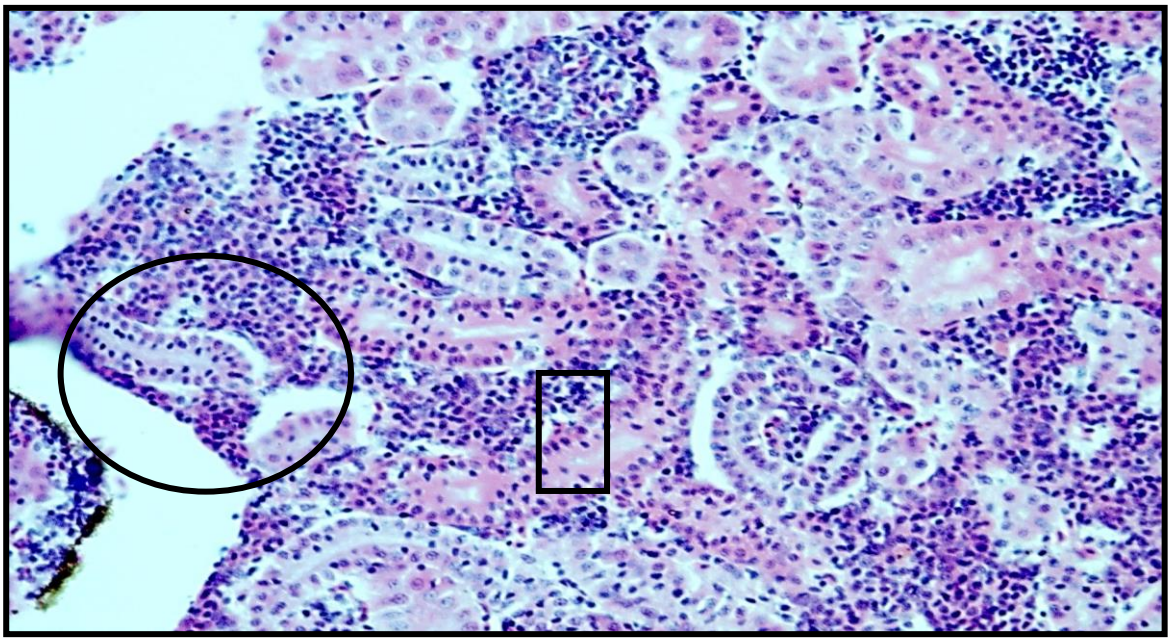
Photomicrograph of T2 liver shows (A-10X) Mild increase in the size of hepatocyte (curved arrow), maximum normal hepatocyte with less cellular presence in the sinusoidal capillaries (Oval), healthy hepatocytes with glycogen vacuolisation (Rectangle), (B-40X) Eosinophilic granular cells (Blank arrow head) & melanomacrophages aggregation (black arrow head)



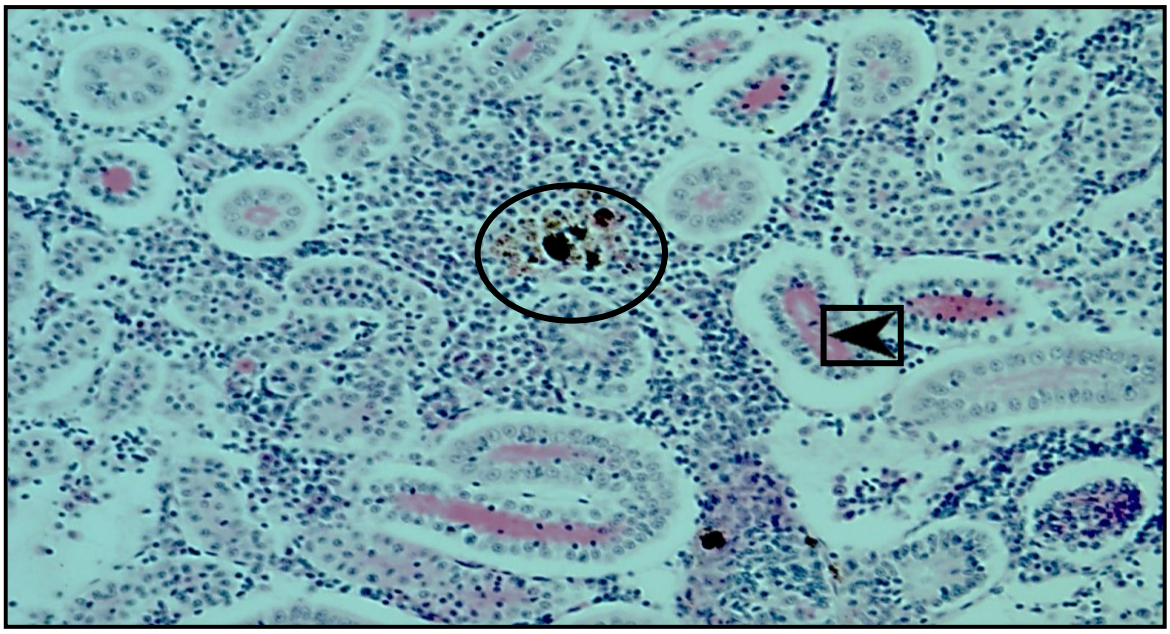
Photomicrograph of T3 liver (60X) showing fibrosis with fat deposits & narrowing of sinusoidal capillaries (arrow head)

Plate 11. Showing photomicrograh of liver of T2 and T3

PLATE 12



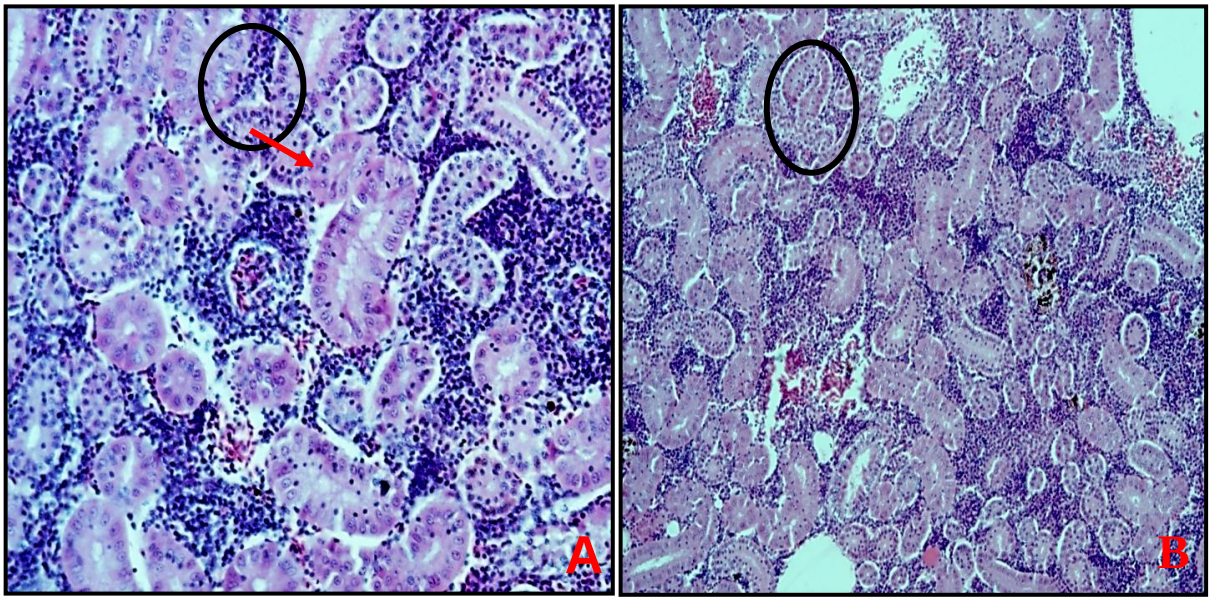
Photomicrograph of transverse section of CT group kidney (40X) showing various sized tubular lumens with normal haematopoietic tissue (rectangle)



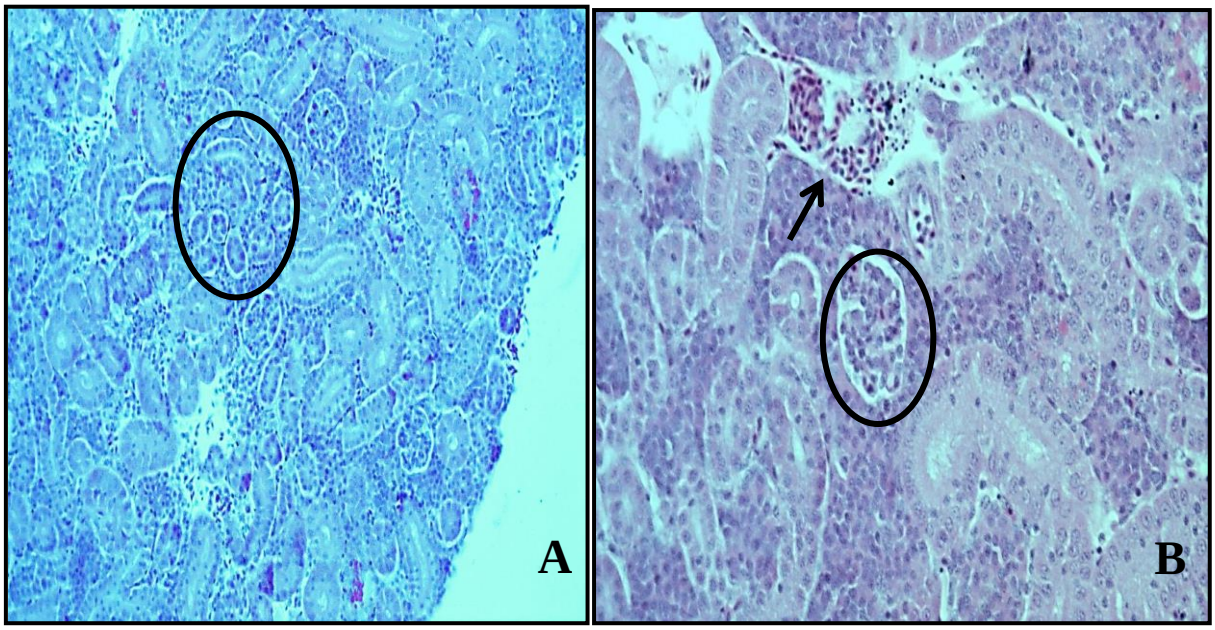
Photomicrograph of transverse section of T1 kidney (40X) showing irregular brush border structure in different tubular lumens (arrow head) with expression of melanin pigmentation (circle) further resulting into matured MMC

Plate 12. Showing photomicrograph of transverse section of kidney of CT and T1 groups

PLATE 13



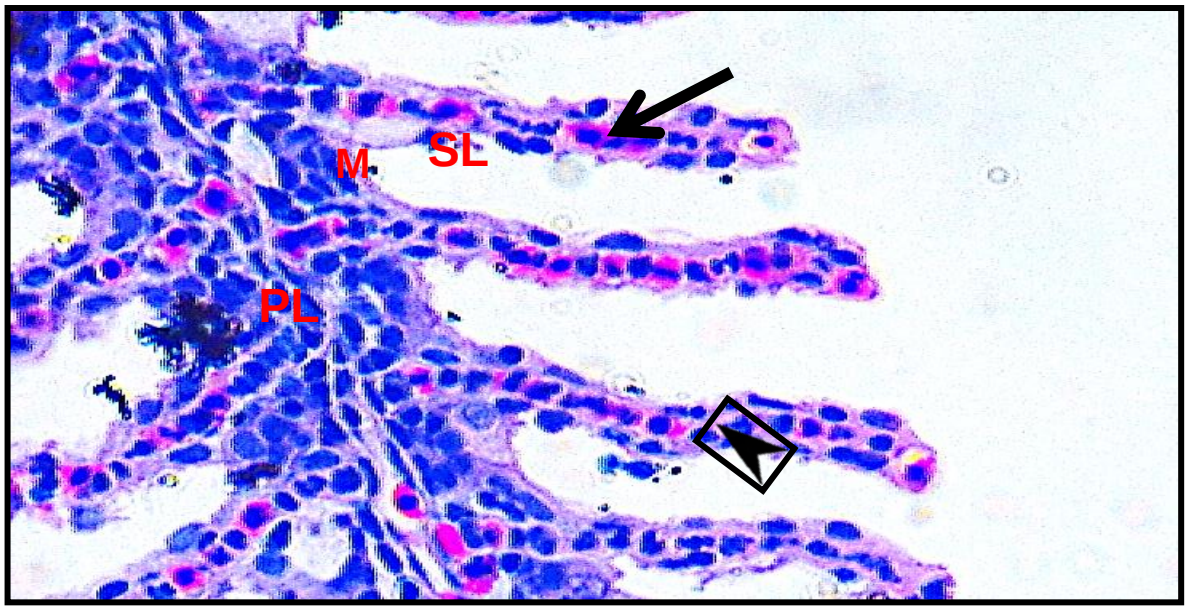
Photomicrograph of transverse section of T2 kidney showing (A-40X) unaltered tubular lumen with moderate alteration in cell types. Melanomacrophage aggregation (red arrow) (B-20X) tubular lumen alteration in structure (round)



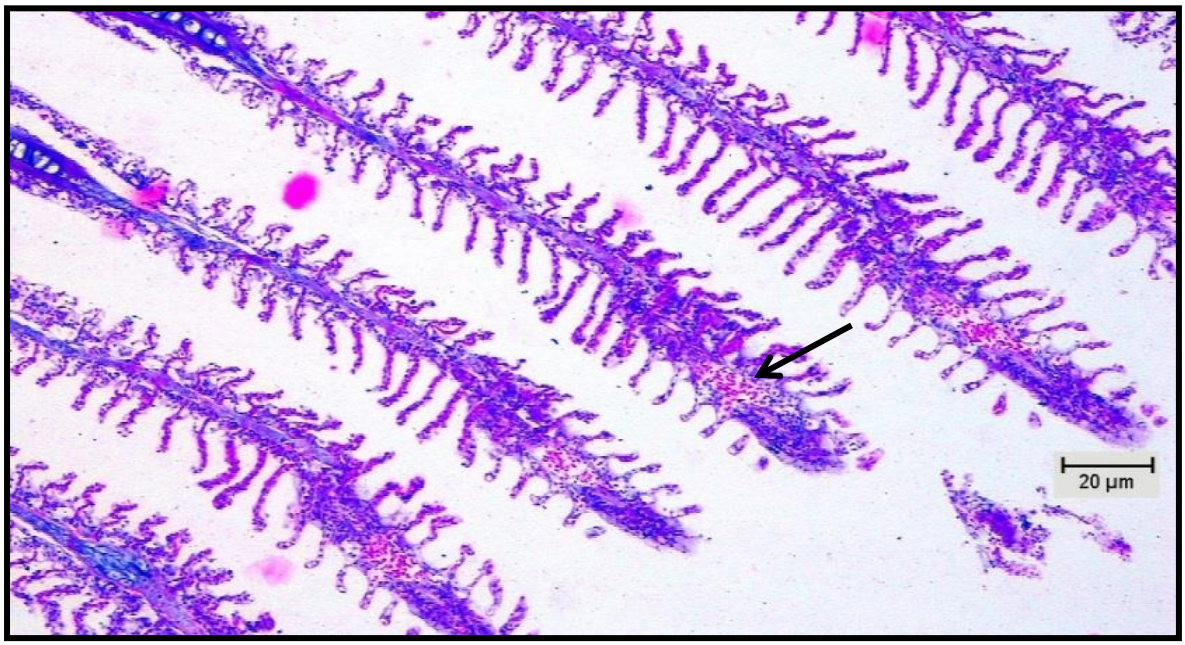
Photomicrograph of transverse section of T3 kidney showing (A-40X) Lumen necrosis and narrow luminar tubules (round in A& B), (B-60X) Infiltration of monocytes (arrow)

Plate 13. Showing photomicrograph of transverse section of Kidney of T2 and T3 groups

PLATE 14



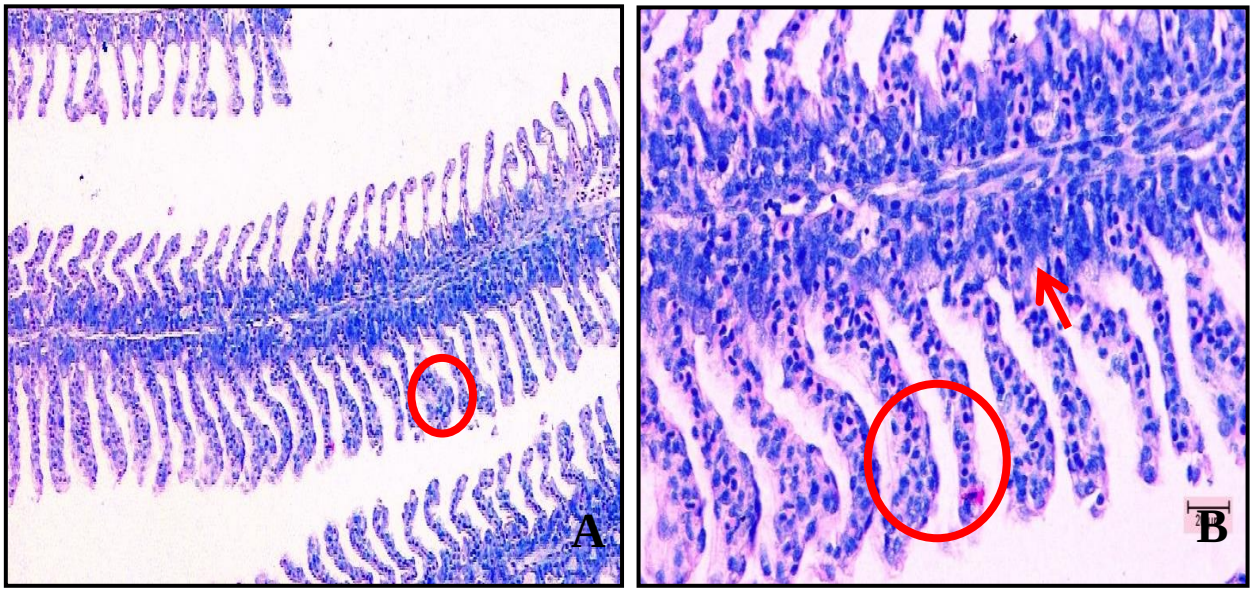
Photomicrograph of gill histology of CT group (60X), showing its normal architecture with primary lamellae (PL), secondary lamellae (SL), mucous cell (M), pillar cell (Arrow head), erythrocyte within capillary lumen (long arrow)



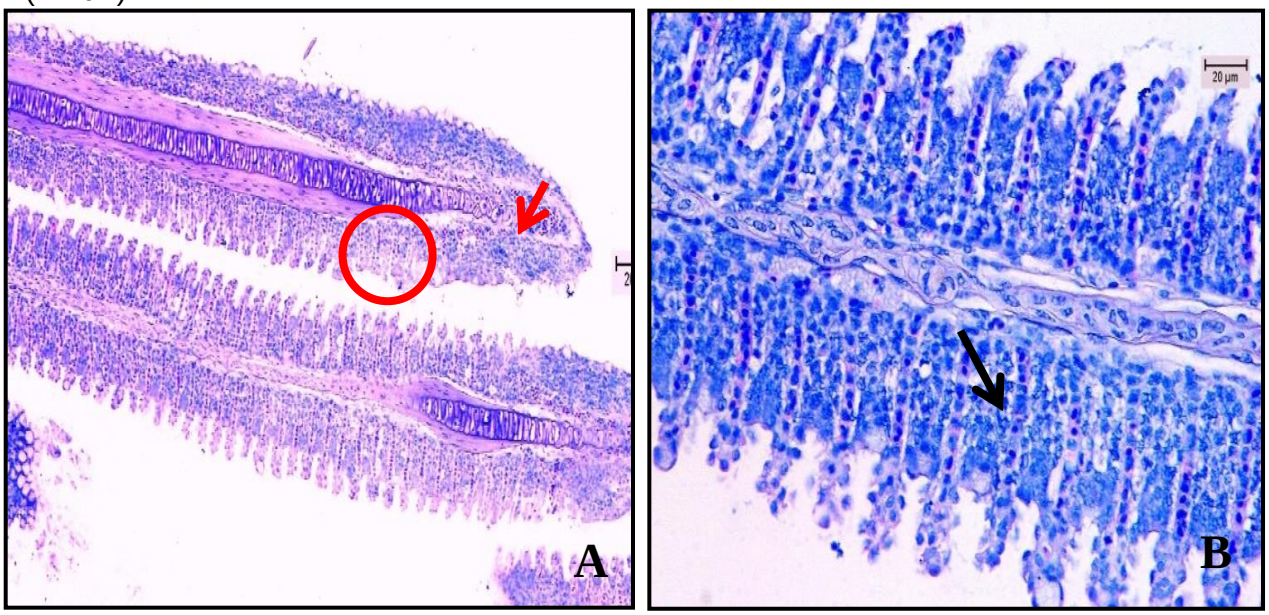
Photomicrograph of gill histology of T1 showing (40X) normal secondary lamellae with erythrocytes aggregation at the tip of the lamellae (arrow)

Plate 14. Showing photomicrograph of gill of CT and T1 groups

PLATE 15



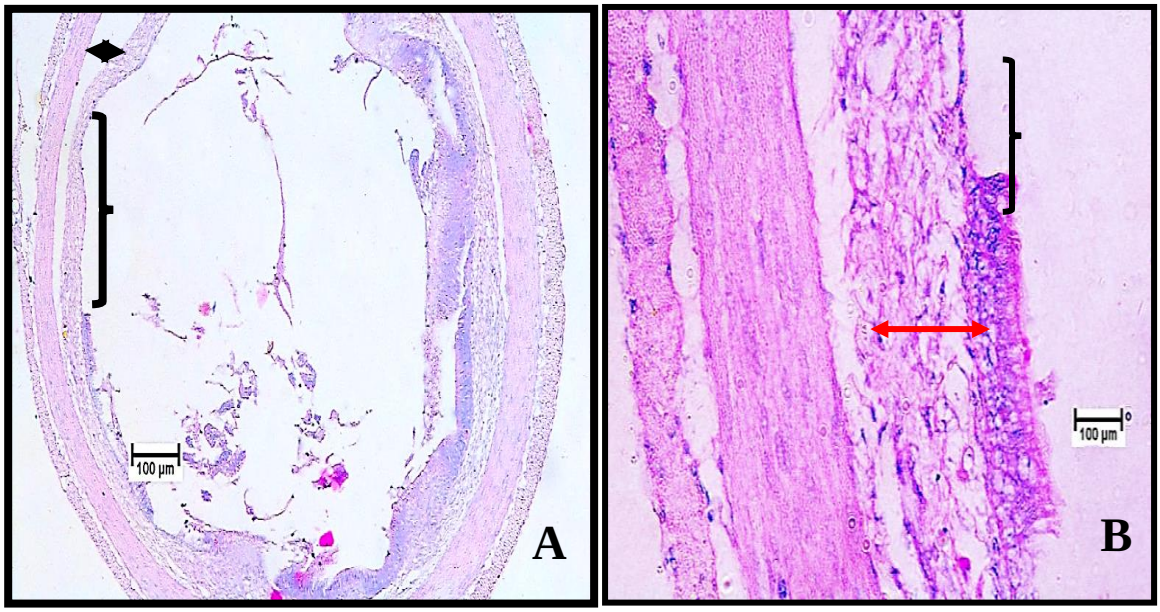
Photomicrograph of gill histology of T2 showing secondary lamellae thickening, erythrocytes (A-10X) & pillar cell proliferation and fusion at the base of the secondary lamellae red arrow (B-40X)



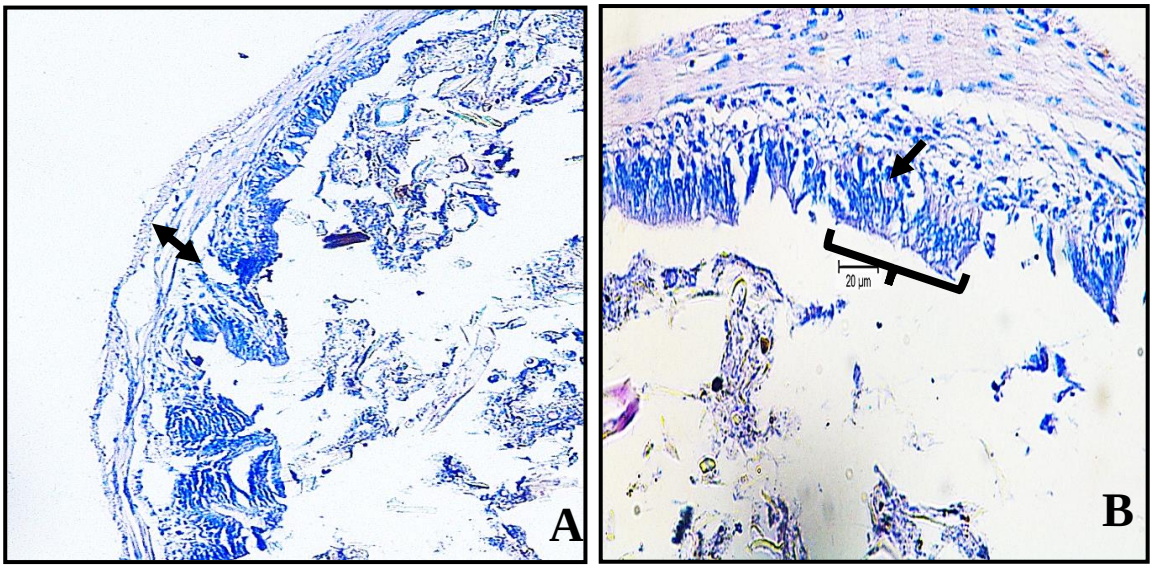
Photomicrograph of histology of gill of T3 showing (A-10X) & (B-40X) fusion of the secondary lamellae (red circle), erythrocytes (red arrow) and mononuclear cell proliferation (black arrow)

Plate 15. Showing photomicrograph of gill of T2 and T3 groups

CHALLENGE STUDY- PLATE 16



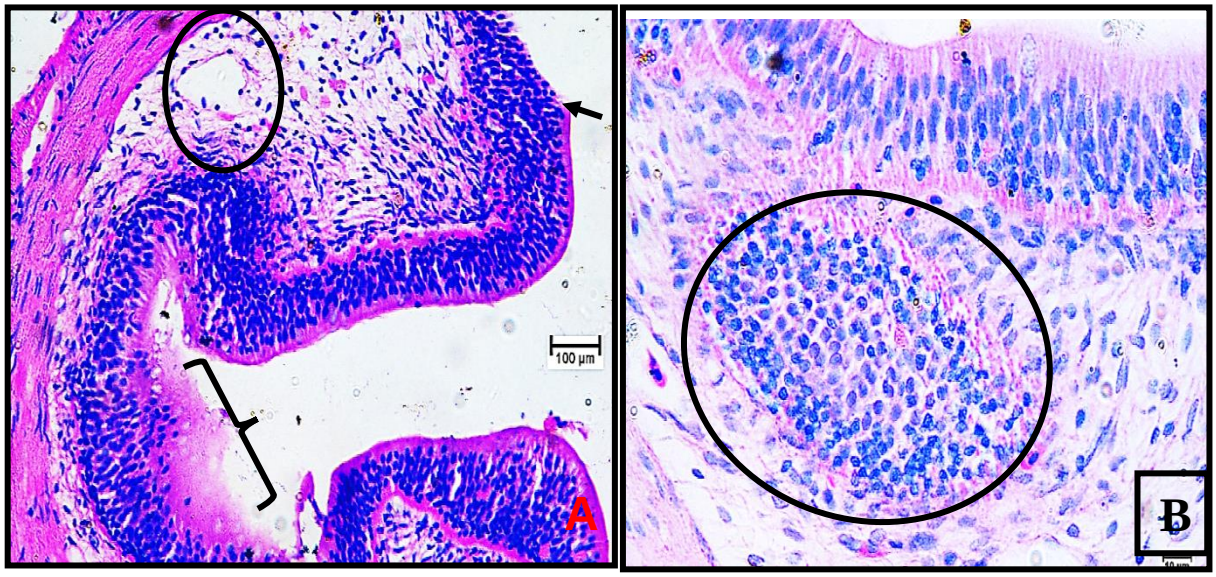
Photomicrgraph of transverse section of fish intestine (mid gut) of CT experimentally challenged with *Aeromonas hydrophila* (A) showing complete necrosis of Mucosal epithelial layer (right brace A-10X & B-60X) & detachment of submucosal layer from muscularis (arrow double A) (B) Magnified view shows necrosis in submucosal layer (arrow double)



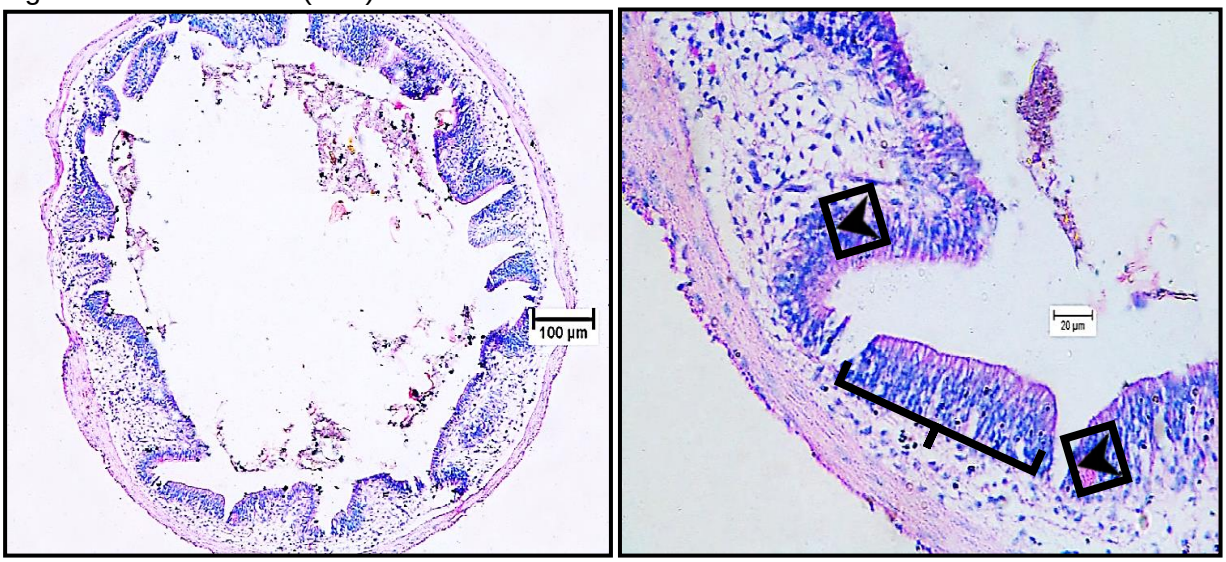
Photomicrograph of transverse section of T1 intestine (mid gut) experimentally challenged with *A. hydrophila* (A-40X) moderate necrosis in muscularis, submuscularis, but the architecture is maintained in comparison of control (double arrow) (B-60X) moderate necrosis of microvilli (right brace) & expression of EGC (arrow)

Plate 16. Showing photomicrograph of transverse section of intestine of CT and T1 groups of challenged with *A. hydrophila*

PLATE 17



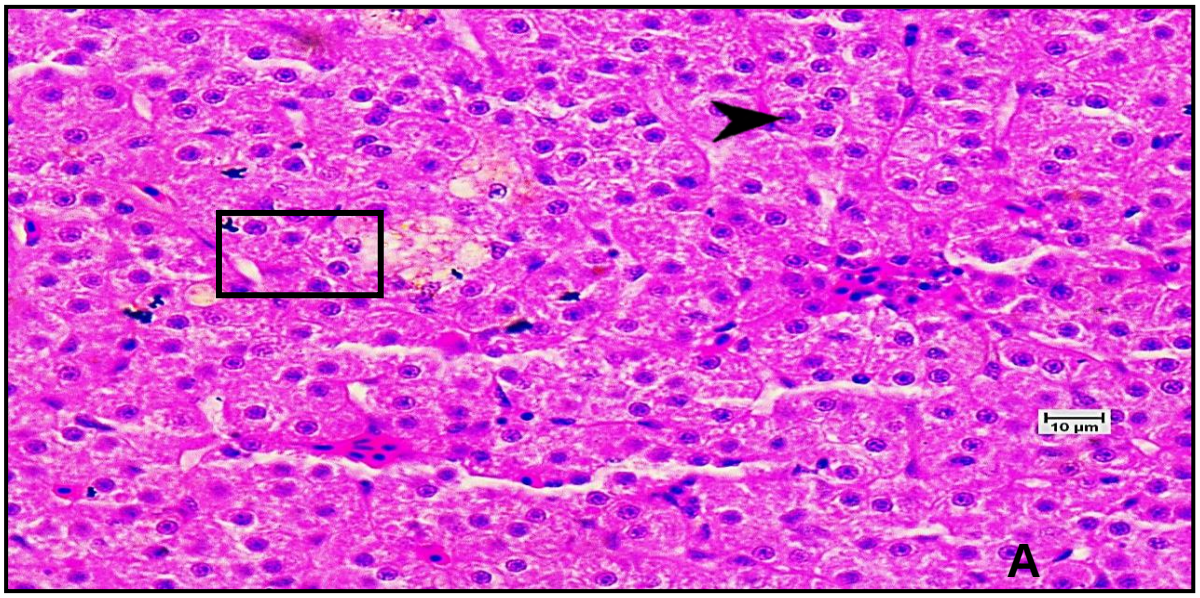
Photomicrograph of transverse section of T2 intestine (mid gut) experimentally challenged with *A. hydrophila* shows (A-40X) intact microvilli with mild necrosis (right brace) & focal necrosis (round) (B-60X) aggregation of monocytes & erythrocytes encysting for granuloma formation (oval)



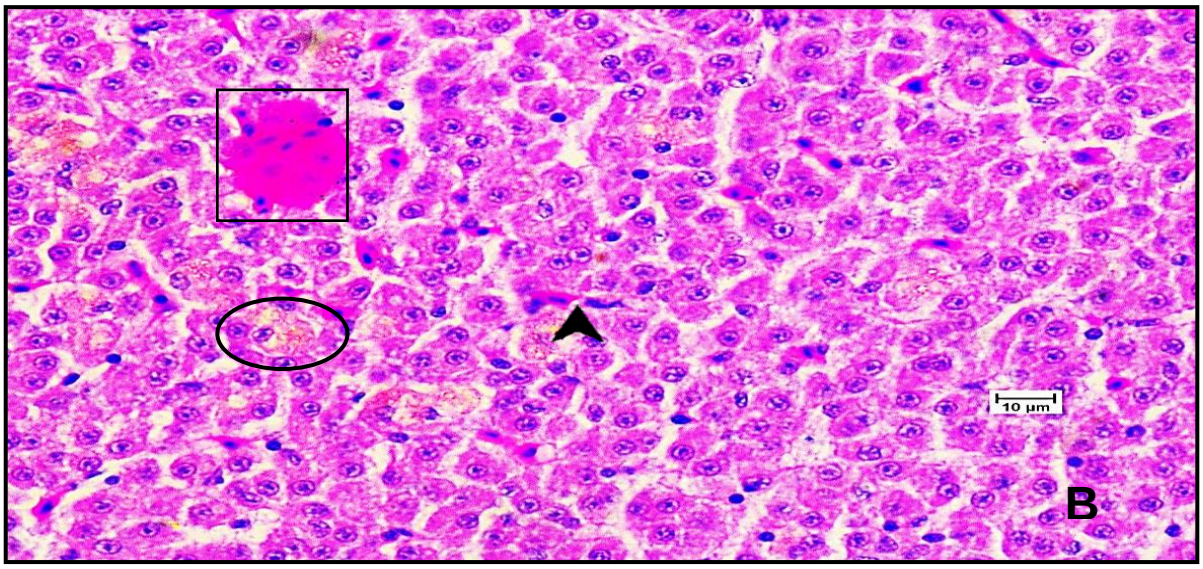
Photomicrograph of transverse section of T3 intestine (mid gut) experimentally challenged with *A. hydrophila* shows (A-10X) Less necrosis of microvilli as compare to CT & T1, (B-40X). Expression of EGC and hypertrophoid goblet cell expression (Arrow head)

Plate 17. Showing photomicrograph of transverse section of intestine of T2 and T3 groups challenged with *A. hydrophila*

PLATE 18



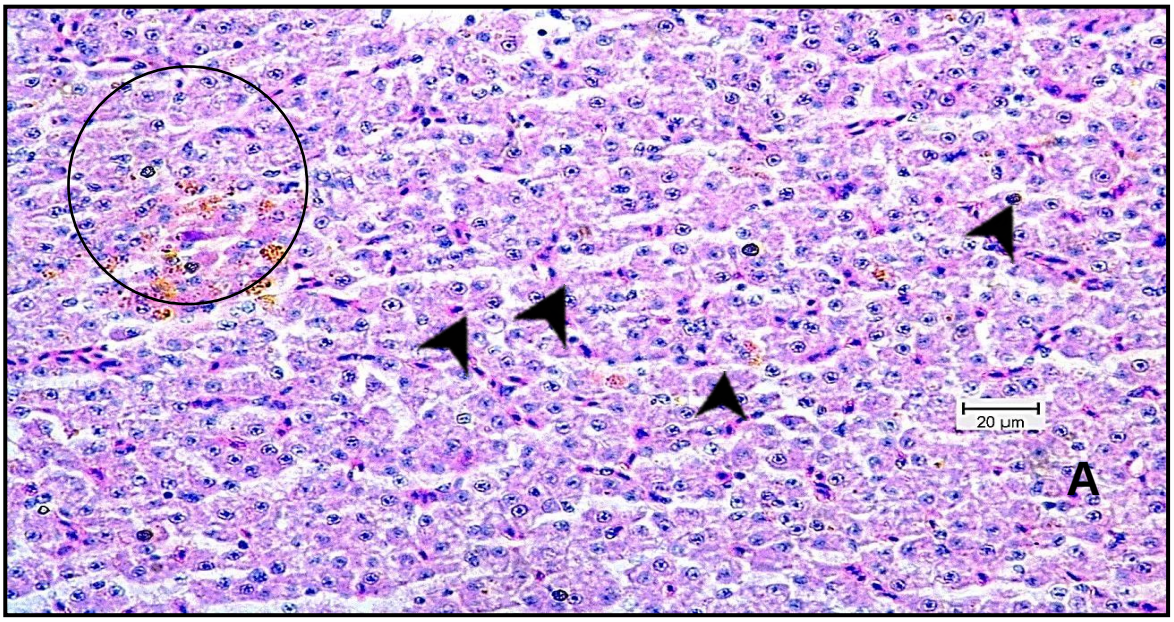
Photomicrograph of histology of CT liver (A-60X) experimentally challenged with *A. hydrophila showing* multifocal necrosis(square), inflammatory cellular aggregation & narrowing of sinusoidal channel (arrow head)



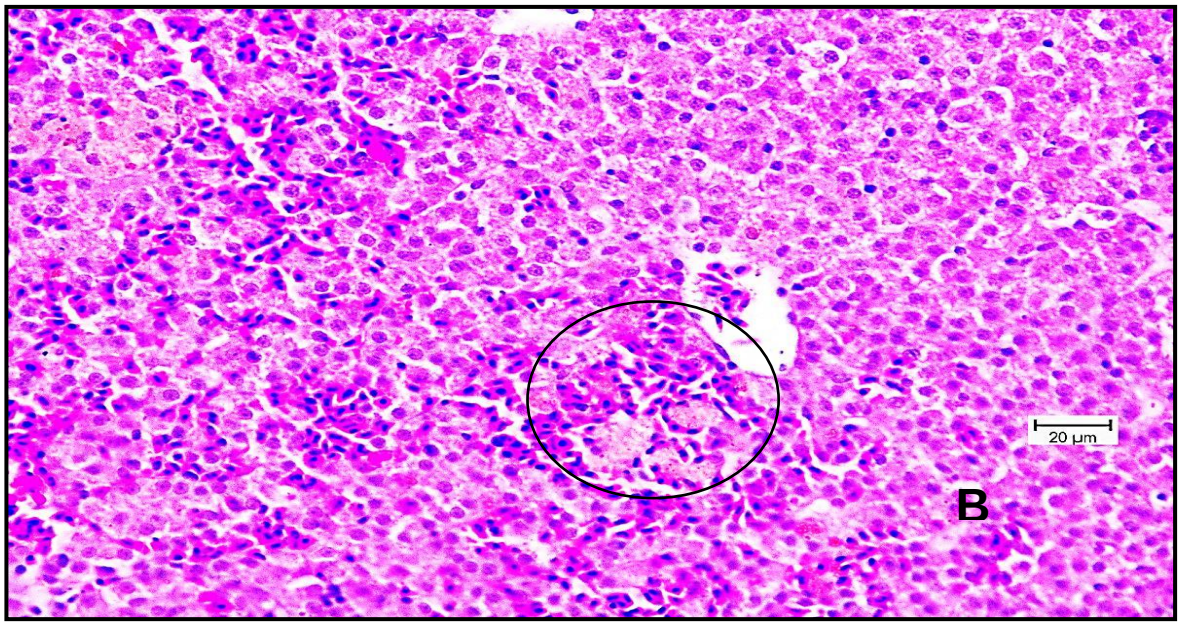
Photomicrograph of histology of T1 liver (B-60X) experimentally challenged with *A. hydrophila* showing expression of inflammatory cellular responses (square), necrosis of hepatocytes(arrow), less prominent sinusoidal channel (arrow head), expression of pigmentation (oval)

Plate 18. Photomicrograph of liver of CT and T1 groups challenged with *A. hydrophila*

PLATE 19



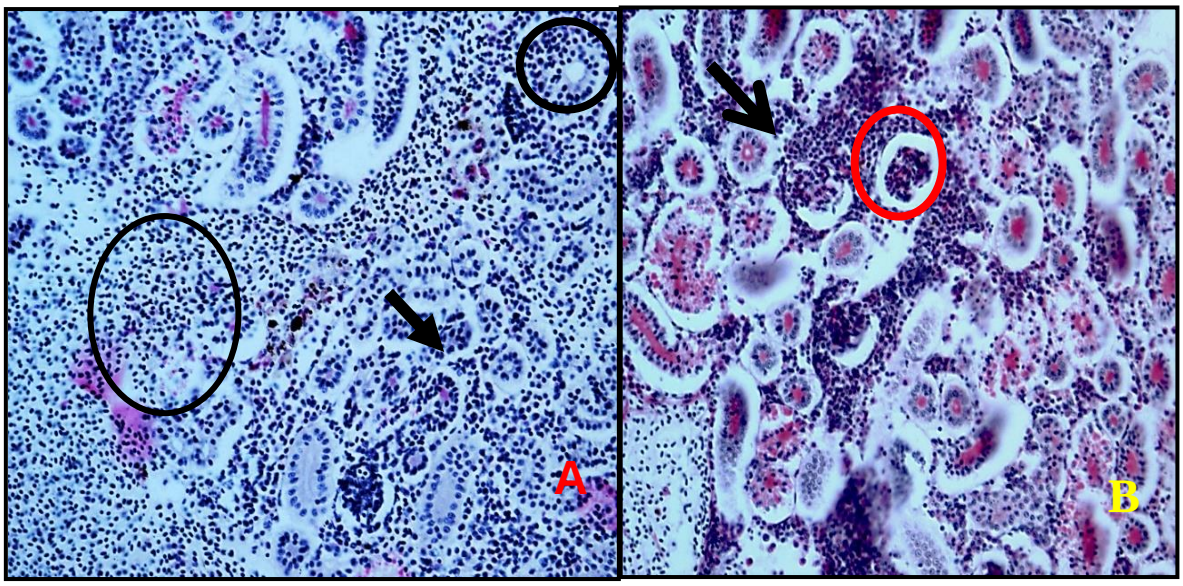
Photomicrograph of T2 liver (A-40X) experimentally challenged with *Aeromonas hydrophila* showing Prominent melanin pigmentation expression (round), normal polygonal hepatocytes & Prominent sinusoidal channel (arrow head)



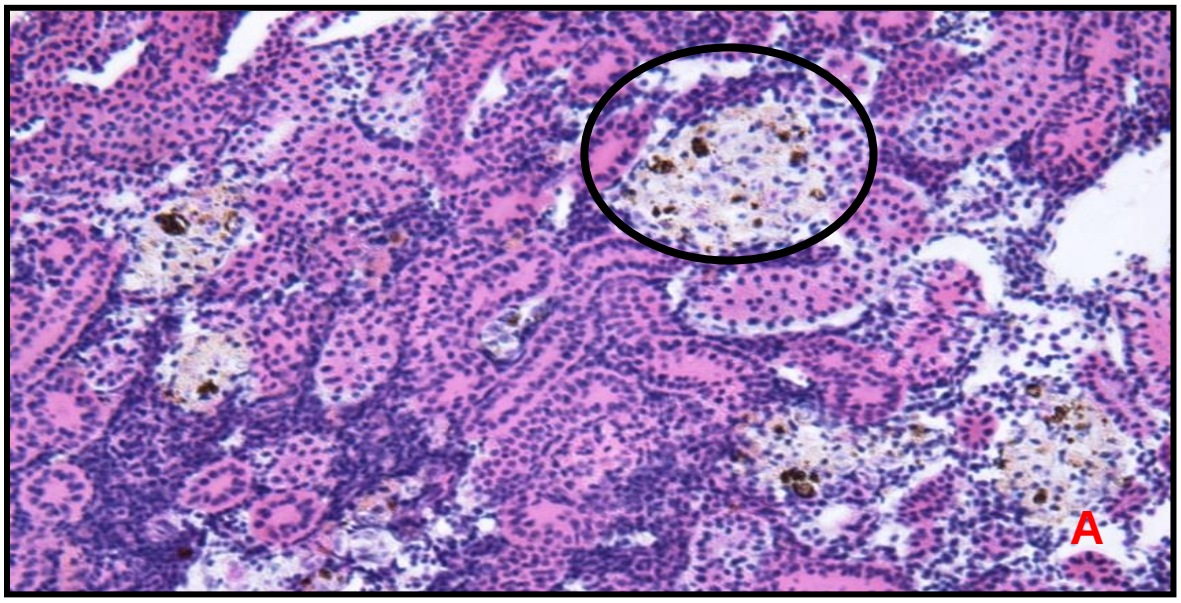
Photomicrograph of T3 liver (B-40X) experimentally challenged with *Aeromonas hydrophila showing* larger inflammatory responses in the form of a cellular aggregation surrounding the severely necrotic hepatocytes (round)

Plate 19. Showing photomicrograph of liver of T2 and T3 groups challenged with *A. hydrophila*

PLATE 20



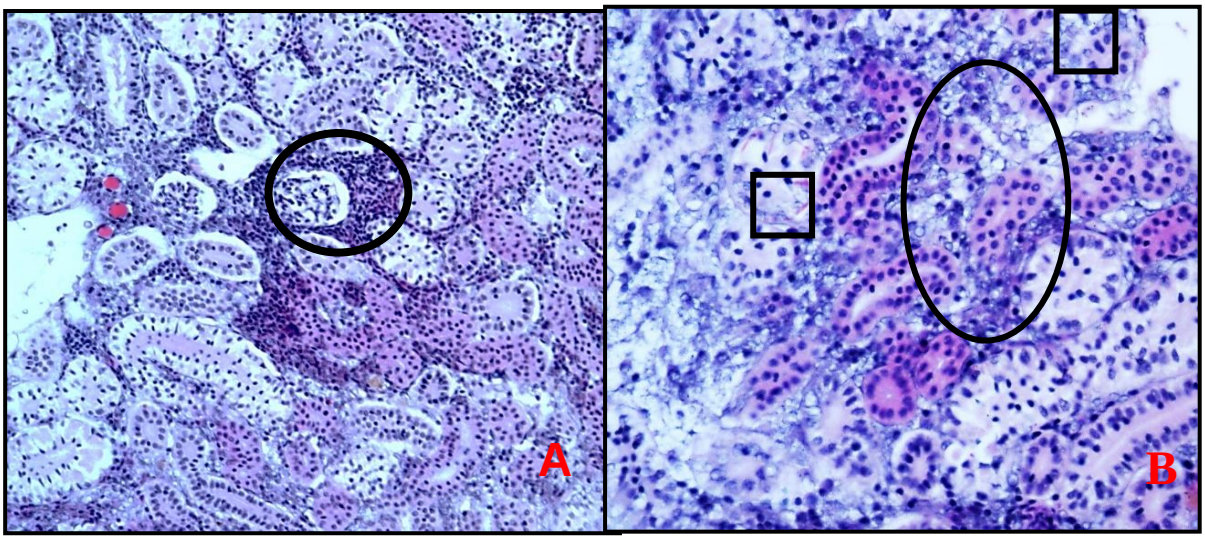
Photomicrograph of histology of CT kidney experimentally challenged with *A. hydrophila* showing (A-40X) Complete necrosis of proximal & distal tubules (circle), haemopoietic tissue necrosis (Arrow in A, B) & (B-60X) Compression of brush borders (round) and haemorrhages



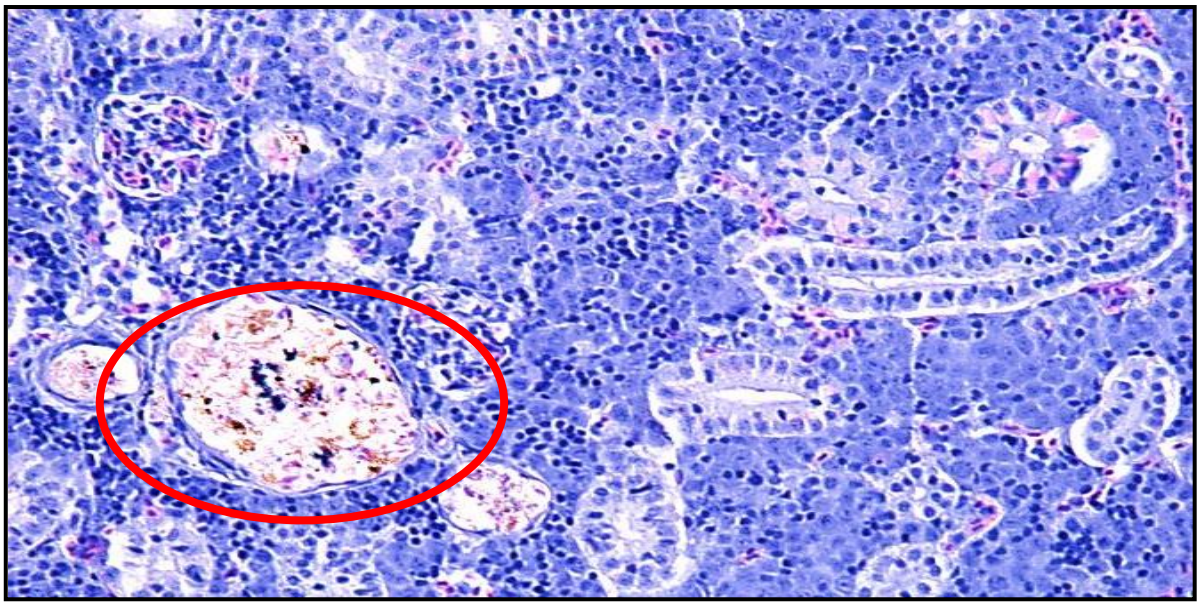
Photomicrograph of histology of kidney of T1 experimentally challenged with *A. hydrophila* (A-60X) showing expression of melanomacrophages centres (circle) with extensive inflammatory cell aggregation and intensity of necrosis is less than CT

Plate 20. Showing photomicrograph of kidney of T1 challenged with *A. hydrophila*

PLATE 21



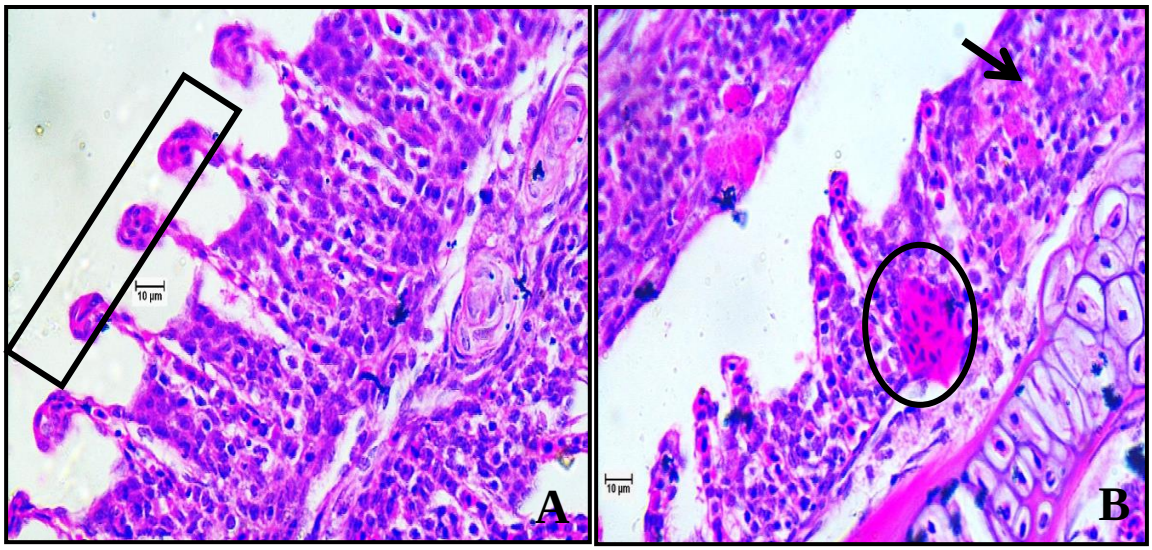
Photomicrograph of histology of T2 kidney experimentally challenged with *A. hydrophila* Showing (A-40X) Distal tubular necrosis with macrophage aggregation (oval) (B-60X) negligible necrosis in proximal & distal tubules(oval), vacuolar degeneration of haemopoietic tissue (square), Haemopoietic tissue necrosis (arrow)



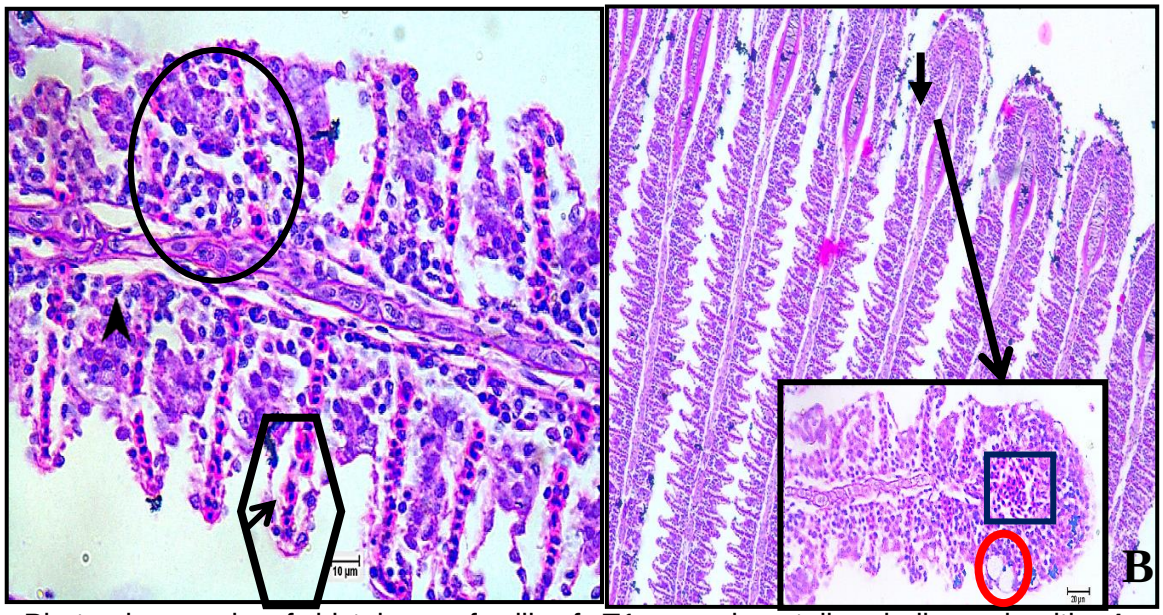
Photomicrograph of histology of T3 kidney (60X) experimentally challenged with *A. hydrophila* showing monocytes, MMC proliferation, inflammatory cells massive infiltration and severe necrosis in proximal & distal tubules

Plate 21. Showing photomicrograph of Kidney of T2 and T3 challenged with *A. hydrophila*

PLATE 22



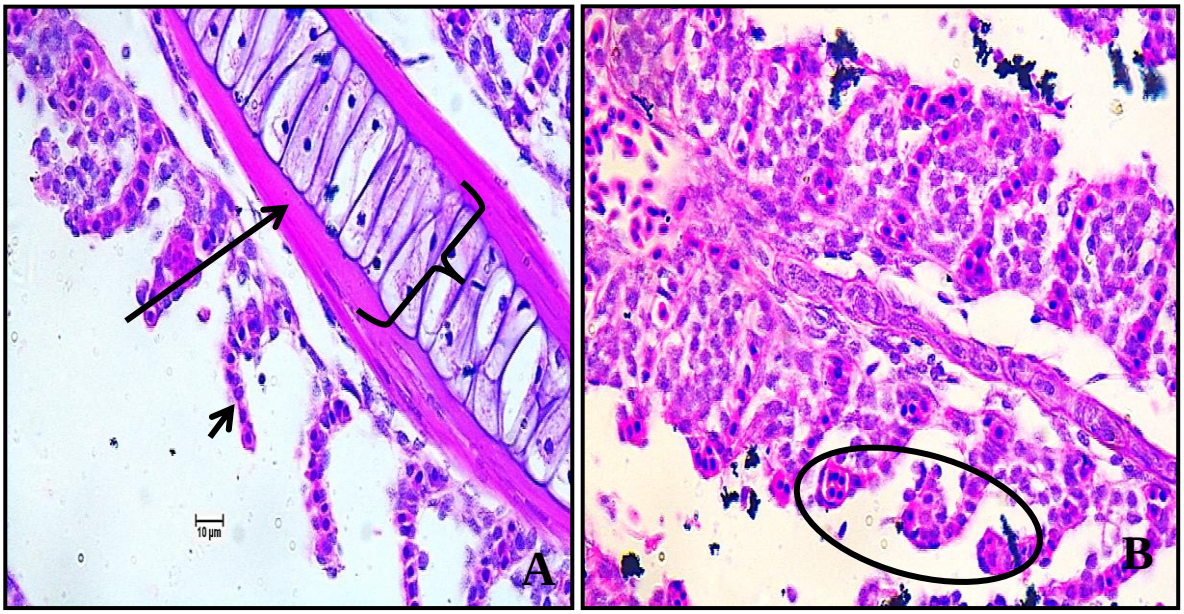
Photomicrograph of histology of gill of CT experimentally challenged with *Aeromonas hydrophila* showing (A-40X) telangiectasis (Rectangle) lesions characterized by focal, blood filled(aneurysmal) distension via lamellar thrombosis (B-60X) hemorrhages (round) with mononuclear cell aggregation, diffuse lamellar fusion (arrow)with mononuclear inflammatory cell infiltration



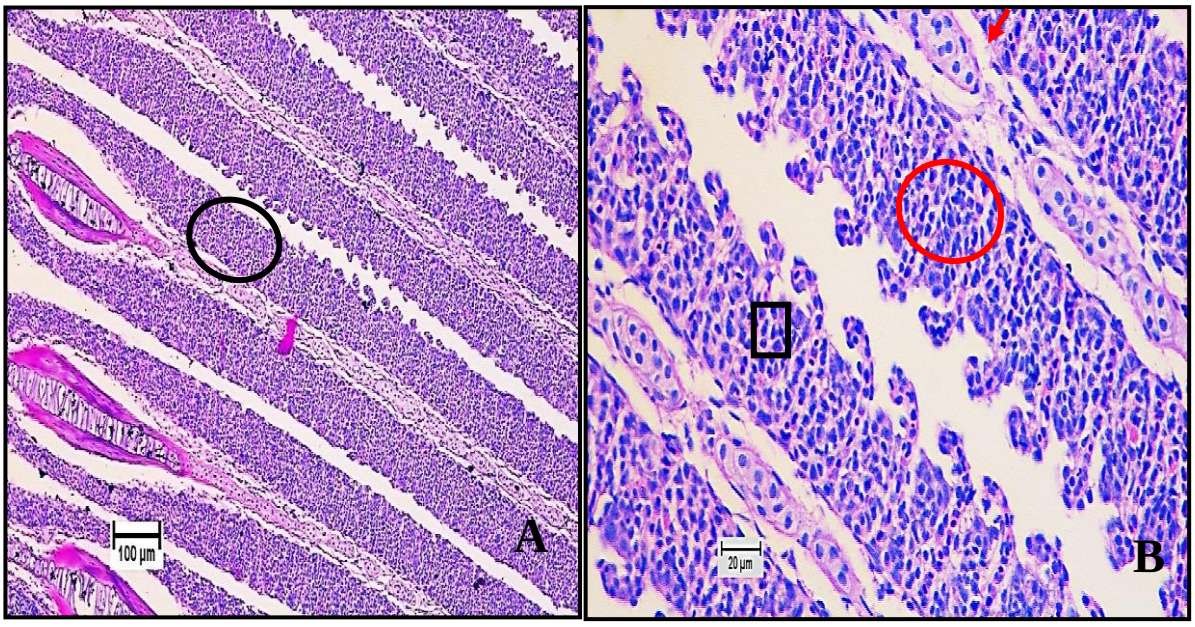
Photomicrograph of histology of gill of T1 experimentally challenged with *A. hydrophila* showing (A-40X) mild lamellar edema (Hexagon)with flocculent material(arrow within hexagon) within the swollen space , thinning of cartilaginous matrix(arrow head), inter lamellar fusion(circle), (B-10X) diffused secondary lamellar fusion(small arrow),hyperplasia and hypertrophy of goblet cells within tip of the lamellae(red circle inset),mononuclear erythrocytic aggregation (square) at the tip of the diffused secondary lamellae .

Plate 22. Showing photomicrograph of gill of CT and T1 challenged with *A. hydrophila*

PLATE 23



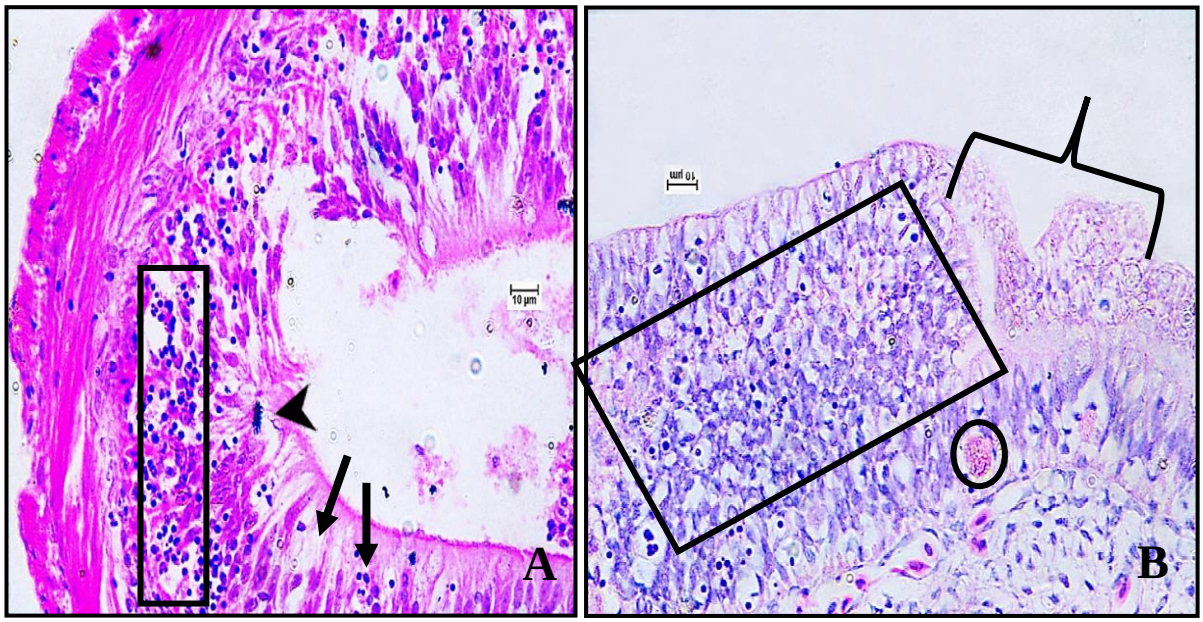
Photomicrograph of histology of gill of T2 experimentally challenged with *A. hydrophila showing* (A-10X) detachment of secondary lamellae from the cartilaginous matrix (Long arrow), hypertrophoid erythrocytes and normal chondrocytes structure (bracket), comparatively healthy secondary lamellae (small arrow) with less inflammatory responses than control & treatment 1 (B-40X) Fusion of secondary lamella at the tip of the filament, negligible telangiectasis (oval) and thinning of primary lamellae



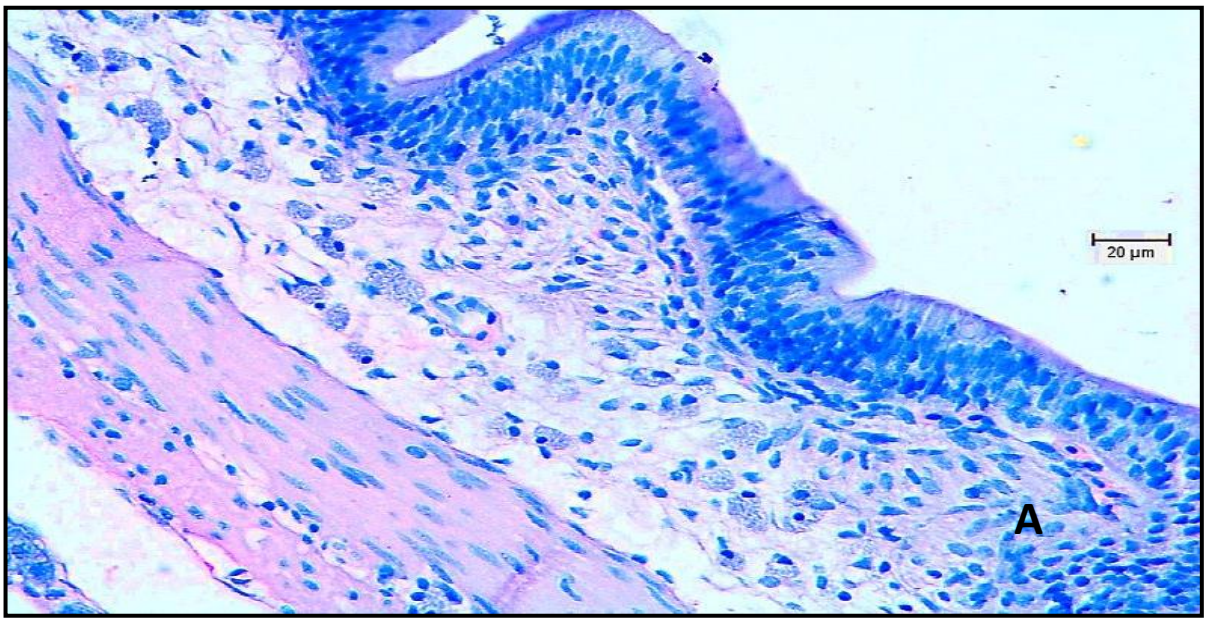
Photomicrograph of histology of gill of T3 experimentally challenged with *A. hydrophilla* showing (A-10X) massive fusion of secondary lamellae (oval), (B-40X) discontinuation of primary lamellae (red oval) with hyperplasial chondrocytes, severe lamellar epithelial hyperplasia (LEH, small arrow), telangiectasia with less mononuclear cell

Plate 23. Showing photomicrograph of gill of T2 and T3 challenged with *A. hydrophila*

PLATE 24



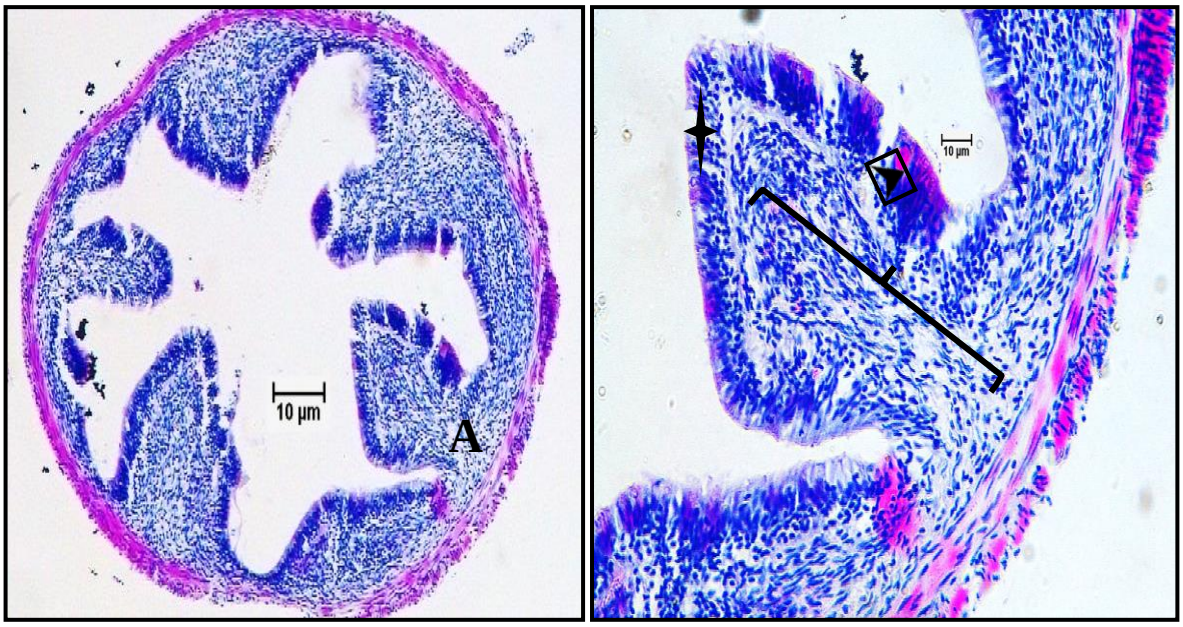
Photomicrograph of transverse section of intestine of CT experimentally challenged with *E. tarda* shows (A-40X) erythrocytic aggregation(rectangle) in sub mucosal layer, necrosis (arrow head) & hyperplasia of enterocytes (Long arrow) in mucosal epithelial layer (B-60X) necrosis of sub mucosal layer with hemorrhage(rectangle), necrotic luminar epithelial cells (right brace) & hypertrophic mucus cell(round) in the mucosal epithelial layer



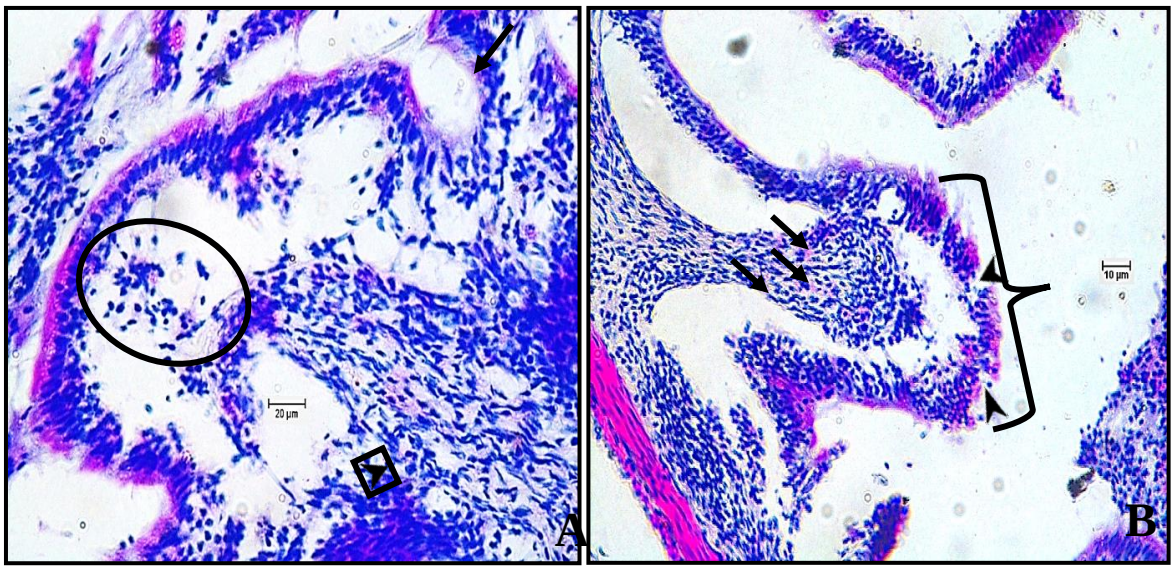
Photomicrograph of transverse section of intestine of T1 (A-40X) experimentally challenged with *E. tarda* shows numerous bacterial occlusion bodies, migration of inflammatory cells and expression of eosinophilic granular cells the subrepithelial layer of microvilli. Muscularis layer showed prolific migration of leucocytes

Plate 24. Showing photomicrograph of intestine of CT and T1 groups infected with *E. tarda*

PLATE 25



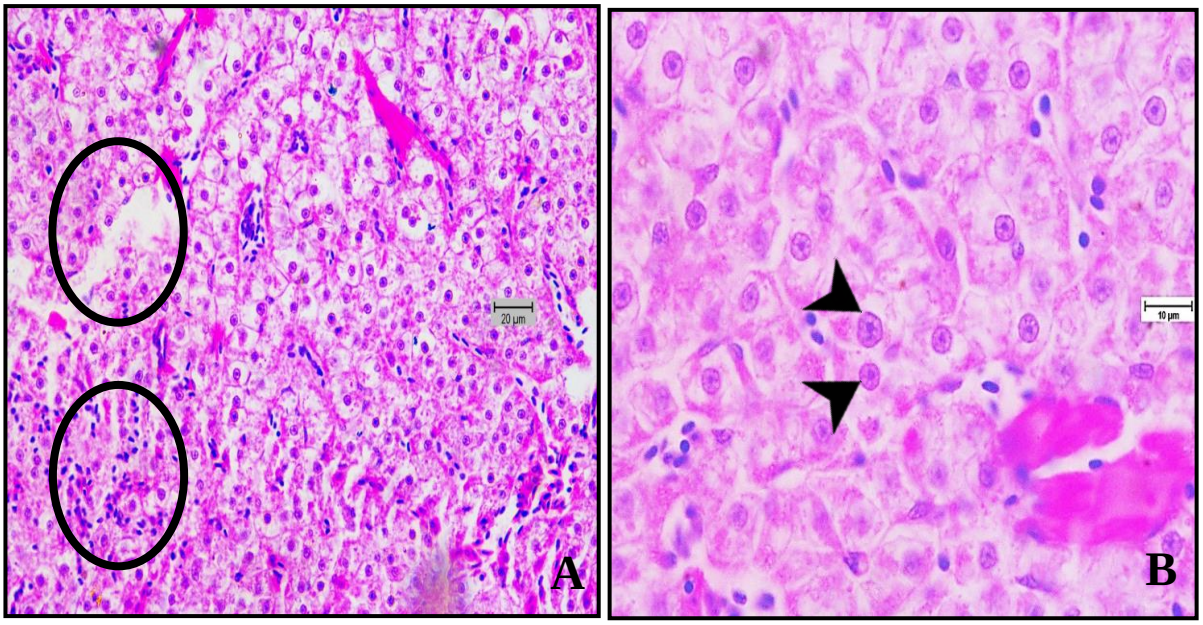
Photomicrograph of T2 intestine (mid gut) experimentally challenged with *E. tarda* shows (A-10X) moderate necrosis of enterocytes, fully replacement of leucocytes in the mucosal& sub mucosal layer of the intestine compared to other treatment. (B-40X) mucosal(star), sub mucosal layer (right bracket) & EGC expression (arrow head) in the mucosal epithelial layer. However, the mucosal fold remained intact.



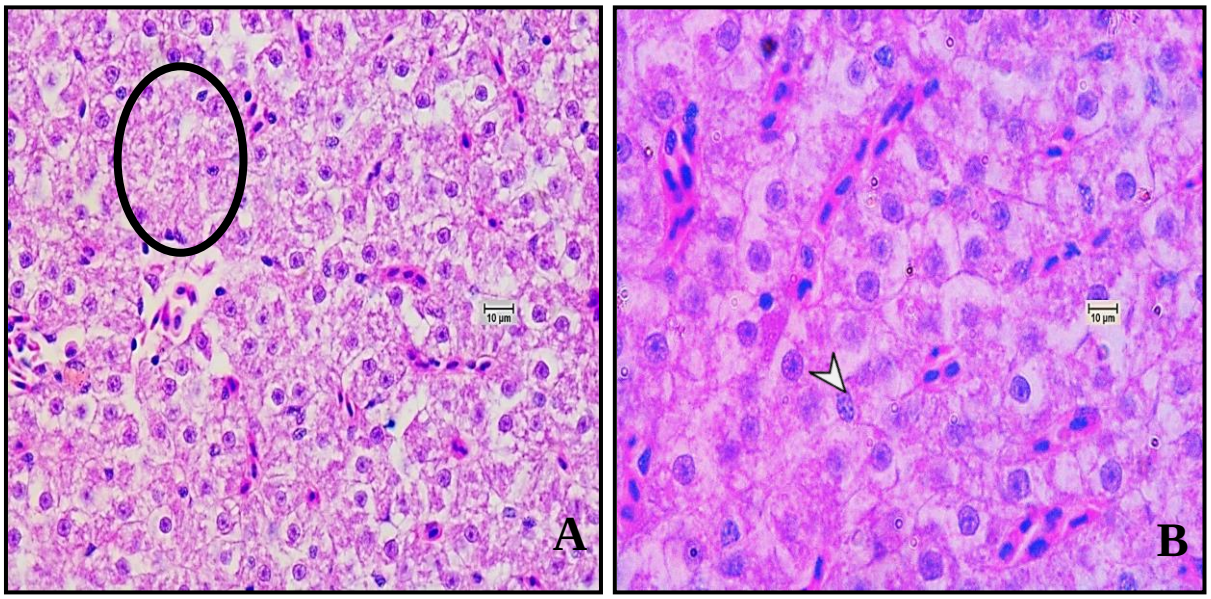
Photomicrograph of T3 intestine (mid gut) experimentally challenged with *Edwardsiella tarda* shows (A-60X) heavy necrosis in the enterocytes, and in the lamia propia, detachment of epithelial layer from the submucosal layer and heavy infiltration of leucocytes. (B-40X) Magnified view shows intact structure of microvilli (right brace) with enormous EGC expression (arrow) & goblet cell proliferation (arrow head) in the mucosal epithelial layer

Plate 25. Showing photomicrograph of intestine of T2 and T3 infected with *E. tarda*

PLATE 26



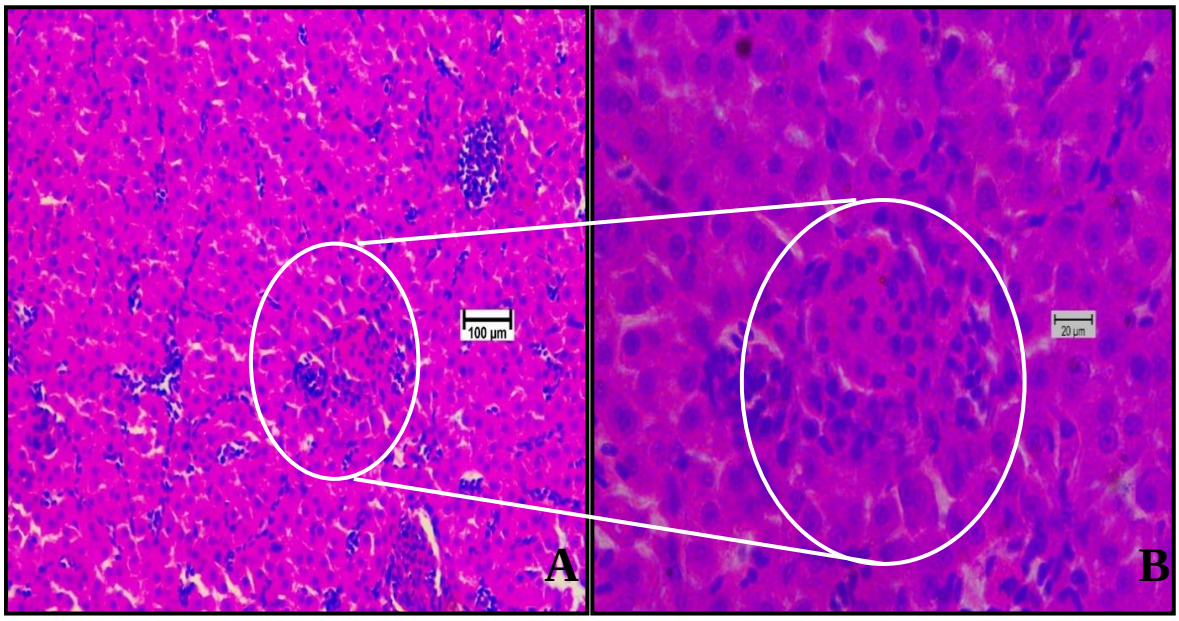
Photomicrograph of histology of CT liver experimentally challenged with *E. tarda* showing (A-40X) necrosis of Hepatocytes and sinusoidal channel (round) (B-60X) Hypertrophied hepatocytes (Arrow head)



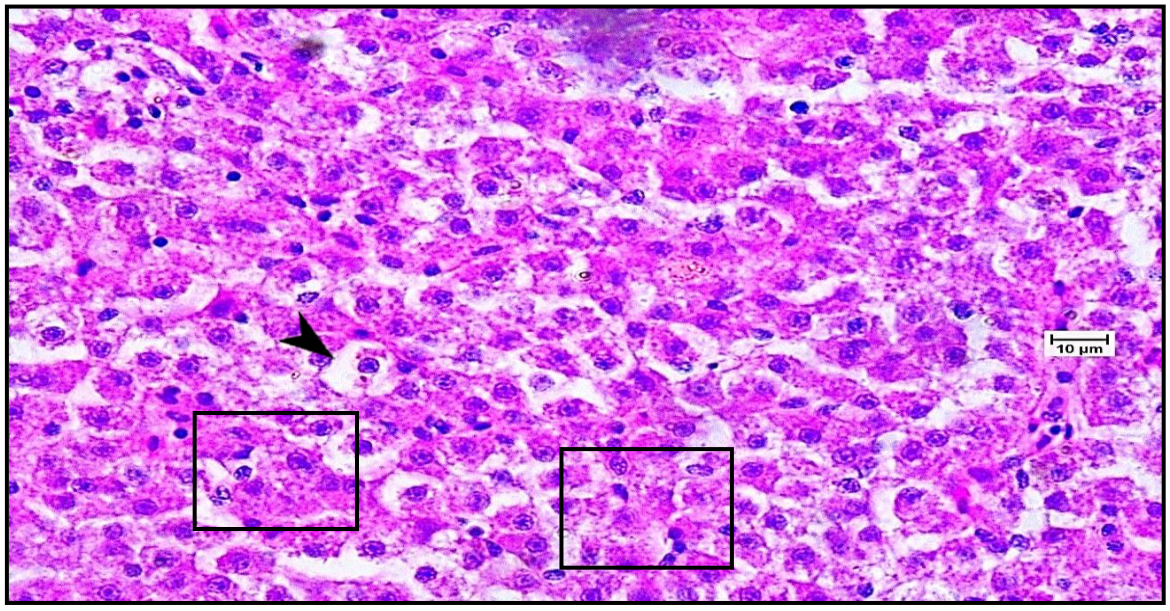
Photomicrograph of histology of T1 liver experimentally challenged with *E. tarda* showing (A-40X) Hepatic necrosis (round) (B-60X) karyorrhexis of hepatocytes (blank arrow head)

Plate 26. Showing Photomicrograph of liver of CT and T1 infected with *E. tarda*

PLATE 27



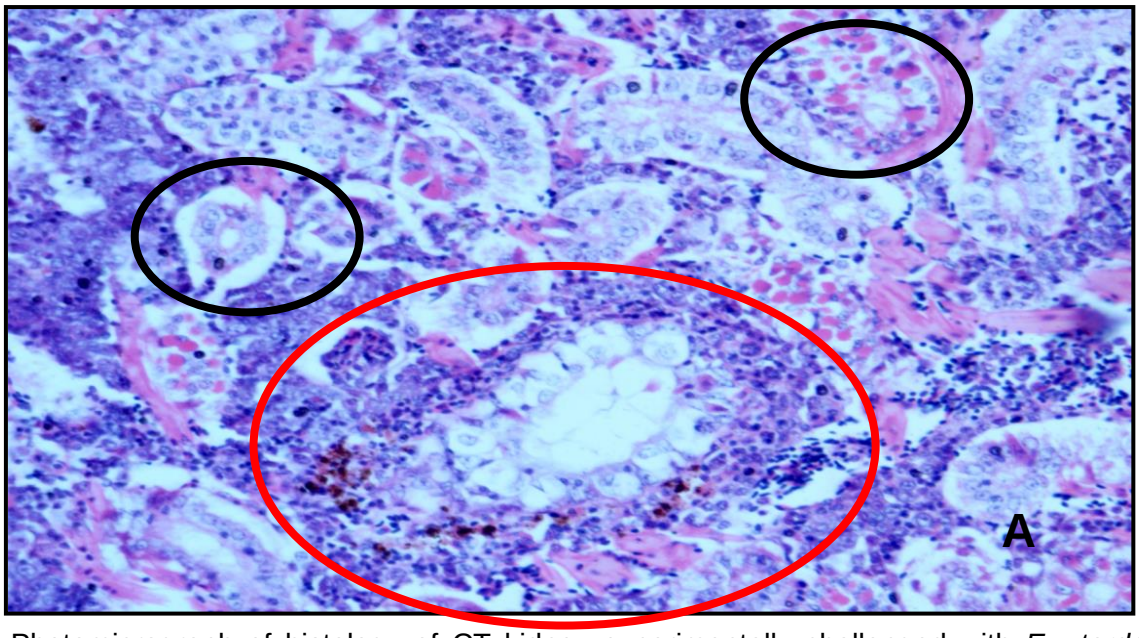
Photomicrograph of histology of T2 liver experimentally challenged with *Edwardsiella tarda* showing (A-40X) Intensive cellular migration in the sinusoidal channel in the form of granuloma (B-60X) Magnified view of A



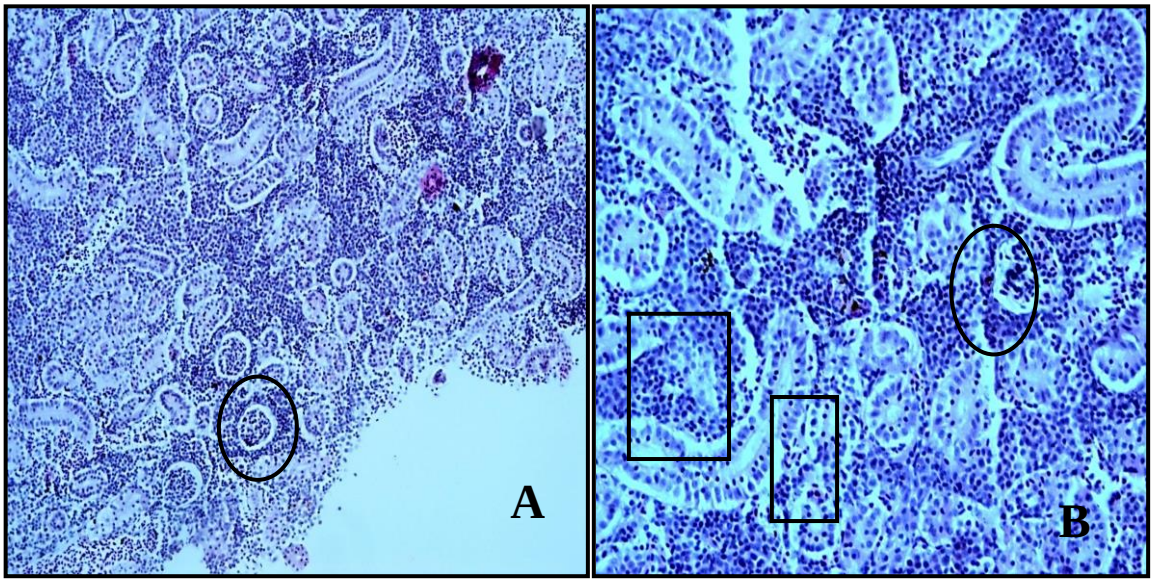
Photomicrograph of histology of T3 liver (60X) experimentally challenged with *Edwardsiella tarda showing* detachment of hepatocytes from the basement membrane(square) & necrosis of hepatocytes (arrow head) and vacuolization

Plate 27. Showing photomicrograph of histology of liver of T2 and T3 infected with *E. tarda*

PLATE 28



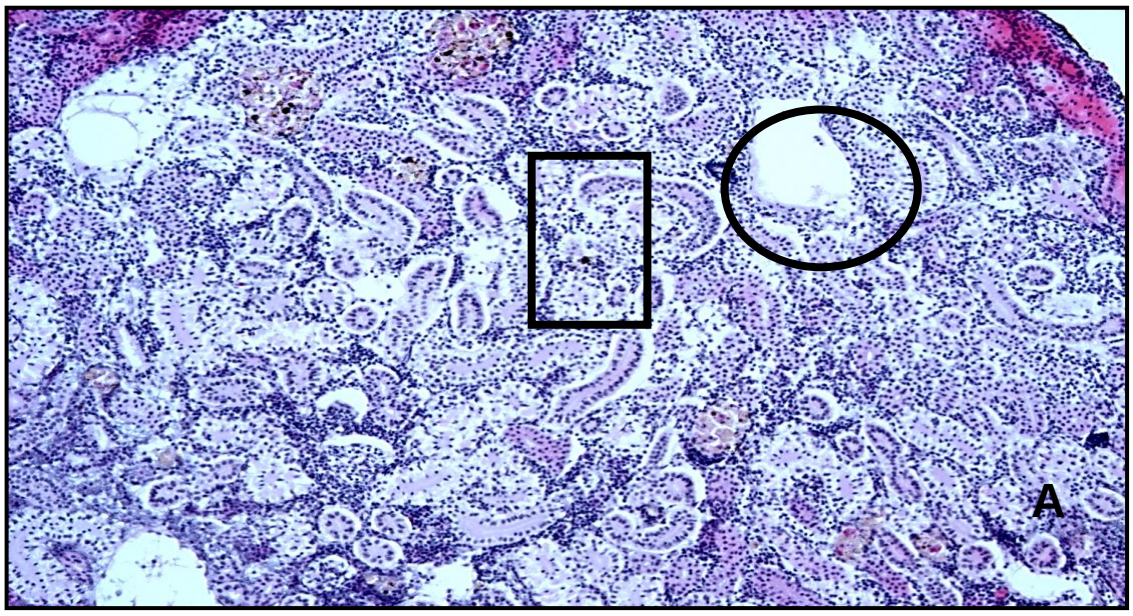
Photomicrograph of histology of CT kidney experimentally challenged with *E. tarda* showing granuloma type macrophage aggregation at the tubular lumen (red circle), laminar and haemopoietic tissue necrosis and haemorrhages (black circle) (A-60X)



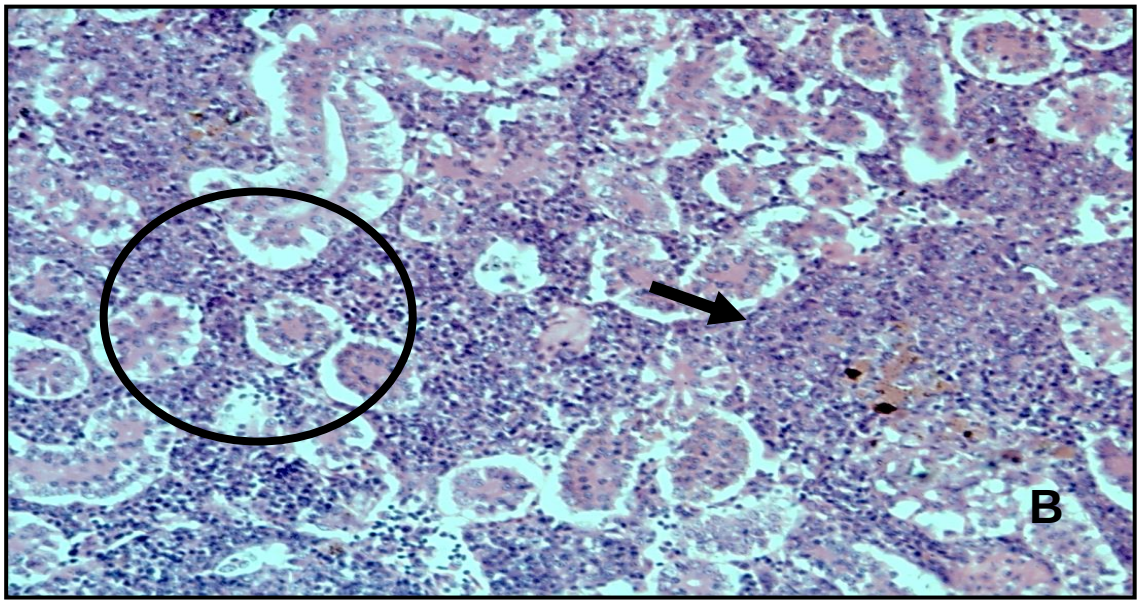
Photomicrograph of histology of T1 kidney experimentally challenged with *E. tarda* showing (A-40X) Proximal & distal tubular necrosis with macrophage aggregation (round in A & B), expression of MMC (B-60X) Haemopoietic tissue necrosis (square)

Plate 28. Showing photomicrograph of Kidney of CT and T1 infected with *E. tarda*

PLATE 29



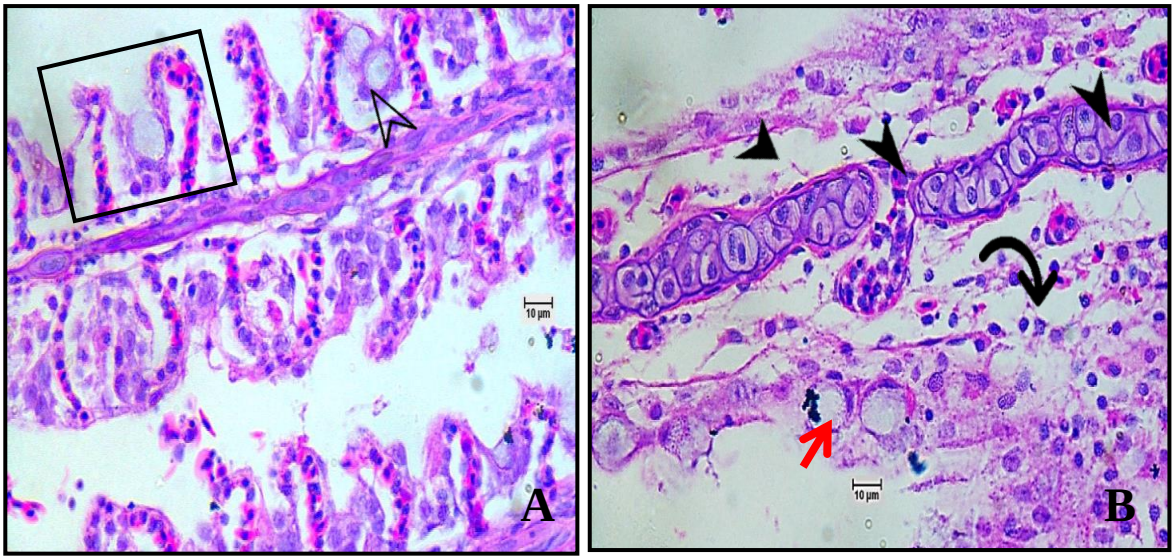
Photomicrograph of histology of T2 kidney (A-60X) experimentally challenged with *E. tarda* showing necrosis in distal & proximal tubules (round) & melanomacrophage pigmentation (arrow)



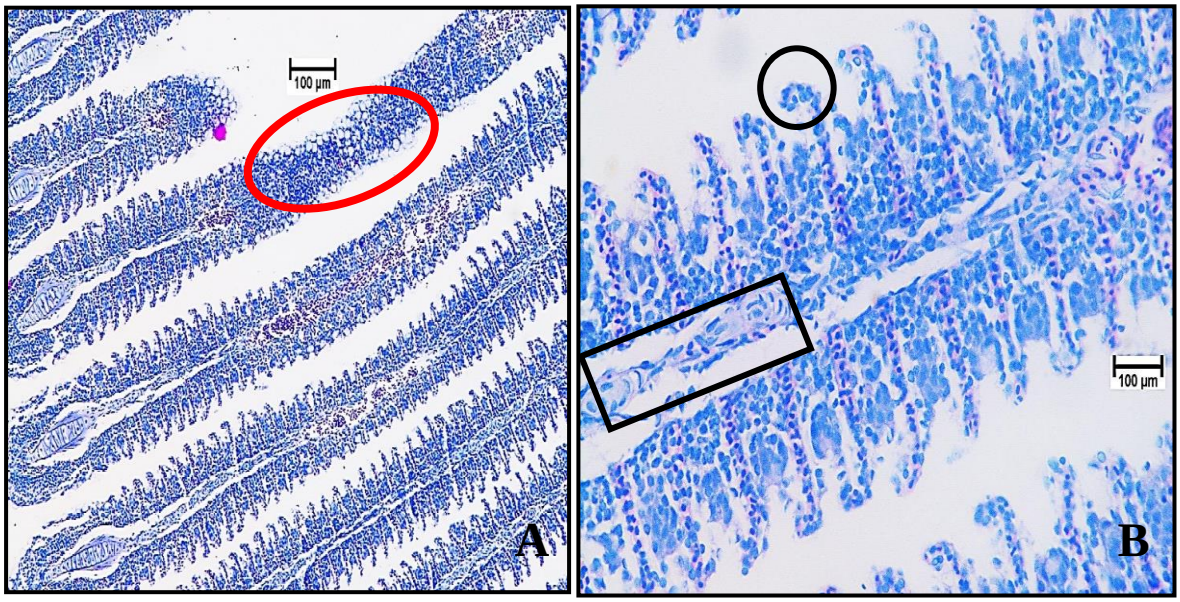
Photomicrograph of histology of T3 kidney (B-60X) experimentally challenged with *Edwardsiella tarda showing* presence of mild distal tubular necrosis but overall tubular architecture is maintained (round), normal lumen structure (rectangle), narrow indicates expression of melanomacrophage pigmentation

Plate 29. Showing Microphotograph of kidney of T2 and T3 infected with *E. tarda*

PLATE 30



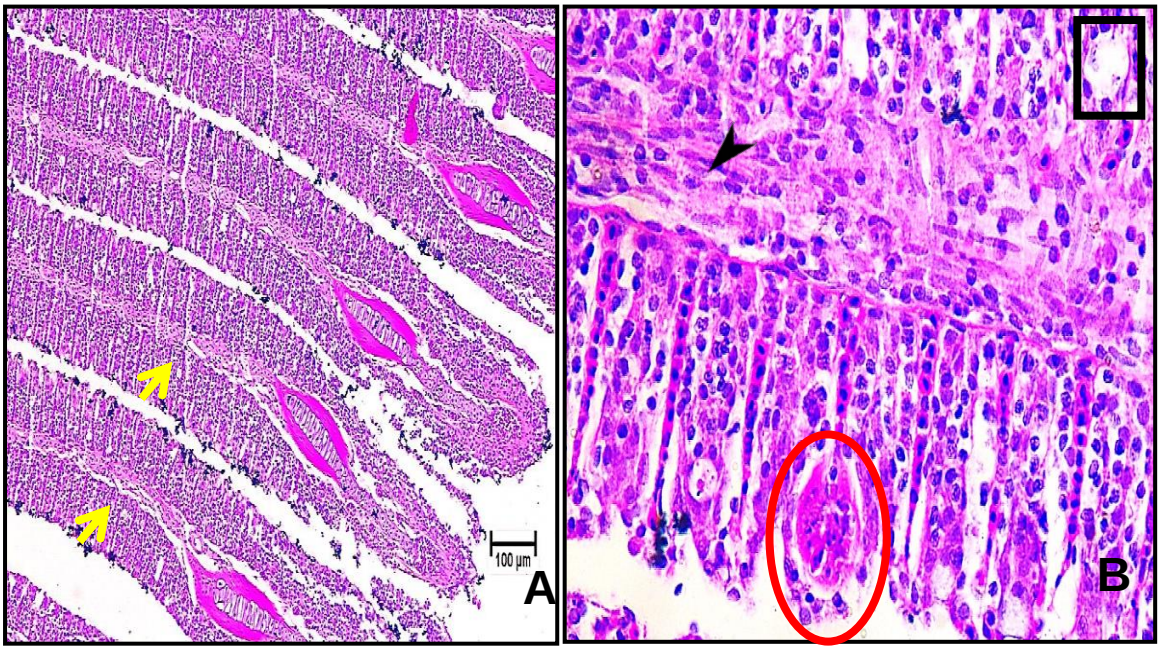
Photomicrograph of histology of CT gill experimentally challenged with *E. tarda* showing (A-40X) Parasagittal section of gill showing mild edema and fusion of tip of secondary lamellae (rectangle), hypertrophy of goblet cells (blank arrow head). (B-60X) complete necrosis of secondary lamellae (curved arrow), fragmentation of primary lamellae with massive infiltration of mononuclear inflammatory cell types in between damaged chondrocytes (arrow head) and excessive hypertrophid mucos cells (Red arrow).



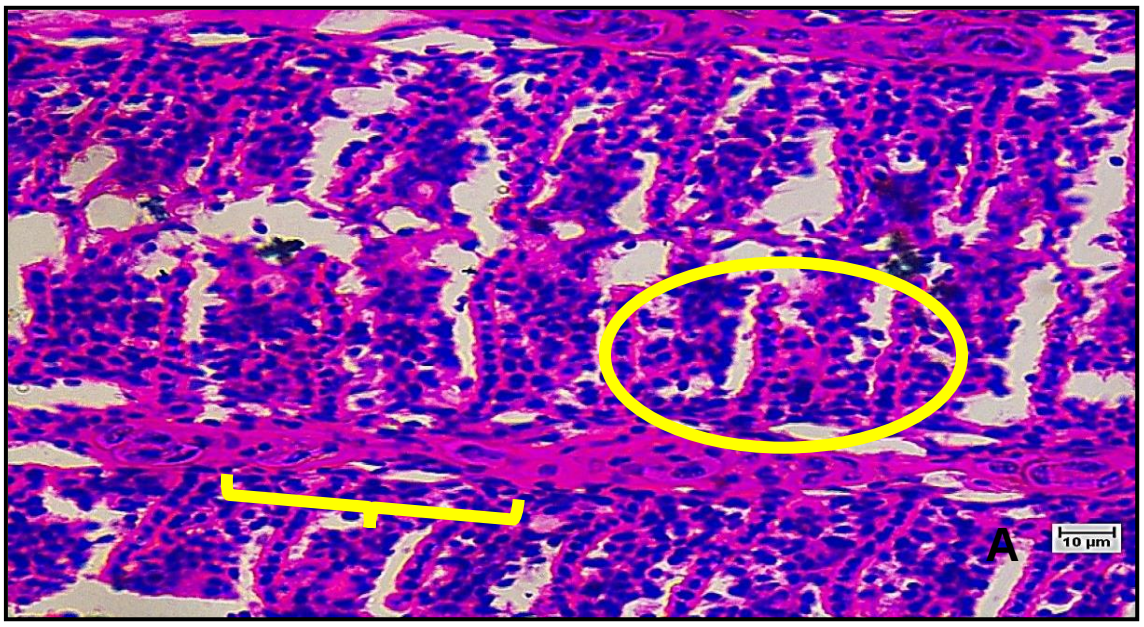
Photomicrograph of histology T1 gill experimentally challenged with *E. tarda* showing (A-10X) Massive mucosal cell hyperplasia and complete destruction of primary lamellae including diffusion of gill lamellae (red oval), (B-40X) Mild telangiectasia tissue damage of cartilaginous matrix (round), tissue damage of cartilaginous matrix (rectangle)

Plate 30. Showing photomicrograph of gill of CT and T1 infected with *E.*

PLATE 31



Photomicrograph of histology of T2 gill experimentally challenged with *E. tarda* showing (A-10X) complete fusion of secondary lamellae (arrow). (B-60X) intact secondary lamellae with massive infiltration of leucocytes in the cartilaginous matrix (arrow head), the primary lamellae with massive inflammatory mononuclear cell infiltration, telangiectasia with bacterial cell (red circle) & vacuoles



Photomicrograph of histology of T3 gill experimentally challenged with *E. tarda* showing (A-60X) complete fusion of secondary lamellae (arrow) massive inflammation in cell types

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Plate 31. Showing photomicrograph of gill of T2 and T3 infected with *E. tarda*

4.8.2. Outdoor pond feed trial

4.8.2.1. Serum enzymes

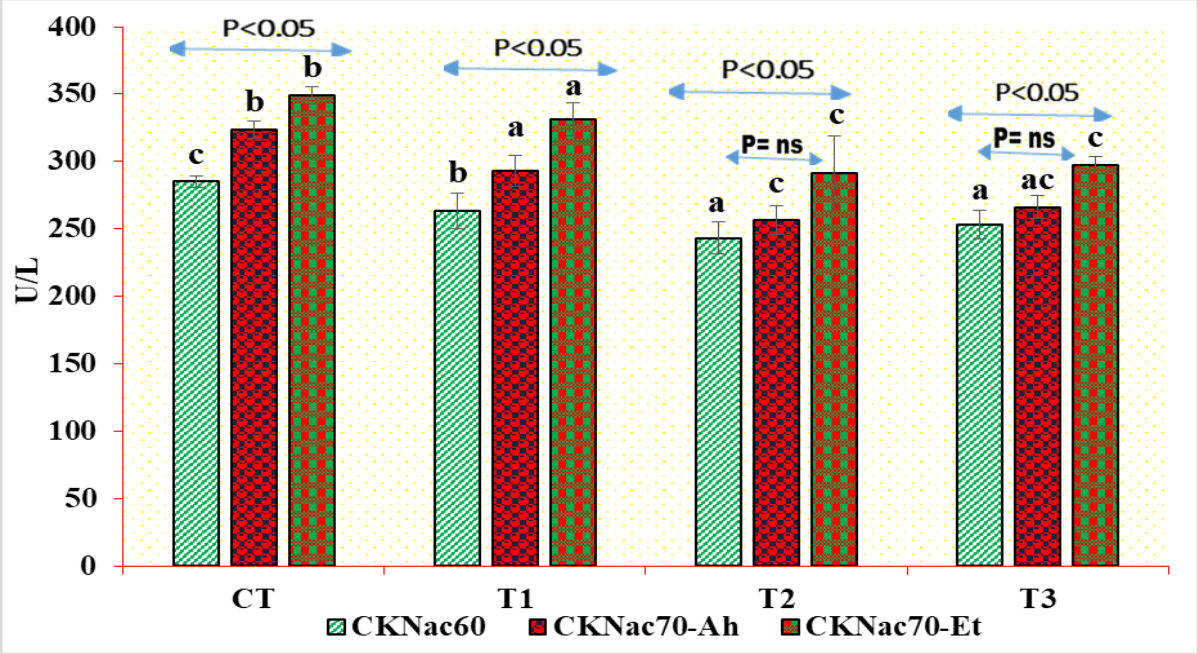

Fig.53. Showing variation in serum creatine kinase (CKNac) of *L. rohita* in outdoor pond feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

When compared to the other groups, T2 and T3 had drastically lower creatine kinase at 60 days (CKNac60) levels ($p<0.05$). The T2 and T3 showed no statistically significance ($p>0.05$). Between the groups, the creatine kinase at 70 days when fish was challenged with *A. hydrophila* (CKNac70-Ah) level differed considerably ($p<0.05$). The T2 and T3, as well as T3 and T1, exhibited no significance ($p>0.05$), however they all differed considerably ($p<0.05$) in CT. In CT and T1, the values are considerably higher for CKNac60, CKNac70-Ah, and ceatine kinase at 70 days when fish was challenged with *E. tarda* (CKNac70-Et) ($p<0.05$), but there was no significance ($p>0.05$) difference between 60 days and infection at 70 days (CKNac60 and CKNac70-Ah) in T2 and T3. There was a considerable change was reported ($p>0.05$) difference with CKNac70-Et in both T2 and T3 (Fig. 53). $Y= 7.89x2-51.10x+329.57$, $R2=0.96$ is CKNac60's polynomial equation for CKNac variables.

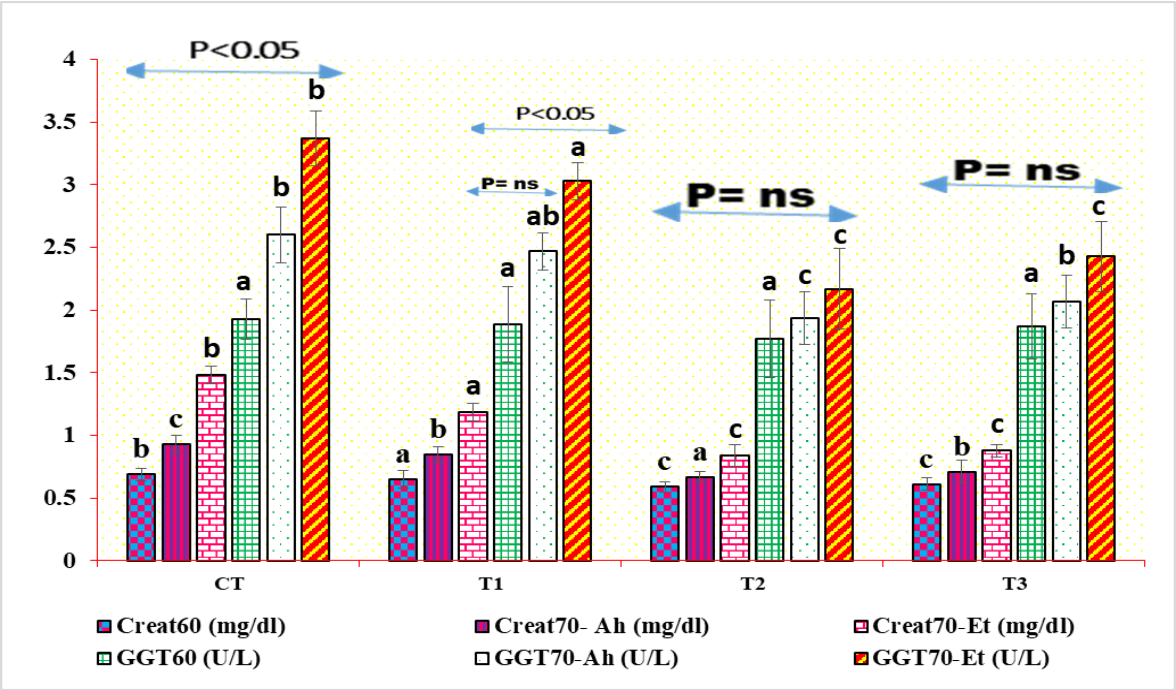

Fig. 54. Showing variation in serum creatinine (Creat) and gamma glutamyl transferase (GGT) of *L. rohita* in outdoor pond feed trial and challenge study

The T2 had a lower creatine at 60 days (Creat60) than the other groups by a considerable ($p<0.05$) margin. While there was a significance in creatine at 70 days when fish was challenged with *A. hydrophila* (Creat70-Ah) between CT, T2, and T3 ($p<0.05$), there was no substantial change was reported between T1 and T3 ($p>0.05$). The creatine at 70 days when fish was challenged with *E. tarda* (Creat70-Et) exhibited a remarkable variation ($p<0.05$) between T3 and the other groups, but not between T2 and T3. The Creat60, Creat70-Ah, and Creat70-Et, as well as, gamma glutamyl transferase at 60 days (GGT60), gamma glutamyl transferase when fish was challenged with *A. hydrophila* (GGT70-Ah), and gamma glutamyl transferase when fish was challenged with *E. trada* (GGT70-Et), had considerable rise within the treatments ($p<0.05$).

In T1, there was a considerable change ($p<0.05$) for both Creat60 and GGT60, as well as infection (70 days) with ET, but no substantial difference was reported ($p>0.05$) for Ah. In both T2 and T3, there was no drastic ($p>0.05$) change was reported (Fig. 54). The polynomial equation for Creat60 is, $Y= 0.015x^2-0.105x+0.785$, $R^2 = 0.92$ and for GGT60 is $Y= 0.035x^2-0.205x+2.115$, $R^2= 0.67$.

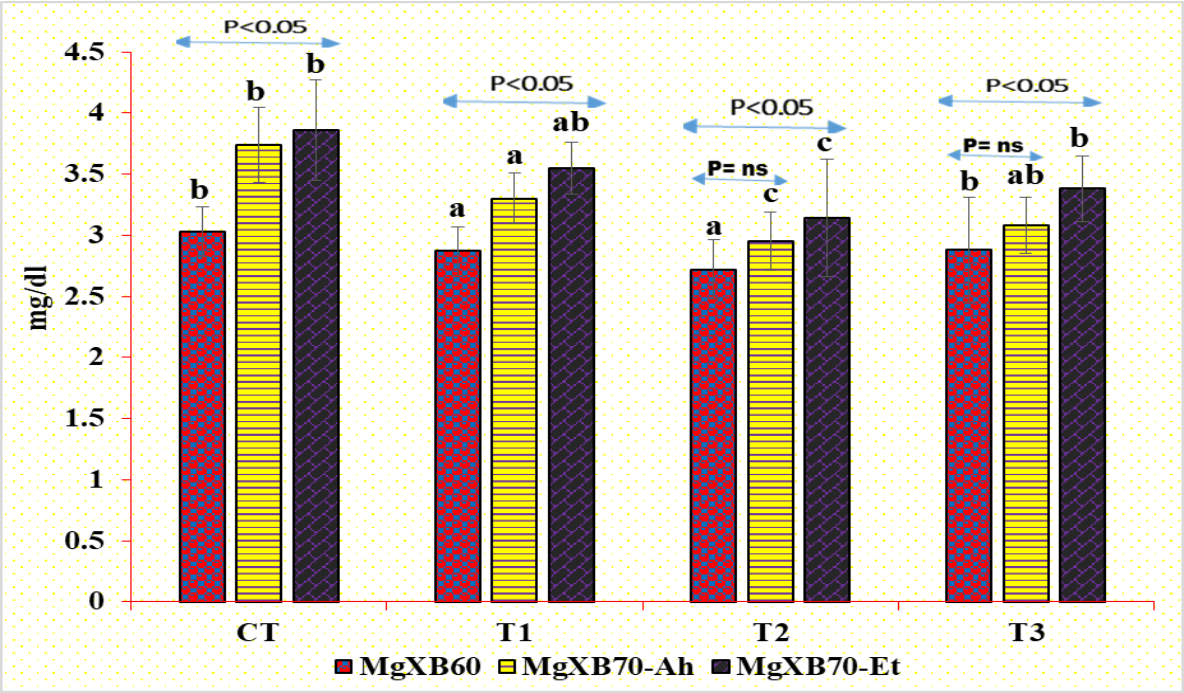

Fig. 55. Showing variation in serum MgXB of *L. rohita* in outdoor pond feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

In MgXB60, T2 showed a drastic ($p<0.05$) drop as compared with other treatments, followed by T3 showing a substantial ($p<0.05$) rise when compared to T2. There was no substantial change was reported ($p>0.05$) between CT and T3, or T1 and T2 for MgXB60. In challenge study, a new pattern emerged: MgXB70-Ah demonstrated significance ($p<0.05$) between CT, T1, and T2, but not between T2 and T3 ($p>0.05$). The MgXB70-Et showed a significance ($p<0.05$) in T2 as compared with other groups. Within the treatments, a considerable ($p<0.05$) rise was found in the values of MgXB60, MgXB70-Ah, and MgXB70-Et in CT and T1. In T2 and T3, between MgXB60 and MGXB70-Ah, no significance ($p>0.05$) was reported but a significance ($p<0.05$) was observed with Et in both T2 and T3 (Fig. 55). The polynomial equation for MgXB60 is as follows, $Y=0.08x^2-0.46x+3.42$, $R^2=0.91$.

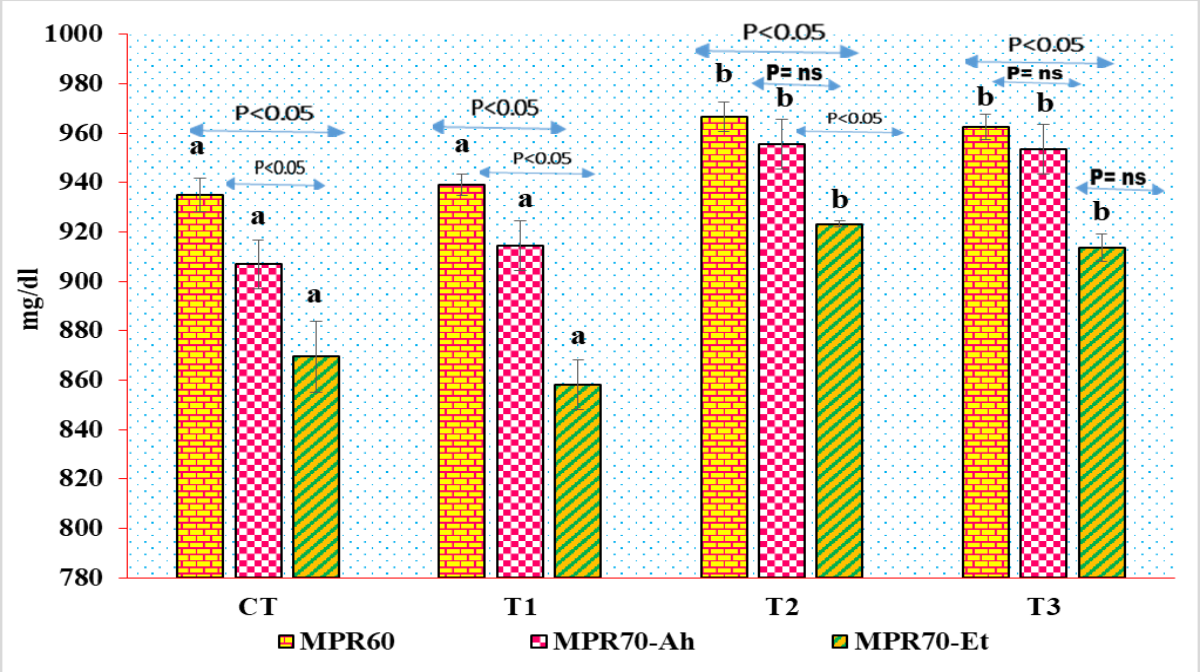

Fig. 56. Showing variation in serum microProtein (MPR) of *L. rohita* in outdoor pond feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The microProtein at 60 days (MPR60) values exhibited a drastic rise ($p<0.05$) in T2 but no significance ($p>0.05$) with T3 as compared to other group and upon infection, same trend was followed between the treatments. Feed trial followed by challenge study (70 days) in pond conditions showed a considerable ($p<0.05$) decrease within the treatments, between MPR60, microProtein at 70 days when fish infected with *A. hydrophila* (MPR70-Ah) and microProtein at 70 days when fish infected with *E. tarda* (MPR70-Et) in CT and T1 while T2 and T3 had no substantial change ($p>0.05$) between MPR60 and MPR70-Ah but it has significance ($p<0.05$) with MPR70-Et in both the treatments (Fig. 56). The linear equation for MPR60 between treatment is as follows; $Y= 11.05x+923.14$, $R^2=0.78$ and within T2 treatment is as under, $Y= -21.67x+991.76$, $R^2=0.92$.

The T1, T2, and T3 all showed a substantial ($p<0.05$) drop in bilirubin total at 60 days (BIT60). Despite the fact that T2 had the lowest value, there was no statistically substantial ($p<0.05$) change between T1, T2, and T3. The CT and T1

demonstrated a considerable ($p<0.05$) rise between the value of 60 days (BIT60) and infection at 70 days (BIT70-Ah and BIT70-Et), whereas T2 and T3 demonstrated no significance ($p>0.05$) between BIT60 and BIT70-Ah but a substantial change was reported ($p<0.05$) with BIT70-Et in both treatments (Fig. 57). The polynomial equation for BIT60 is as follows, $Y=0.03x^2-0.166x+0.415$, $R^2=0.99$.

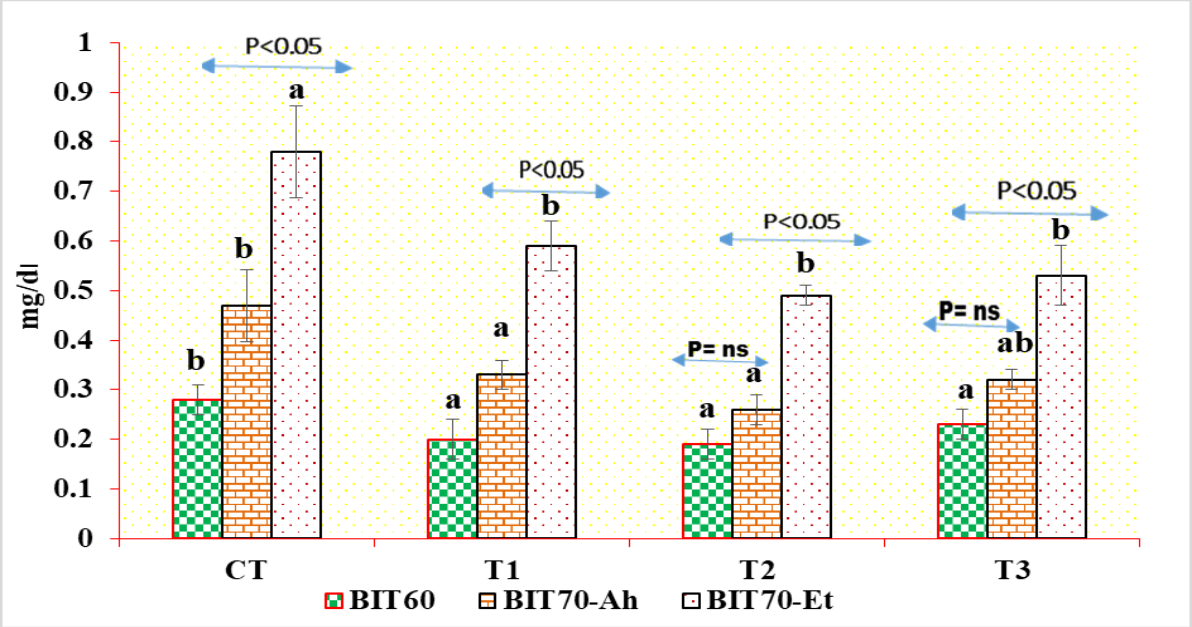

Fig. 57. Showing variation in serum bilirubin total (BIT) of *L. rohita* in outdoor pond feed trial and challenge study

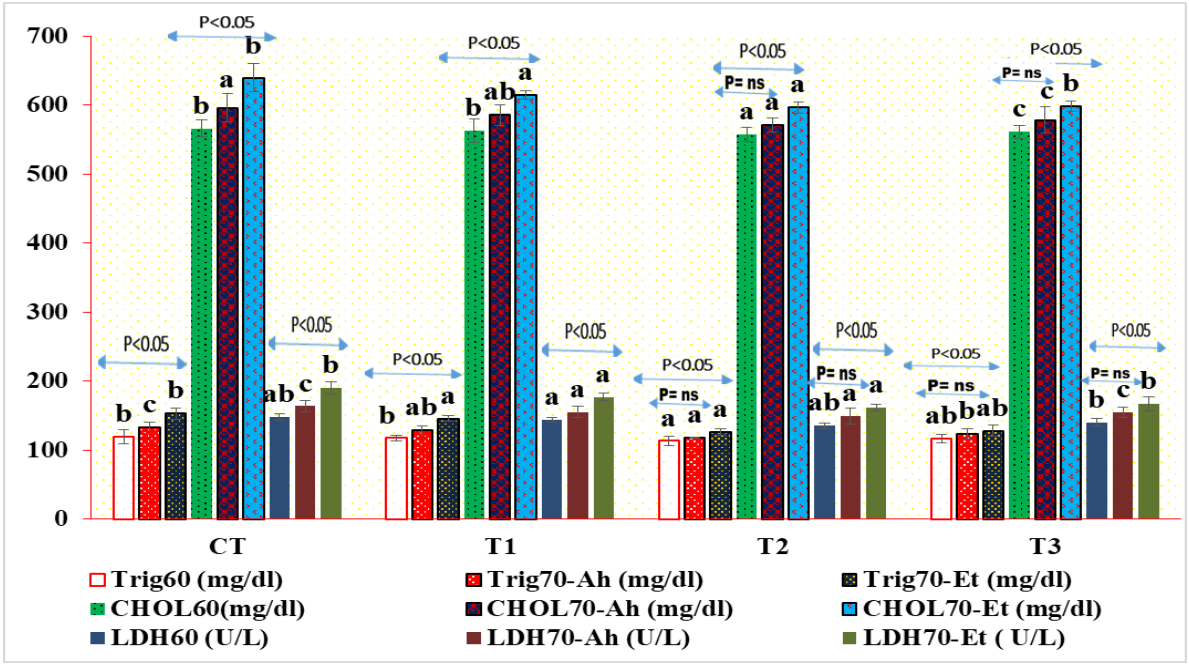

Fig. 58. Showing variation in serum triglycerides (Trig), cholesterol (CHOL) & lactate dehydrogenase (LDH) of *L. rohita* in outdoor pond feed trial and challenge study

The values of Trig60, CHOL60, and LDH60 dropped considerably between treatments in T2 and T3 (p0.05), while there was no substantial change was reported (p>0.05) between CT and T1. The trend was the same in all treatments after infection (70 days). When compared to Ah, Et displayed more values. Within treatments, there was a substantial (p<0.05) rise in CT and T1, but no substantial (p>0.05) rise in T2 and T3. Within the groups, a substantial (p<0.05) rise in CT and T1 was found, but no considerable (p>0.05) change was reported in T2 and T3 (Fig. 58). The polynomial equation for Trig60 is as follows, $Y= -1.142x^2 -6.99x+125.51$, $R^2=0.75$. The polynomial equation for CHOL60 is as under $Y=1.685x^2 -10.44x+575.57$, $R^2=0.82$ and for LDH60 it is $Y=2.165x^2-13.82x+160.17$, $R^2=0.82$.

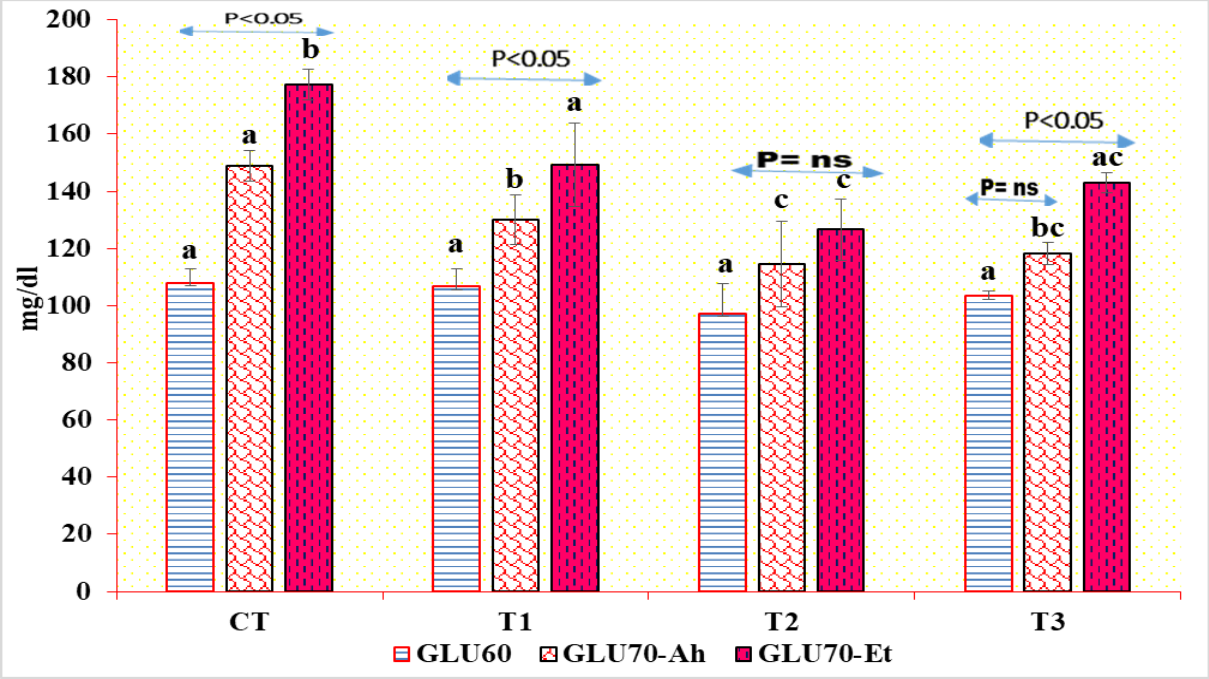

Fig. 59. Showing variation in serum glucose (GLU) of *L. rohita* in outdoor pond feed trial and challenge study

The glucose at 60 days (GLU60) levels did not alter substantially across treatments (p>0.05). While, glucose at 70 days when fish was challenged with *A. hydrophila* (GLU70-Ah) and glucose at 70 days when fish was challenged with *E. tarda* (GLU70-Et) exhibited a significance (p>0.05) between CT, T1, T2, but not between T2, T3, and T1 and T3. In CT and T1, there was a substantial (p<0.05) change between the treatments for GLU60, GLU70-Ah, and GLU70-Et. There was no considerable (p>0.05) variation between GLU60, GLU70-Ah, and GLU70-Et in T2

and T3, however there was a substantial ($p<0.05$) difference between GLU60 and GLU70-Ah and GLU70-Et in T3 (Fig. 59). The equation for GLU60 of treatments is as $Y=- 1.837x^2-11.54x+118.86$, $R^2=0.61$.

The serum glutamic oxaloacetic transaminase (SGOT60) declined little towards T2, then increased dramatically ($p<0.05$) in T3. As, serum glutamic oxaloacetic transaminase at 70 days when fish was challenged with *A. hydrophila* (SGOT70-Ah) values decreased in T2, considerably ($p<0.05$) when compared with other groups. There was no substantial ($p>0.05$) difference between CT and T1, or T2 and T3 for SGOT70-Ah, and the similar pattern was seen in serum glutamic oxaloacetic transaminase at 70 days when fish was challenged with *E. tarda* (SGOT70-Et).

Within the treatments, SGOT60 drastically ($p<0.05$) increased in response to Ah and Et infection in CT and T1, but there was no substantial ($p>0.05$) change between SGOT60 and SGOT70-Ah but a considerable (p0.05) variation was reported with SGOT70-Et in T2 and T3. When compared to the other groups, AMY60 reduced considerably ($p<0.05$). There was no statistically significance was reported ($p>0.05$) between CT, T1, T2, and T3. There was a drastic rise ($p<0.05$) in CT and T1 within treatments, but no substantial rise ($p>0.05$) in T2 and T3 after infection (Fig. 60).

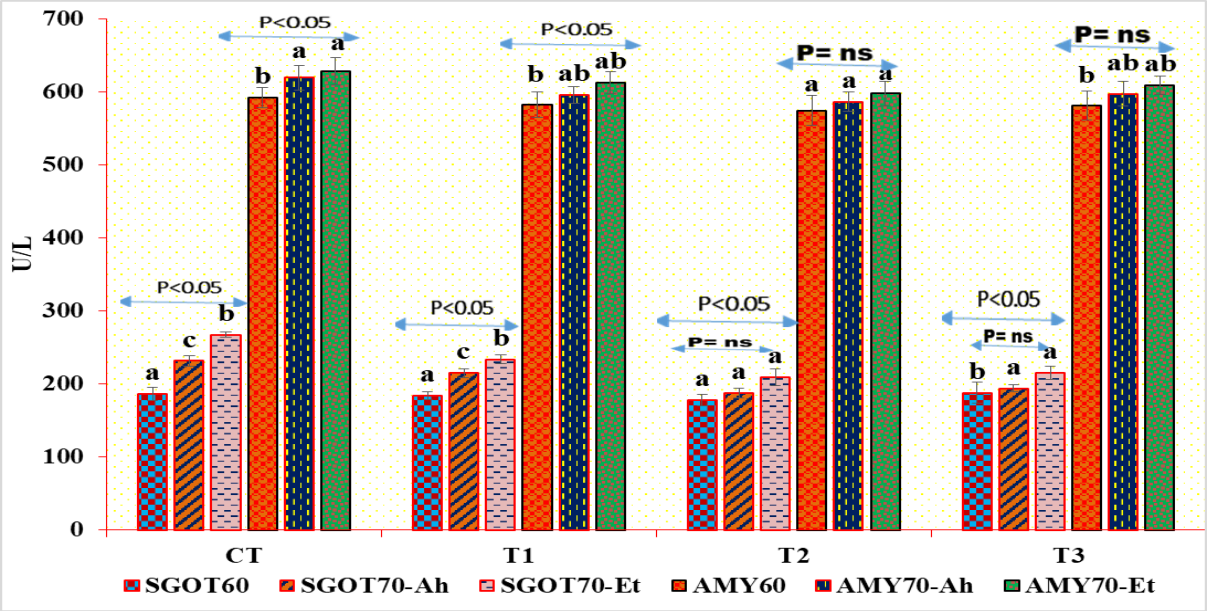

Fig. 60. Showing variation in serum glutamic oxaloacetic transaminase (SGOT) and amylase (AMY) of *L. rohita* in outdoor pond feed trial and challenge study

The pond experiment showed that serum glutamic pyruvic transaminase at 60 days (SGPTD60) differed considerably ($p<0.05$) in T3 from T2, and that that serum glutamic pyruvic transaminase at 70 days when fish was challenged with *A. hydrophila* (SGPTD70-Ah) and serum glutamic pyruvic transaminase at 70 days when fish was challenged with *E. tarda* (SGPTD75-Et) differed considerably ($p<0.05$) in T1 and T2 from other treatments (Figure 61).

When SGPTD70-Ah and SGPTD70-Et were compared to SGPTD60 in CT after infection with bacterial pathogens, there was a substantial change ($p<0.05$) was reported within treatment. Between SPTD60 and SGPTD70-Ah, there was no significance ($p>0.05$) in T1, T2, and T3, but it was remarkably different ($p<0.05$) in SGPTD-Et. When compared to the other treatments, ALPU60 was considerably ($p<0.05$) reduced in T2 and T3. When compared to other treatments, ALPU70-Ah differed considerably ($p<0.05$) in T1 and T2. Within treatments, ALPU60, ALPU70-Ah, and ALPU70 Et exhibited a substantial ($p<0.05$) variation in CT and T1, but there was no significance ($p>0.05$) between ALPU60 and ALPU70-Ah in T2 and T3, but there was significance ($p<0.05$) with ALPU-Et in T2 and T3.

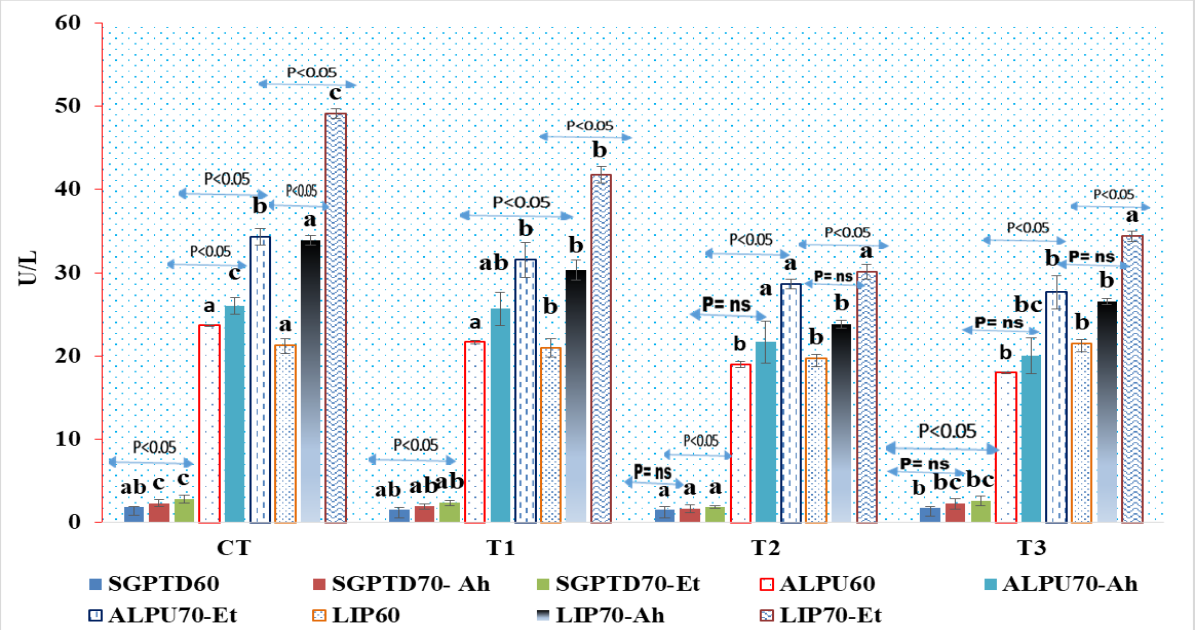

Fig. 61. Showing variation in serum glutamic pyruvic transaminase (SGPTD), lipase (LIP) and alkaline phosphatase (ALPU) of *L. rohita* in outdoor pond feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The lipase at 60 days (LIP60) differed remarkably ($p<0.05$) in T2 and T3 when compared to other treatments. Although, lipase at 70 days when fish was challenged with *E. tarda* (LIP70-Et) was statistically ($p<0.05$) different between treatments, CT had the greatest LIP70-Ah, followed by T1>T3, and T2. After infection, there was a substantial variation ($p<0.05$) between LIP60, lipase at 70 days when fish was challenged with *A. hydrophila* (LIP70-Ah), and LIP70-Et in CT and T1, but no significance was seen ($p>0.05$) in T2 and T3, but a considerable ($p<0.05$) change was reported with LIP70-Et in both treatments. The polynomial equation of SGPTD60 between treatment is as follows, $Y=-0.0127x^2-0.6745x+2.422$, $R^2=0.99$. The linear equation for ALPU60 is as follows, $Y=-1.98x+25.50$, $R^2=0.97$ and the polynomial equation for LIP60 is as under, $Y=-0.547x^2-2.78x+23.72$, $R^2=0.62$.

4.8.2.2. Digestive enzymes

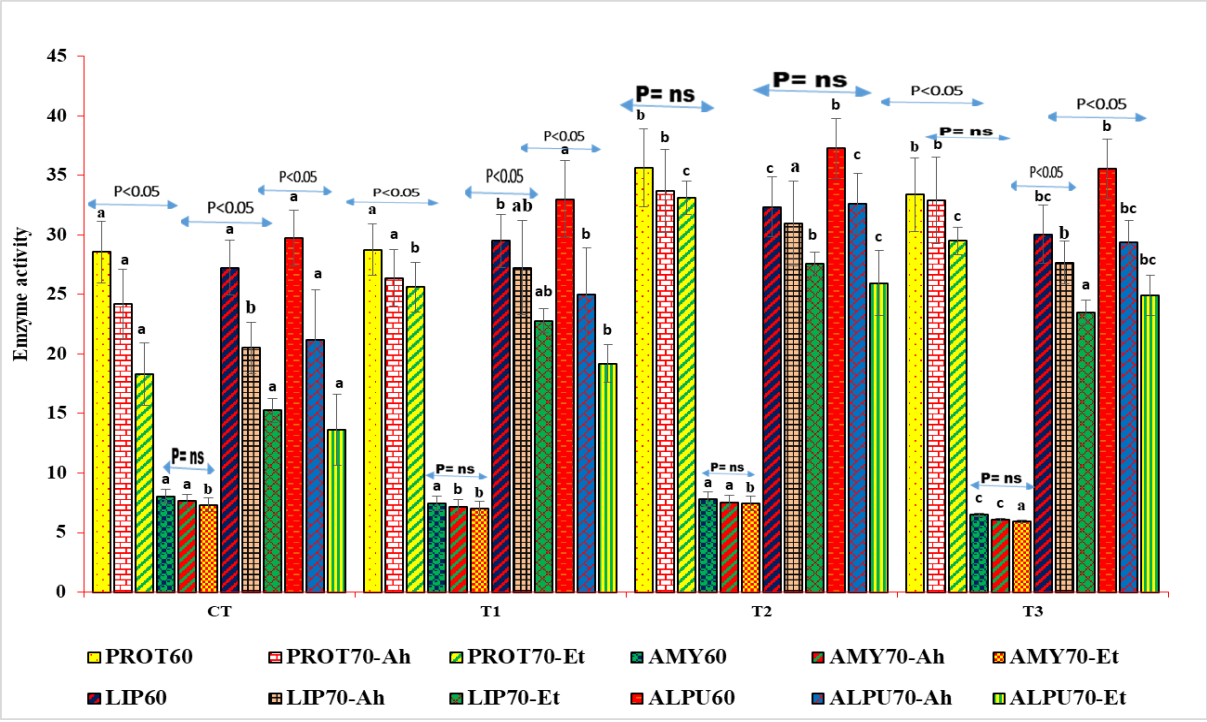

Fig. 62. Showing variation in digestive protease (PROT), amylase (AMY), lipase (LIP) and alkaline phosphatase (ALPU) of *L. rohita* in outdoor pond feed trial and challenge study

Here, protease activity expressed as micromoles of tyrosine released/min/mg protein at 37 ℃; Maltose activity expressed as micromoles of maltose released/min/mg protein at 37 ℃; Lipase activity expressed as units/mg protein at 37℃; Alkaline phosphatase activities expressed as nanomoles p-nitrophenol released/min/mg protein at 37 ℃.

The activity of protease at 60 days (PROT60) in outdoor pond feed trial exhibited no significance ($p>0.05$) between CT and T1, however T3 and T2 had considerably higher values ($p<0.05$) than CT and T1. The lipase at 60 days (LIP60) activity did not differ between CT, T1, or T3, however it was substantially ($p<0.05$) higher in T2 than in the other groups. Between CT and T1, and T2 and T3, ALPU60 revealed no significance ($p>0.05$). The activity of AMY60 was as follows. CT> T1>T2>T3. The value of AMY did not differ substantially ($p>0.05$) among the treatments when challenged with Ah and Et, and the value of all enzyme activity in CT and T1 continues to decrease. The activity of all enzymes did not alter considerably ($p>0.05$) in T2, however it declined with Ah and Et. In T3, there was no remarkable change ($p>0.05$) between 60-day activity and Ah infection, however there was a substantial ($p<0.05$) drop-in activity after Et infection (Fig. 62).

4.8.2.3. Enzyme of oxidative damage

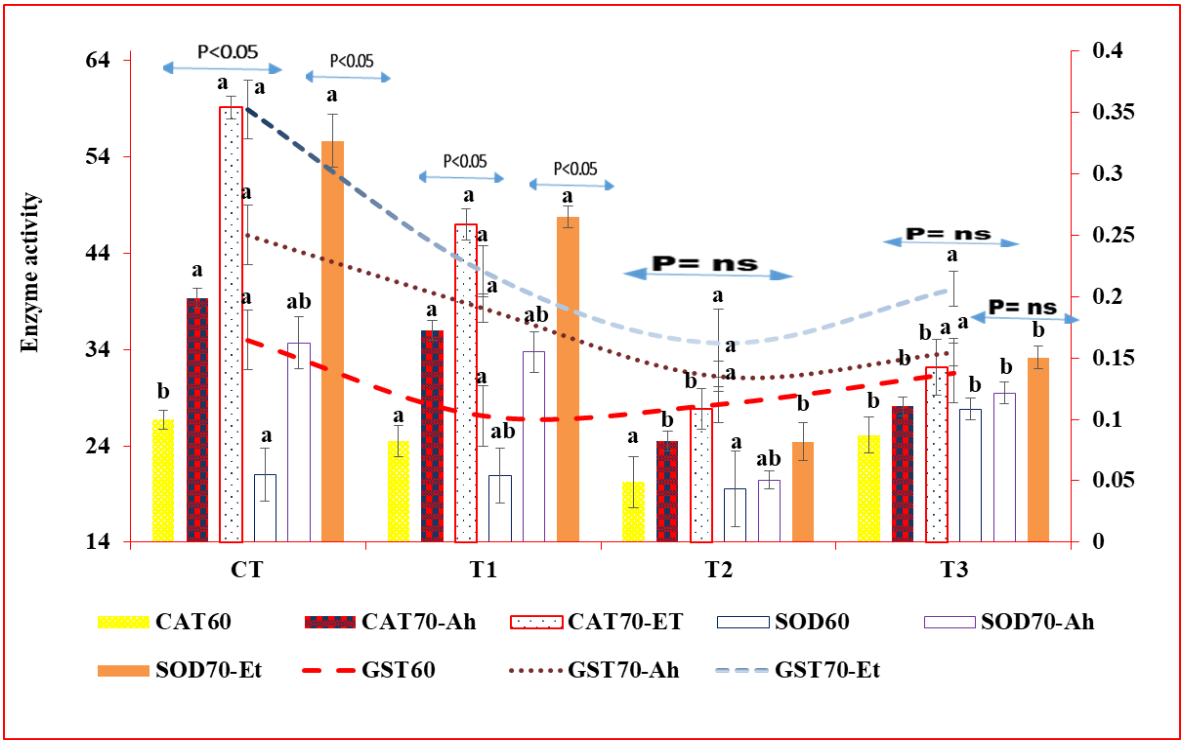

Fig. 63. Showing variation in catalase (CAT), superoxidedismutase (SOD) and glutathione-s-transferase (GST) of *L. rohita* in outdoor pond feed trial and challenge study

Here, Catalase: mmol H_2O_2 decomposed /min/ mg protein at 37 ℃; SOD (superoxide dismutase): µmol mg^{-1} protein/min at 37 ℃; GST: Glutathione-S-transferase Units/mg protein

The catalase at 60 days (CAT60) did not differ substantially ($p>0.05$) between T1 and T2, as well as T3 and CT, in the outdoor pond feed trial. There was no considerable change was reported between CT and T1, and T2 and T3 after infection with Ah ($p>0.05$). Enzymatic activity increased drastically ($p<0.05$) in CT and T1, but not substantially ($p>0.05$) in T2 and T3 (Fig. 63). The CAT60 polynomial equation is, $Y=1.781x^2 -9.819x+ 35.33$, $R^2=0.73$. SOD60 and GST60 polynomial equation are as follows, $Y= 2.01x^2-8.6183x+ 28.077$, $R^2= 0.85$; $Y=0.0291x^2-0.116x+0.2665$, $R^2=0.94$.

4.8.2.4. Enzymes of neuro-transmitter

According to the results of the outdoor pond feed trial, there were no statistically substantial changes in the levels of acetyl choline esterase at 60 days (AchE60) across the groups ($p>0.05$). Following the infection, the trend persisted. The CT demonstrated a remarkable ($p<0.05$) increase in acetyl choline esterase at 70 days when fish was challenged with *A. hydrophila* (AchE70-Ah) and acetyl choline esterase at 70 days when fish was challenged with *E. tarda* (AchE70-Et) between treatments (Fig. 64). The polynomial equation for AchE90 of different treatment is as follows, Y= 0.0394x2-0.2852x+2.9033, R2=0.81.

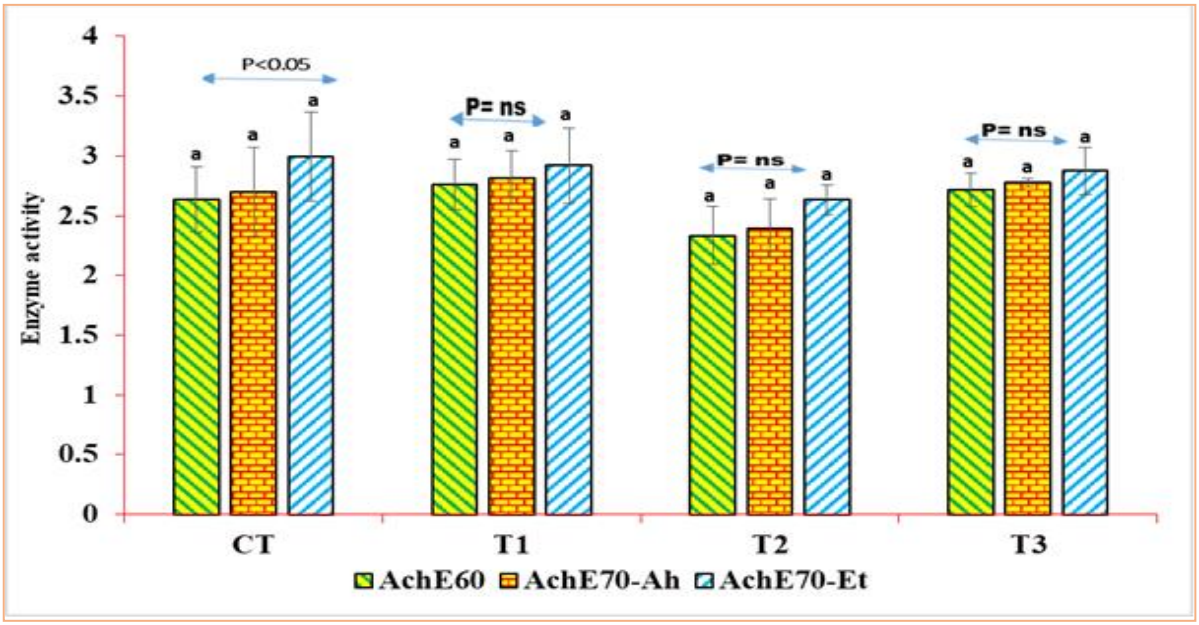

Fig. 64. Showing variation in acetyl choline esterase (AchE) of *L. rohita* in outdoor pond feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.8.2.5. Triiodothyronine (T3), thyroxine (T4) and cortisol

The cortisol at 30 days (CORT-30) did not differ substantially ($p>0.05$) among the treatments, although, cortisol at 60 days (CORT-60) differed considerably ($p<0.05$) among the treatments.

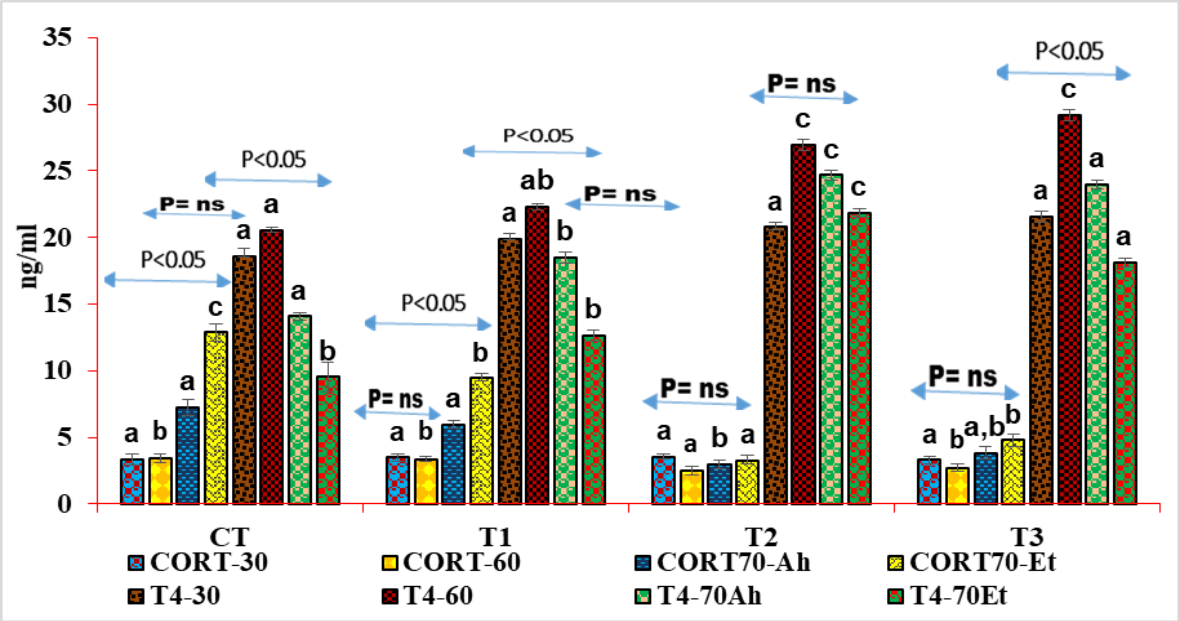

Fig. 65. Showing variation in serum cortisol (CORT) and throxine (T4) level of *L. rohita* in outdoor pond feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

Additionally, thyroxine at 30 days (T4-30) did not differ statistically substantially across the treatments, while thyroxine at 60 days (T4-60) differed substantially ($p<0.05$) among the treatments, with the greatest variance in T3 ($p<0.05$) but no statistical significance with T2.

When infected with bacterial pathogens, the values of CORT-30 and CORT-60 showed a substantial change with CORT70-Ah and CORT70-Et in CT and T1, however the values of CORT-30 and CORT-60 did not differ substantially ($p>0.05$) in T2 and T3, respectively. In challenged study, a substantial ($p<0.05$) differences was reported in the levels of T4 in each of the treatment groups (Fig. 65).

4.8.2.6. Antibody titre

The IgM level exhibited no statistically substantial difference between the treatments. When T2 was compared to the other groups after 60 days, a substantial ($p<0.05$) rise was seen, while there was no considerable increase ($p>0.05$) inT3 as compared with T2. Up to T2, the IgM level was considerably ($p>0.05$) enhanced with Ah and Et, although a modest reduction was noted in T3, 70 days after the infection. A non- substantial ($p>0.05$) relationship between Ah and the treatments was detected in CT and T1. However, when comparing T2 and T3, a modest drop was reported as compared to other groups ($p>0.05$) (Fig. 66).

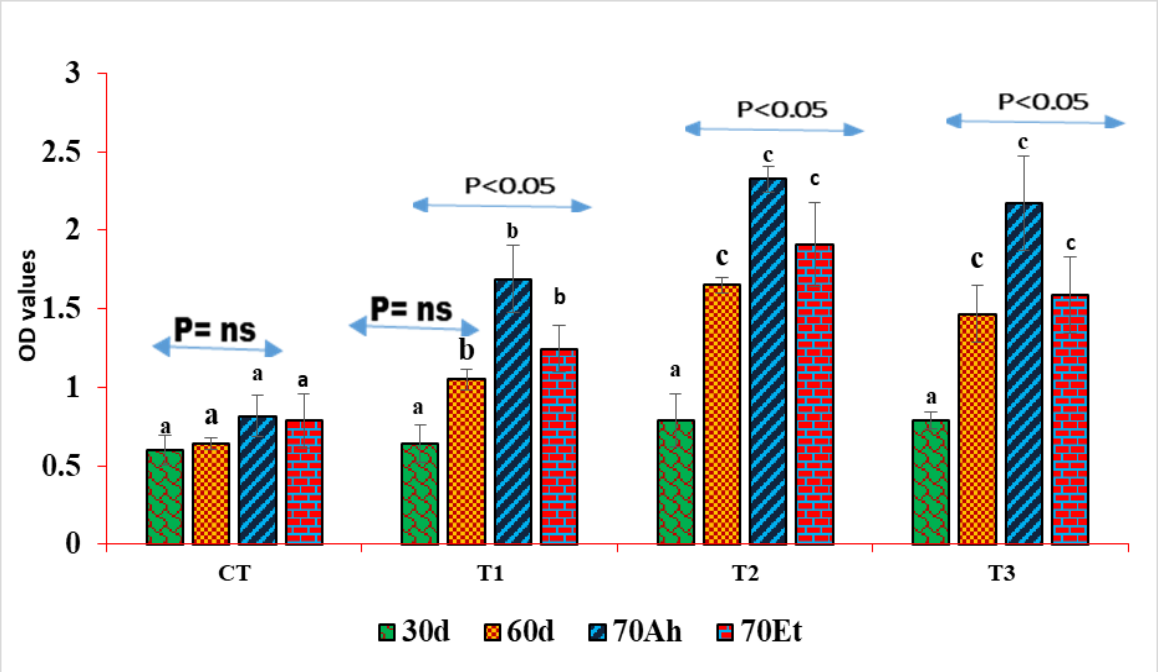

Fig. 66. Showing variation in serum immunoglobulin M (IgM) level of *L. rohita* in outdoor pond feed trial and challenge study

There was a considerable ($p<0.05$) variation between the treatments in T3-30, T3-60, T3-70Ah, and T3-70Et, with the exception of T3-60 in T1. There was a substantial ($p<0.05$) change in T3-60 in T2 as compared with the other treatments. There had been a substantial rise in values in all the treatments. Remarkably upon infection, T2 did not show any substantial ($p>0.05$) differences, in any of the parameters (Fig. 67).

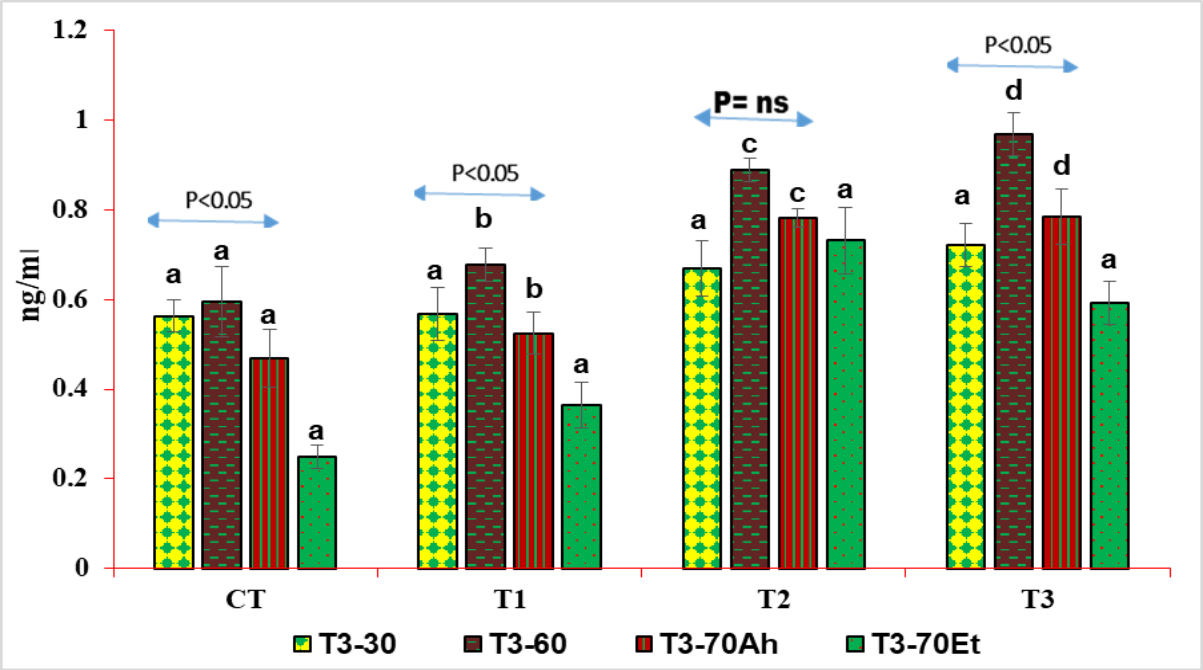

Fig. 67. Showing variation in serum triiodothyronine (T3) level of *L. rohita* in outdoor pond feed trial and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.8.2.7. Gut-metagenomics

In the pond experiment, a stack diagram was used to demonstrate the differences between treatments. The Bacillaceae occupy a larger area in TP2 than any of the other classifications. Across all treatments, the aeromonadaceae take up the most space, with the Clostridiaceae occupying the greatest space in TP3. The Residues of the Ruminococcaceae family were also identified in TP2. Other germs and bacteria that have not been grown can be found in all categories (Fig. 68).

When it comes to pond trials, bacteria can be classified into two primary clusters: cluster1 contains CP and TP1, while cluster2 includes TP2 and TP3. Clusters one and two had bootstrap values of 0.20 and 0.21, respectively, while cluster three had a value of 0.20. (Fig. 69).

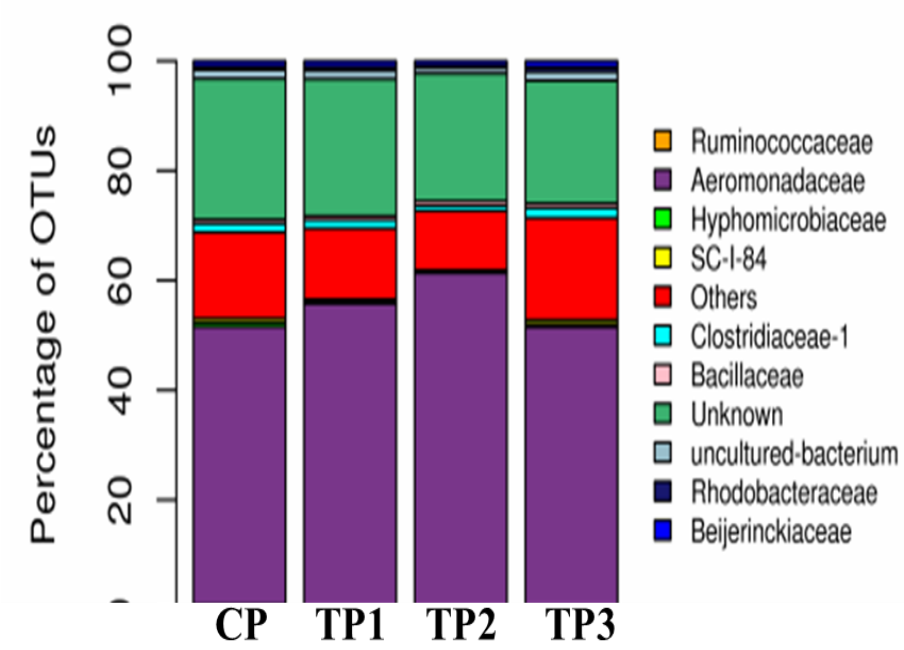

Fig. 68. Showing variation in gut-microbiome of *L. rohita* in outdoor pond feed trial

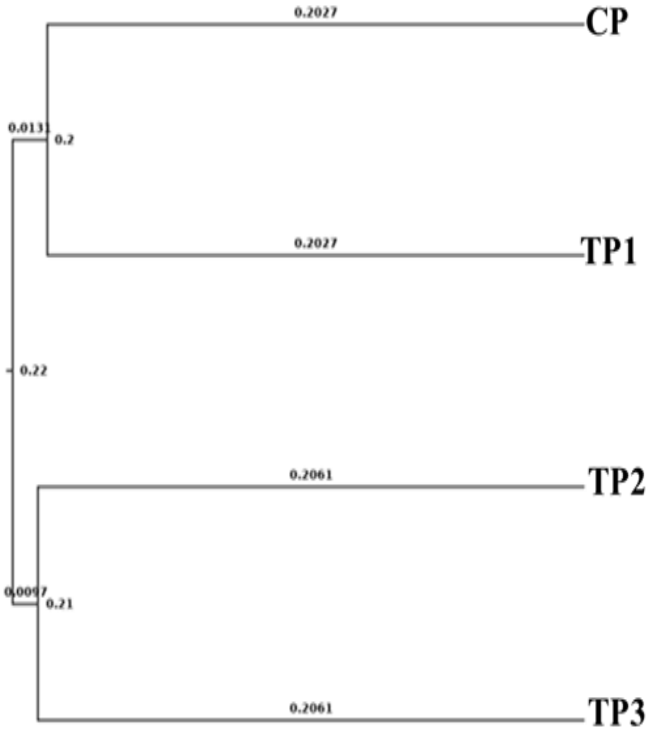

Fig. 69. Showing variation in association pattern of gut-micrbiome of *L. rohita* in outdoor pond feed trial

When comparing the comparative bacterial community structure based on their numerical abundance, dominance, and architectures, it was found that the pattern was different from the pattern found in their individual analyses. The combined stacked diagram revealed that the pattern of variation was the same in both trials. When comparing the pond experiment and the feed trial, it was discovered that the T2 treatment had a greater area of aeromonadaceae (Fig. 70).

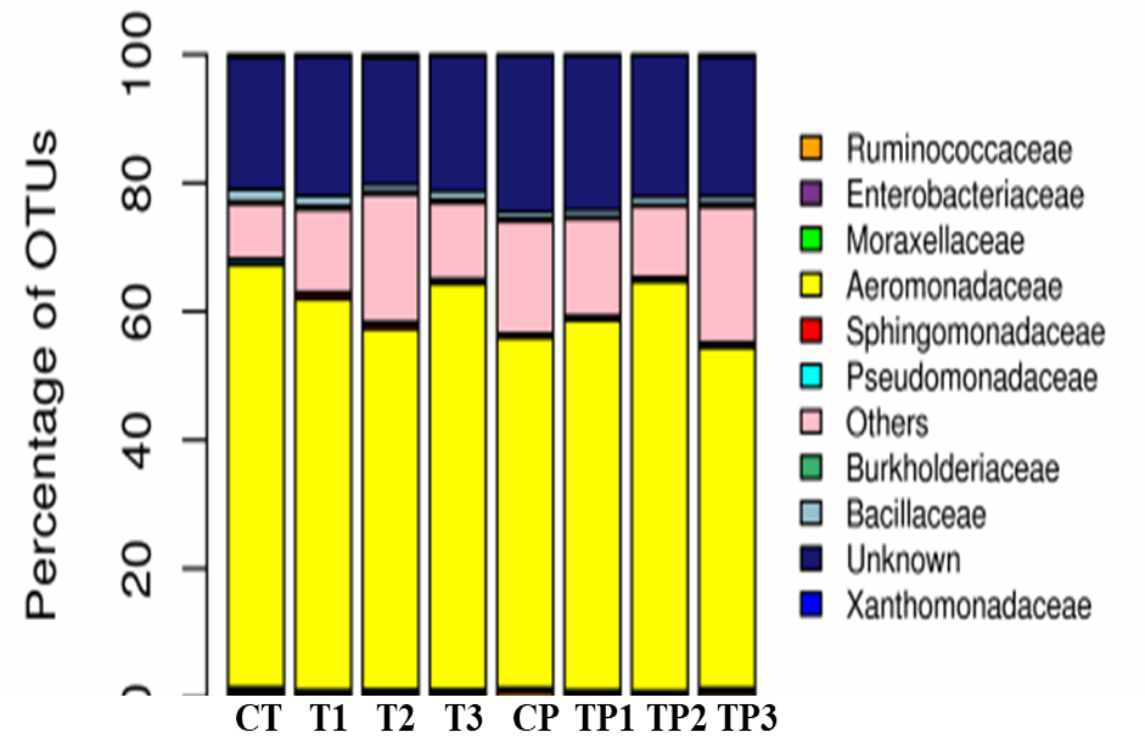

Fig. 70. Showing variation in combined gut-microbiome of pond and indoor feed trial of *L. rohita* in outdoor pond feed trial

Two primary clusters were identified in both the pond and feed trials; however, two subclusters were identified in the feed trial in cluster two. In the pond experiment, the bootstrap values were 0.22 and 0.23, and in the feed trial, the bootstrap values were 0.24, 0.25, and 0.27 for CT and T1, T2, and T3, respectively (Fig. 71).

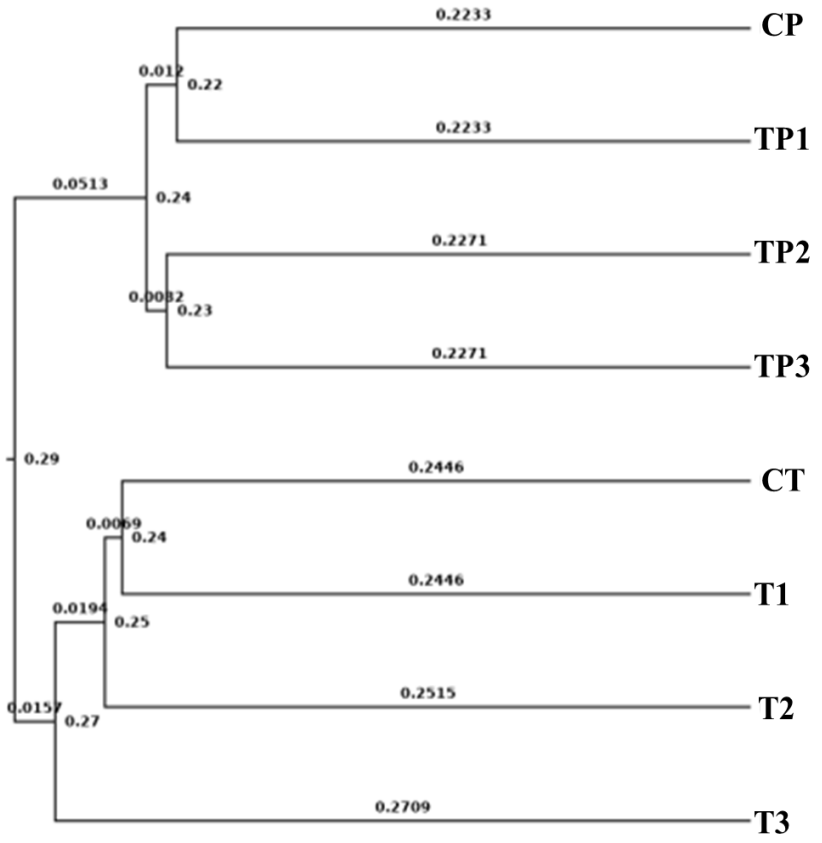

Fig. 71. Showing variation in association pattern of combined gut-micrbiome of *L. rohita* in indoor and outdoor pond feed trials

4.8.3. Intraperitoneal inoculation experiment

4.8.3.1. Serum enzymes

No significance ($p>0.05$) was reported in creatine kinase at 15 days (CKNac15), creatine kinase at 30 days (CKNac30) between the groups, but upon infection with bacterial pathogens an increasing trend was reported. Within treatment, CT showed substantial ($p<0.05$) increased among variables. T1 showed significance ($p<0.05$) among CKNac15, CKNac30 and creatine kinase at 25 days when fish was challenged with *E. tarda* (CKNac25-Et) but no significance ($p>0.05$) with creatine kinase at 25 days when fish was challenged with *A. hydrophila* (CKNac25-Ah). In T2 and T3, no significance ($p>0.05$) was reported among the variables. In CT, a significance was also reported ($p<0.05$) between CKNac25-Ah and CKNac25-Et (Fig. 72). The linear equation of variable of CKNac in T3 group is, $Y=10.037x+269.18$,

R^2=0.86. The linear equation of CKNac25-Ah and CKNac25-Et among CT, T1, T2 and T3 are; Y=-19.93x+367.67, R^2=0.92 and Y=-27.853x+418.58, R^2=0.89, respectively.

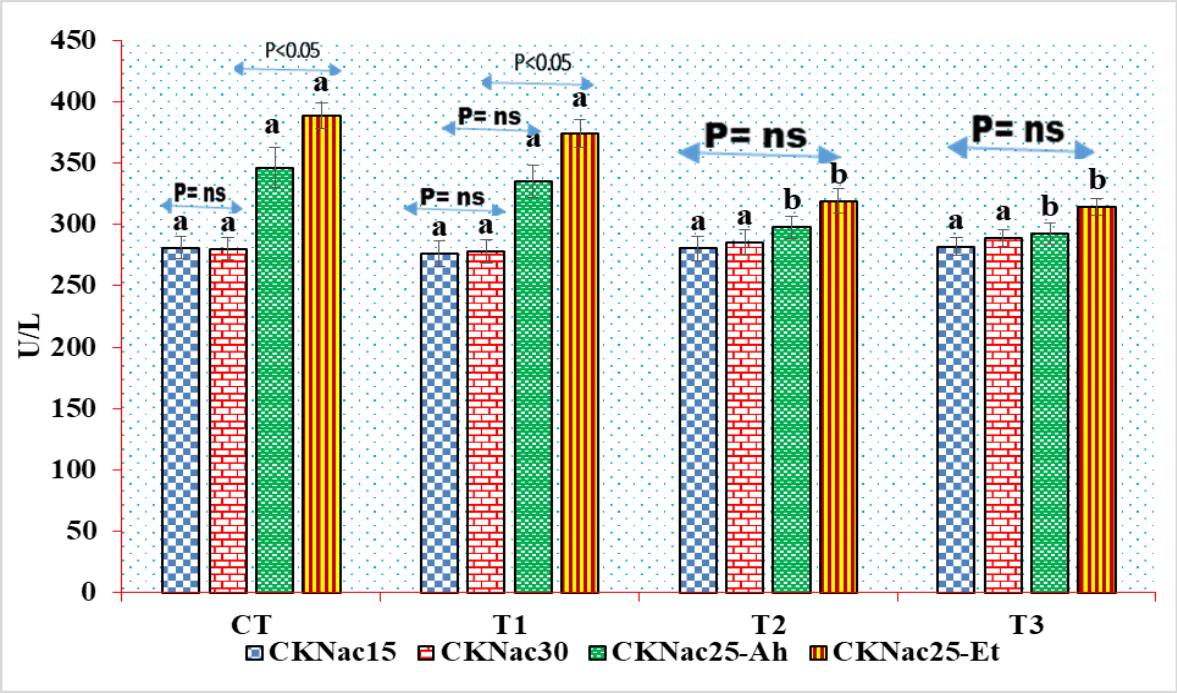

Fig. 72. Showing variation in serum creatine kinase (CKNac) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

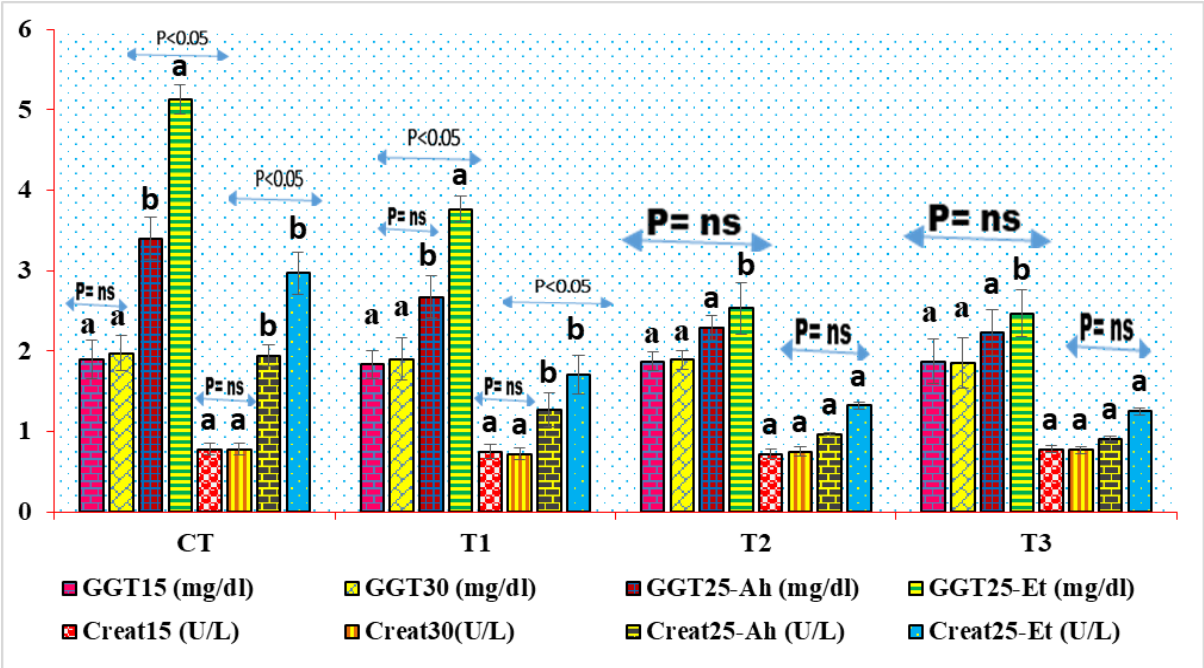

Fig. 73. Showing variation in serum creatine (Creat) and gamma glutamyl transferaseGGT values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

No statistically substantial differences (p>0.05) were seen between the treatments in the GGT15, Creat15, GGT30, and Creat30 measurements. In the comparisons between CT and T1, and T2 and T3, there was no substantial change was reported (p>0.05) in the levels of GGT25-Ah, Creat25-Ah, GGT25-Et, and Creat25-Et. When compared to the other groups, the values in T2 and T3 were considerably lower (p<0.05). In challenge study, the values levels increased in all treatments.

The GGT25-Ah and GGT25-Et showed a substantial (p<0.05) increase in CT when compared to GGR15 and GGT30. In comparison to Creat15 and Creat30, Creat25-Et and Creat25-Et followed the significance (p<0.05). While T2 and T3 exhibited remarkable (p<0.05) changes in both parameters within the groups, T1 and T2 did not differ considerably (Fig. 73). In the T3 group, the linear equations for GGT and Creat are Y=0.389x+3.65, R2=0.92, and Y=0.202x+0.43, R2=0.89. GGT25-Ah and Creat25-Et linear equations for CT, T1, T2, and T3 are Y=-0.389x+3.65, R2=0.87 and Y=-0.33x+2.12, R2=0.86, respectively.

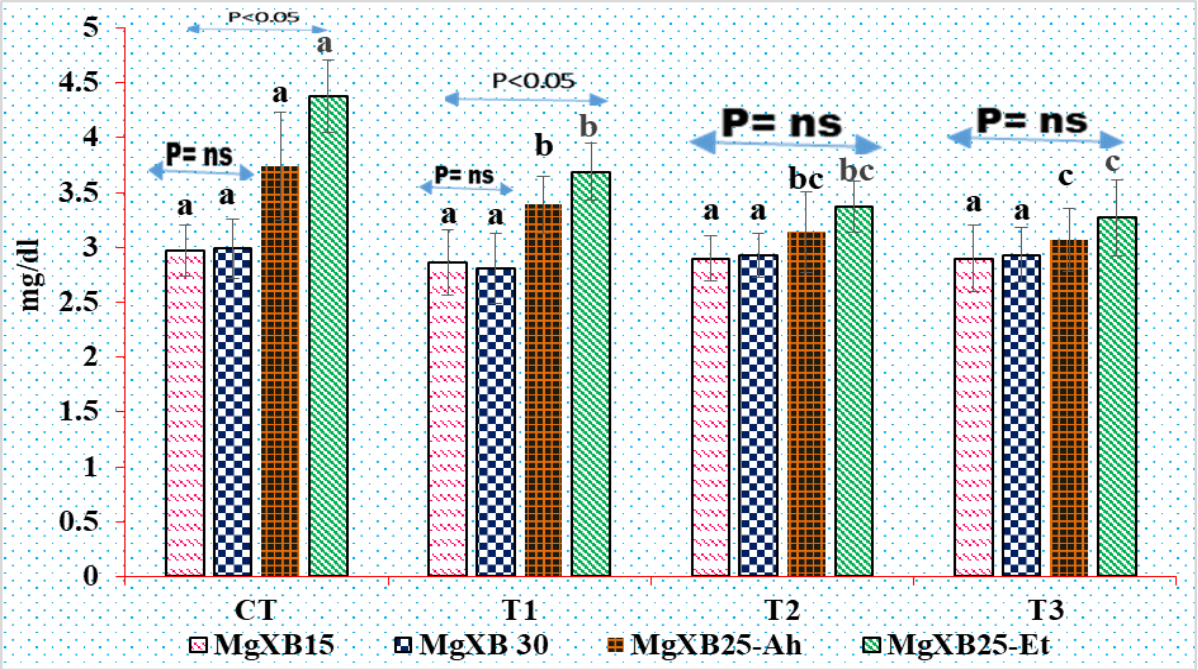

Fig. 74. Showing variation in serum MgXB values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The MgXB15, MgXB30, MgXB25-Ah and MgXB25-Et showed substantial ($p<0.05$) changes between all the treatments except MgXB25-Ah and MgXB25-Et in T2. In CT, substantial change was reported ($p<0.05$) between MgXB15 and MgXB30 with MgXB25-Ah and MgXB25-Et. In T1, no significance ($p>0.05$) was reported among MgXB15, MgXB30 and MgXB25-Ah but it has significance ($p<0.05$) with MgXB25-Et, whereas in T2 and T3 no significance ($p>0.05$) was reported among the MgXB variables (Fig. 74).

The linear equation of variable of MgXB in T3 group is, $Y=0.125x+2.73.18$, $R^2=0.91$. The linear equation of MgXB25-Ah and MgXB25-Et among CT, T1, T2 and T3 are; $Y=-0.226x+3.9$, $R^2=0.93$ and $Y=-0.365x+4.59$, $R^2=0.88$, respectively.

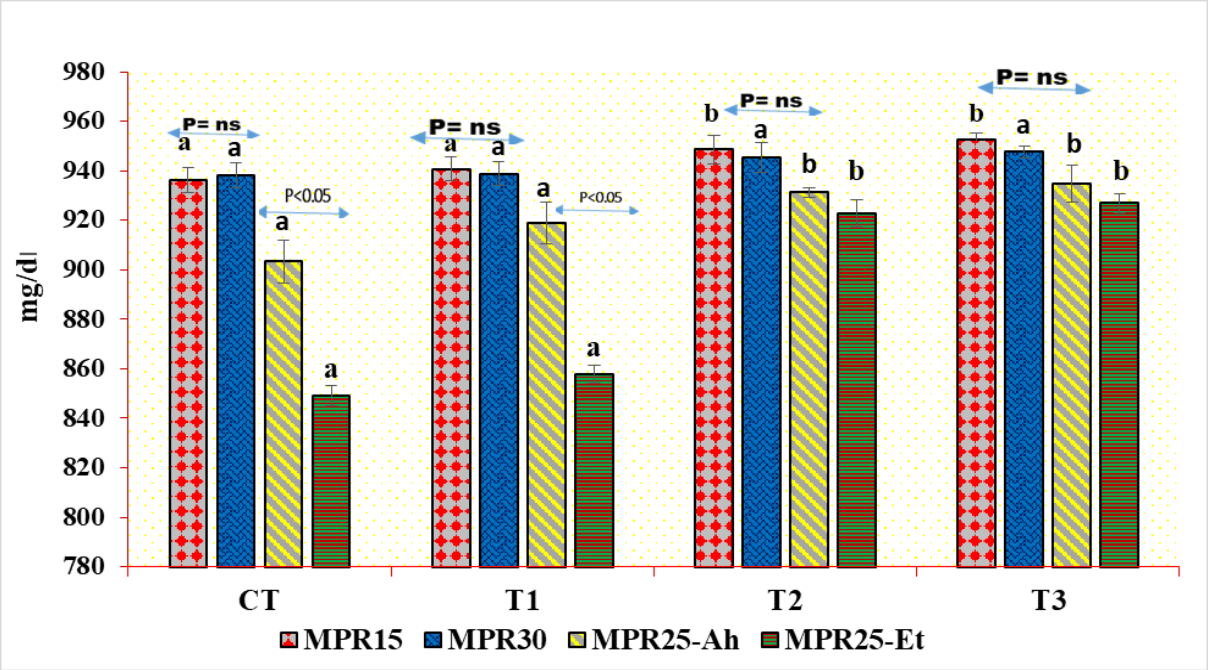

Fig. 75. Showing variation in serum microProtein (MPR) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

The microProtein at 15 days (MPR15) and microProtein at 30 days (MPR30) did not differ substantially ($p>0.05$) between CT and T1, but were considerable ($p<0.05$) in T2 and T3 when compared to other groups. The MPR30 levels in T2 and T3 were noticeably lower than MPR15 values. The microProtein at 25 days when fish was challenged with *A. hydrophila* (MPR25-Ah) and microProtein at 25 days when fish was challenged with *E. tarda* (MPR25-Et) exhibited a significance ($p<0.05$) in CT

and T1 compared to MPR15 and MPR30, while T2 and T3 did not show any significance ($p<0.05$) among the variables (Fig. 75). The linear equation of variable of MPR in T3 group are, $Y=-12.68x+967.91$, $R^2=0.95$. The linear equation of MPR25-Ah and MPR25-Et among CT, T1, T2 and T3 are; $Y=8.729x+898.92$, $R^2=0.79$, and $Y=26.82x+819.64$, $R^2=0.80$, respectively.

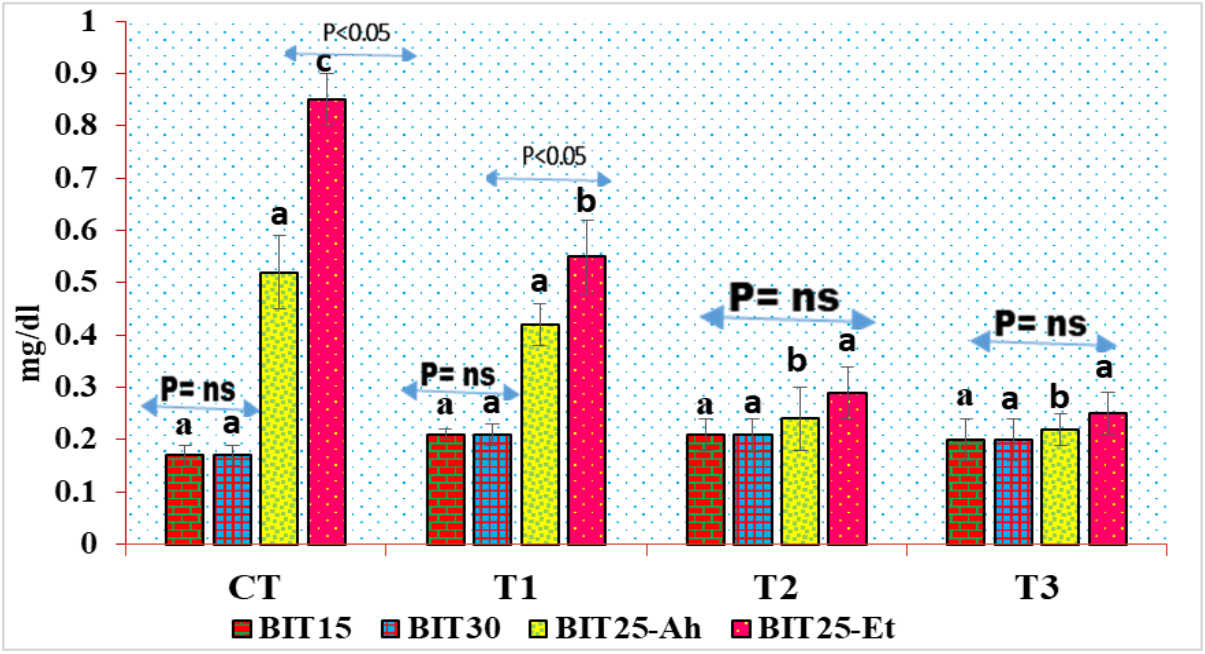

Fig. 76. Showing variation in serum bilirubin total (BIT) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

The bilirubin total at 15 days (BIT15) and bilirubin total at 30 days (BIT30) did not differ substantially between groups ($p>0.05$). The bilirubin total at 25 days when fish was challenged with *A. hydrophila* (BIT25-Ah) did not differ between CT and T1, nor between T2 and T3. The bilirubin total at 25 days when fish was challenged with *E. tarda* (BIT25-Et) differed across CT, T1, T2, and T3. The BIT15, BIT30, BIT25-Ah, and BIT25-Et differed considerably in CT and T1 ($p<0.05$). the values of BIT variables in T2 and T3 did not differ substantially ($p>0.05$) between groups (Fig. 76). In the T3 group, the linear equation of variable BIT is $Y=0.027x+0.15$, $R2=0.86$. $Y=-0.206x+1$, $R2=0.92$ and $Y=-0.108x+0.62$, $R2=0.93$, respectively, for BIT25-Ah and BIT25-Et among CT, T1, T2, and T3.

The triglycerides at 15 days (Trig15), triglycerides at 30 days (Trig30), lactate dehydrogenase at 15 days (LDH15), lactate dehydrogenase at 30 days (LDH30), cholesterol at 15 days (CHOL15), and cholesterol at 30 days (CHOL30) results did not differ substantially ($p>0.05$) between groups; however, values were marginally higher after 30 days than at 15 days. Except for CT and T1, triglycerides at 25 days when fish was challenged with *A. hydrophilla* (Trig25-Ah) varied considerably ($p<0.05$), whereas, triglycerides at 25 days when fish was challenged with *E. tarda* (Trig25-Et) exhibited no significance ($p>0.05$) in T2 and T3.

The lactate dehydrogenase at 25 days when fish was challenged with *A. hydrophilla* (LDH25-Ah) exhibited a considerable ($p<0.05$) relationship between CT and T1, but no relationship ($p>0.05$) between T2 and T3, and the lactate dehydrogenase at 25 days when fish was challenged with *E. tarda* (LDH25-Et) also showed the similar trend (Fig. 77). The CHOL also followed same trend for both LDH25-Ah and LDH25-Et as like as CHOL25-Ah, CHOL25-Et and LDH25-Ah and LDH25-Et. The linear equation of variable of Trig, LDH and CHOL in T3 group are, $Y=6.62x+110.1$, $R^2=0.97$, $Y=26.09x+532.01$, $R^2=0.91$ and $Y=6.934x+136.33$, $R^2=0.93$, respectively.

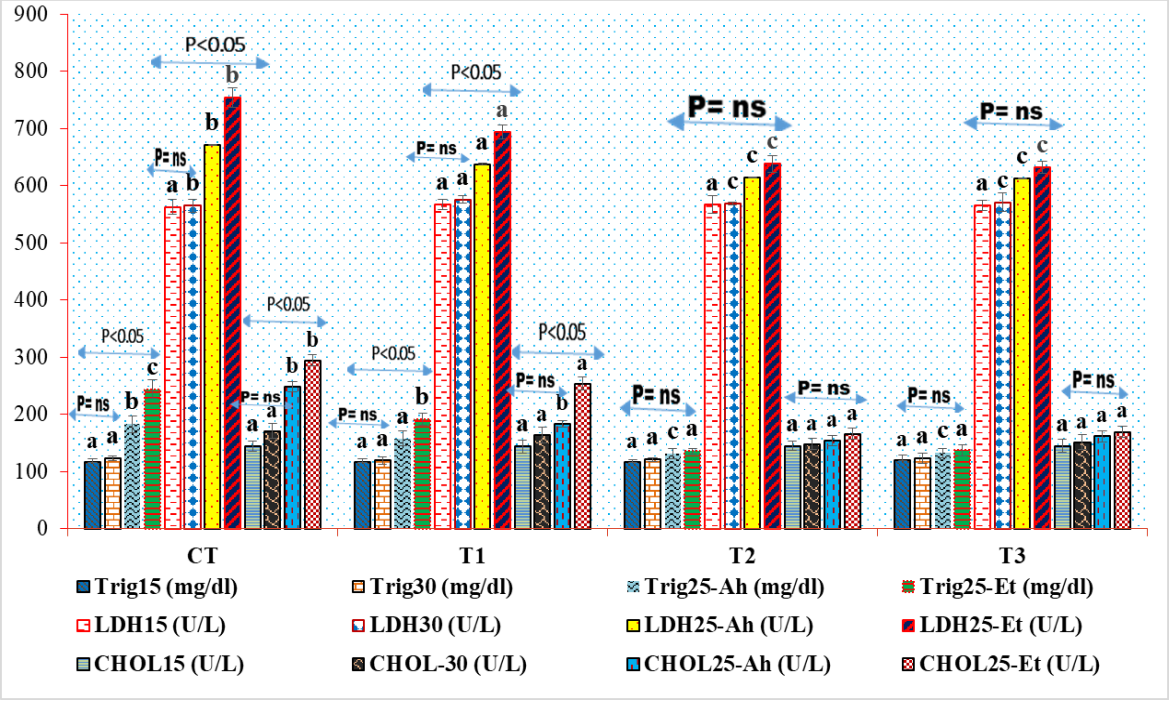

Fig. 77. Showing variation in serum triglycerides (Trig), lactate dehydrogenase (LDH) and cholesterol (CHOL) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

The glucose at 15 days (GLU15), glucose at 30 days (GLU30) and glucose at 25 days when fish was challenged with *A. hydrophila* (GLU25-Ah) exhibited no significance ($p>0.05$) among the groups but glucose at 25 days when fish was challenged with *E. tarda* (GLU25-Et) showed significance ($p<0.05$) with CT. Upon challenge with bacterial pathogens, the values are increasing. In CT, the values are considerably ($p<0.05$) increasing between GLU25-Ah and GLU-25-Et as compared to GLU15. In T1, no significance ($p>0.05$) between GLU15 and GLU25-Ah but it has significance ($p<0.05$) with GLU25-Et. The T2 and T3, exhibited no significance ($p>0.05$) between GLU15, GLU30, GLU25-Ah and GLU25-Et was reported (Fig. 78).

The linear equation of variable of GLU in T3 group is, $Y=4.725x+93.73$, $R^2=0.85$. The linear equation of GLU25-Ah and GLU25-Et among CT, T1, T2 and T3 are; $Y=-12.462x+151.14$, $R^2=0.93$ and $Y=-21.45x+194.17$, $R^2=0.91$, respectively.

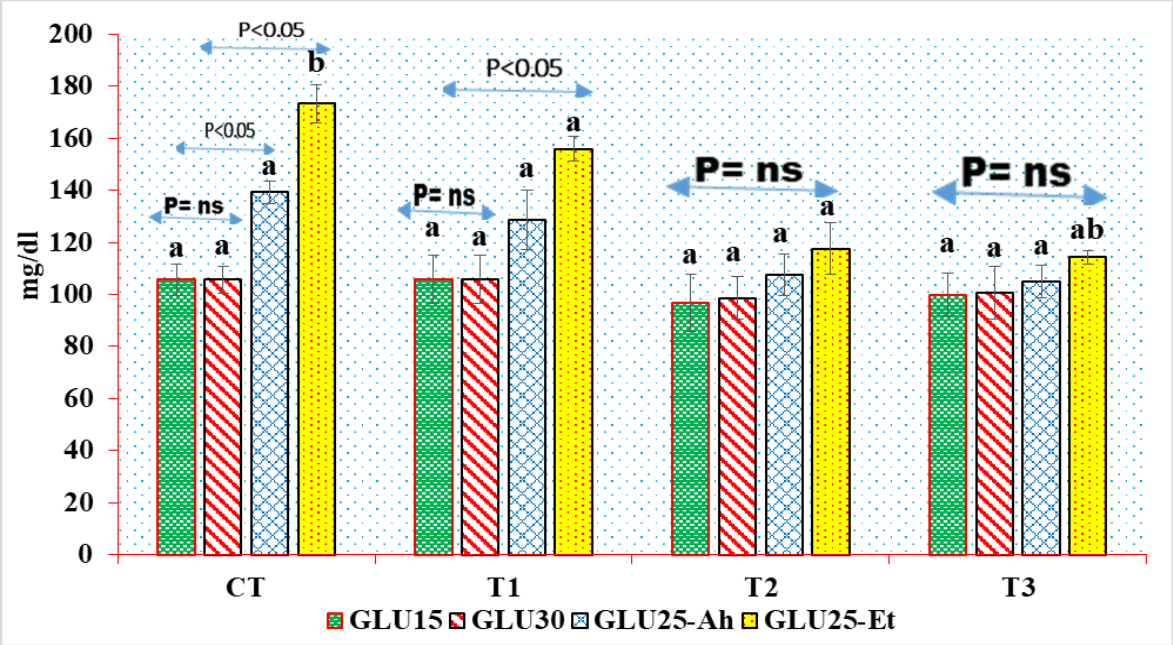

Fig. 78. Showing variation in serum glucose (GLU) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

Except for serum glutamic oxaloacetic transaminase at 30 days (SGOT30), which did not vary considerably ($p>0.05$) in treatments CT and T3, the serum glutamic oxaloacetic transaminase at 15 days (SGOT15), SGOT30, serum glutamic oxaloacetic transaminase at 25 days when fish was challenged with *A. hydrophila* (SGOT25-Ah), and serum glutamic oxaloacetic transaminase at 25 days when fish

was challenged with *E. tarda* (SGOT-25Et) differ considerably ($p<0.05$) between treatments. The enzymes SGOT15, SGOT30, SGOT25-Ah, and SGOT-25Et differ insignificantly ($p>0.05$) in treatments CT, T1 and T2, but not in T3 and T2. The SGOT30 had a substantial ($p<0.05$) variation in T1 among the treatments.

There was no substantial change was reported ($p>0.05$) between treatments T2 and T3 after infection. Except for amylase at 15 days (AMY15) and amylase at 25 days when fish was challenged with *A. hydrophila* (AMY25-Ah) in T1, (AMY30 in T2, and AMY15 in T3, there was a substantial change ($p<0.05$) between treatments for AMY15, AMY30, and amylase at 25 days when fish was challenged with *E. tarda* (AMY25-Et). The AMY15 and AMY25-Et were the only treatments that exhibited a substantial ($p<0.05$) variation in CT. In treatments T2 and T3, there was no significance ($p>0.05$) between AMY15 and AMY25-Et after infection with Ah and Et. There was a remarkable ($p<0.05$) rise in the values for AMY25-Et until T1, then there was a substantial ($p<0.05$) reduction in values. The AMY25-Ah was steady across all treatments (Fig. 79).

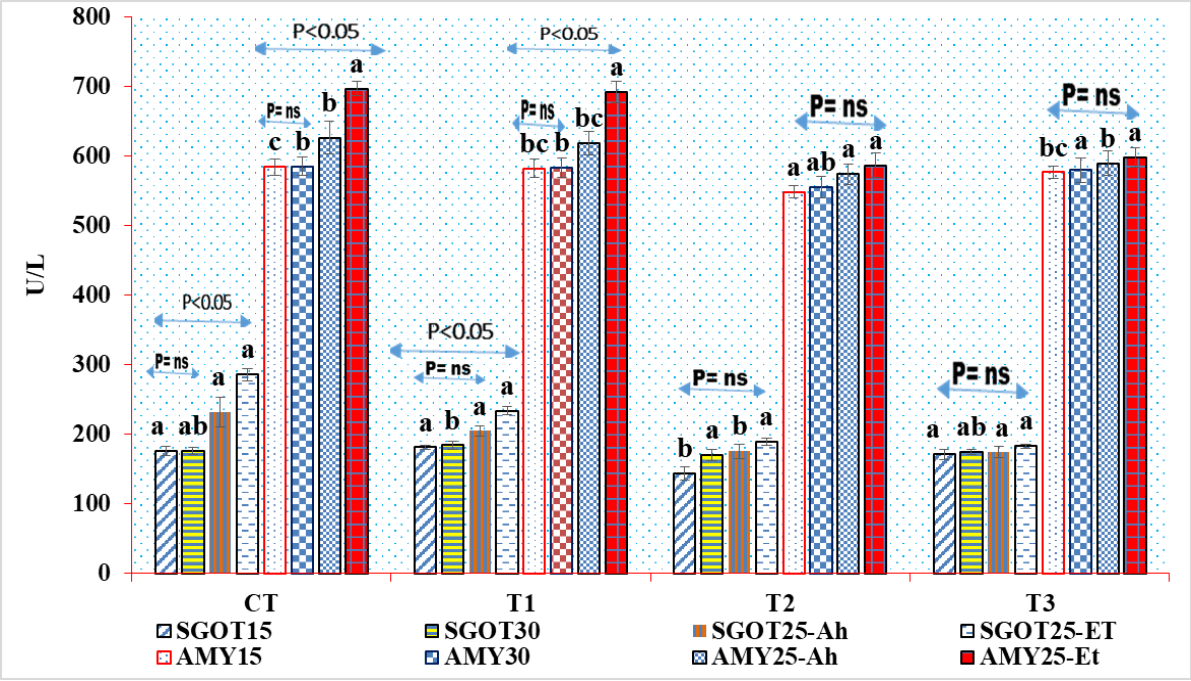

Fig. 79. Showing variation in serum glutamic oxaloacetic transaminase (SGOT) and amylase (AMY) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

The linear equation of variable of SGOT in T2 group is, Y=3.596x+167.18, R^2=0.88. The linear equation of SGOT25-Ah and SGOT25-Et among CT, T1, T2 and T3 are; Y=-19.74x+246.61, R^2=0.89 and Y=-35.353x+311.58, R^2=0.92, respectively. The linear equation of variable of AMY in T3 group is, Y=7.465x+567.18, R^2=0.97. The linear equation of AMY25-Ah and AMY25-Et among CT, T1, T2 and T3 are; Y=-15.346x+640.5, R^2=0.65 and Y=-40.167x+744, R^2=0.77, respectively.

The SGPTD15 decreased considerably ($p<0.05$) in T2, but not in the other treatments ($p>0.05$). The SGPTD30 demonstrated a substantial ($p<0.05$) rise in T2 after 30 days when compared to other treatments. After infection, the values of SGPTD25-Ah and SGPTD25-Et increased remakably ($p<0.05$), but it has no significane ($p>0.05$) in T2 and T3.

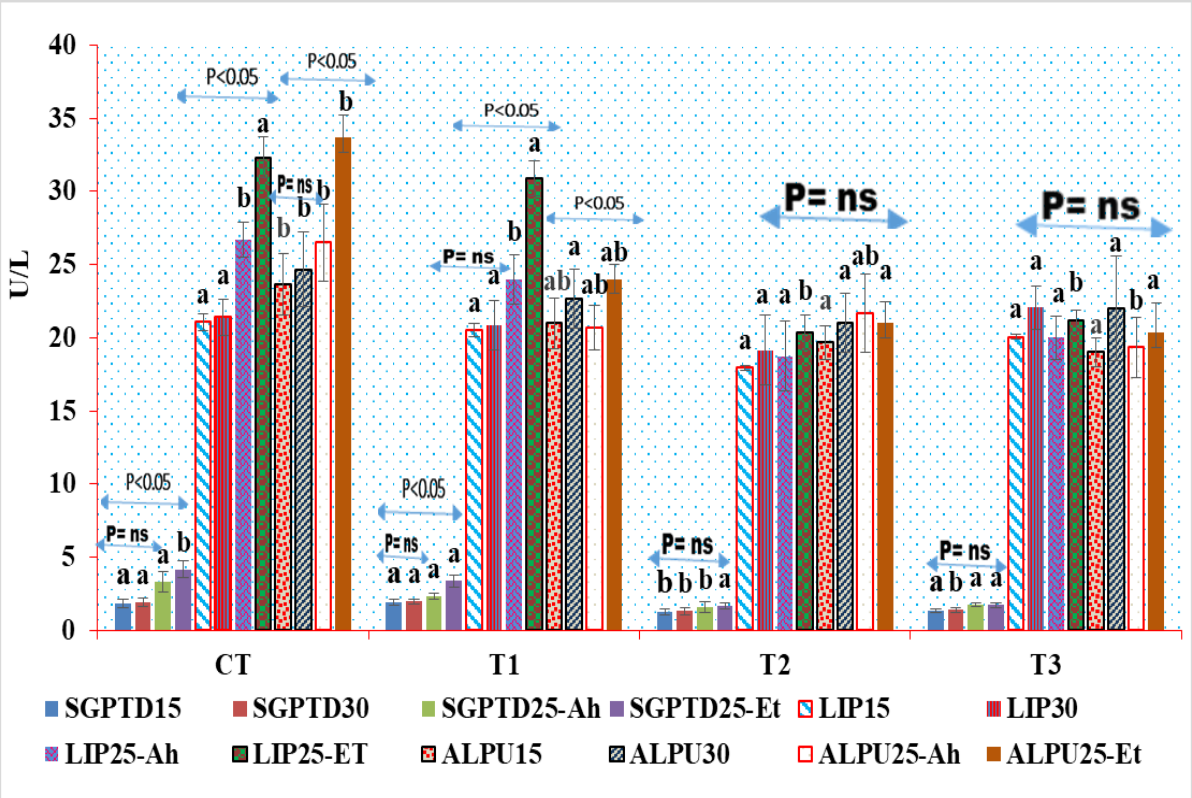

Fig. 80. Showing variation in serum glutamic pyruvic transaminase (SGPTD), lipase (LIP) and alkaline phosphatase (ALPU) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

The LIP15 and LIP30 did not differ substantially ($p>0.05$) between treatments. The LIP25-Ah and LIP25-Et differ considerably ($p<0.05$) in T2 and T3 when

compared to other treatments. Within treatments, the values of LIP25-Ah and LIP25-Et increased considerably when compared to LIP25 and LIP30 in CT, however no significance ($p>0.05$) was seen within the treatments in T2 and T3. ALPU15 varies considerably ($p<0.05$) among treatments when compared to CT, and the same trend with greater values was reported for ALPU30. The ALPU25-Ah and ALPU25-Et values were sustantial ($p<0.05$) among the treatments. Within treatments, CT showed a substantial ($p<0.05$) rise in ALPU25-Ah and ALPU25-Et when compared to ALPU25 and ALPU30 in CT, but T2 and T3 showed no significance ($p>0.05$) (Fig. 80).

The linear equation of variable of SGPTD in T3 group is, $Y=0.1725x+1.83$, $R^2=0.79$. The linear equation of SGPTD25-Ah and SGPTD25-Et among CT, T1, T2 and T3 are; $Y=-0.544x+3.62$, $R^2=0.79$ and $Y=-0.899x+4.97$, $R^2=0.89$, respectively. The linear regression equation of variable of LIP in T3 group is, $Y=0.1725x+1.83$, $R^2=0.79$. The linear equation of LIP25-Ah and LIP25-Et among CT, T1, T2 and T3 are; $Y=-0.544x+3.62$, $R^2=0.79$ and $Y=-0.899x+4.97$, $R^2=0.89$, respectively.

The linear regression equation of variable of ALPU in T3 group is, $Y=0.1725x+1.83$, $R^2=0.79$. The linear equation of ALPU25-Ah and ALPU25-Et among CT, T1, T2 and T3 are; $Y=-0.544x+3.62$, $R^2=0.79$ and $Y=-0.899x+4.97$, $R^2=0.89$, respectively.

4.8.3.2. Digestive enzymes

Except for CT and T1, the values of digestive enzymes decreased insignificantly ($p>0.05$) after intraperitoneal inoculation when compared to enzymatic activity at 15 days. Enzymatic activity increased considerably ($p<0.05$) in all treatments as compared to CT. The T3 had the most activity at 15 days, followed by T2>T1 and CT. Following infection with Ah and Et, the treatments followed the same pattern. The CT and T1 levels were found to be considerably ($p<0.05$) lower in the treatments after infection. In T2 and T3, there was no significance was reported ($p>0.05$), although PROT15 activity after infection exhibited significance ($p<0.05$) with PROT25-Et (Fig. 81).

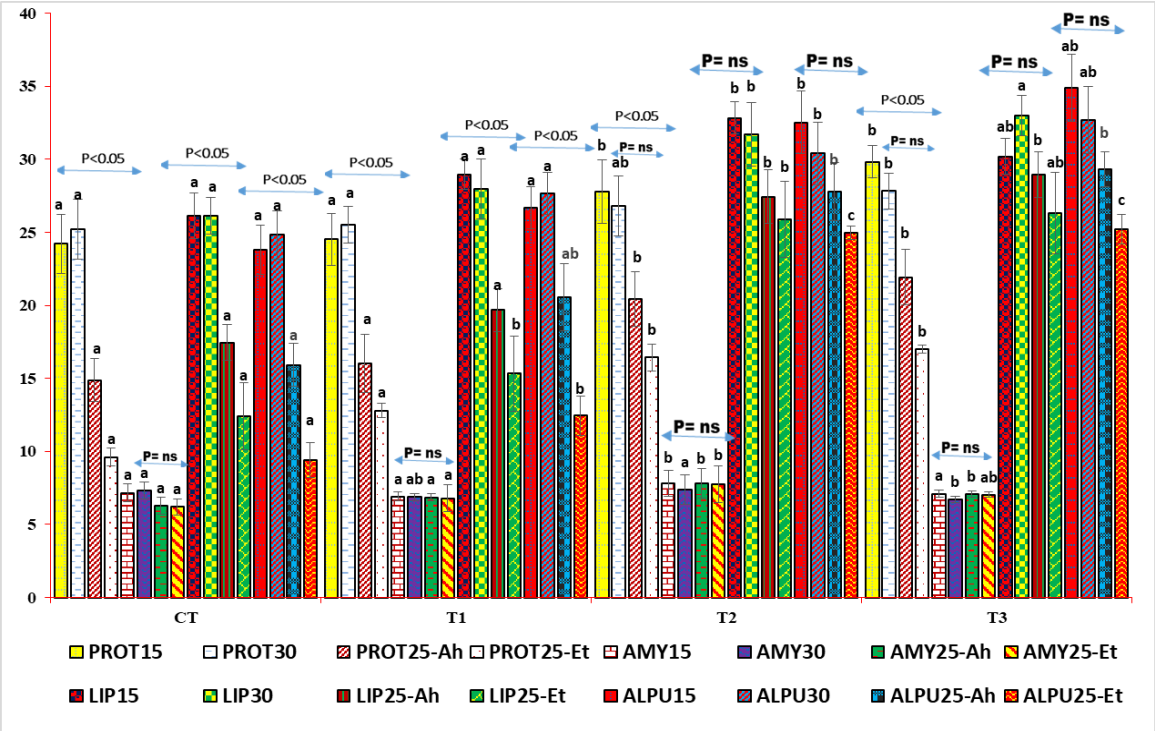

Fig. 81. Showing variation in digestive proteases (PROT), amylase (AMY), lipase (LIP) and alkaline phosphatase (ALPU) values of *L. rohita* in Intraperitoneal inoculation experiment and challenge study

Here, protease activity expressed as micromoles of tyrosine released/min/mg protein at 37 ℃; Maltose activity expressed as micromoles of maltose released/min/mg protein at 37 ℃; Lipase activity expressed as units/mg protein at 37℃; Alkaline phosphatase activities expressed as nanomoles p-nitrophenol released/min/mg protein at 37 ℃.

4.8.3.3. Enzyme of oxidative stress

In intraperitoneal inoculation experiment, catalase at 15 days (CAT15) and catalase at 30 days (CAT30) exhibited no significance ($p<0.05$) among the treatments. While, superoxide dismutase at 15 days (SOD15), superoxide dismutase at 30 days (SOD30), glutathione-s-tranferase at 15 days (GST15) and glutathione-s-tranferase at 30 days (GST30) varied considerably ($p<0.05$) among the treatments. Upon infection, the value of CAT25-Ah, CAT25-Et, SOD15, SOD30, GST15 and GST30 showed significance ($p<0.05$) among the treatments. Within the treatments, values increased substantially ($p<0.05$) in CT and T1, but T2 and T3 showed non-substantial ($p>0.05$) rise in the value. The enzyme activity increases non-substantially ($p>0.05$) at 30 days as compared with 15 days except CT in which the activity was not declined much after 30 days (Fig. 82). The linear equations for

CAT15, SOD15 and GST15 are as follows, Y= -0.7141x + 26.37, R^2=0.98; Y=-0.9082x+24.408, R^2=0.92and Y= -0.0337x+0.3057, R^2=0.89, respectively.

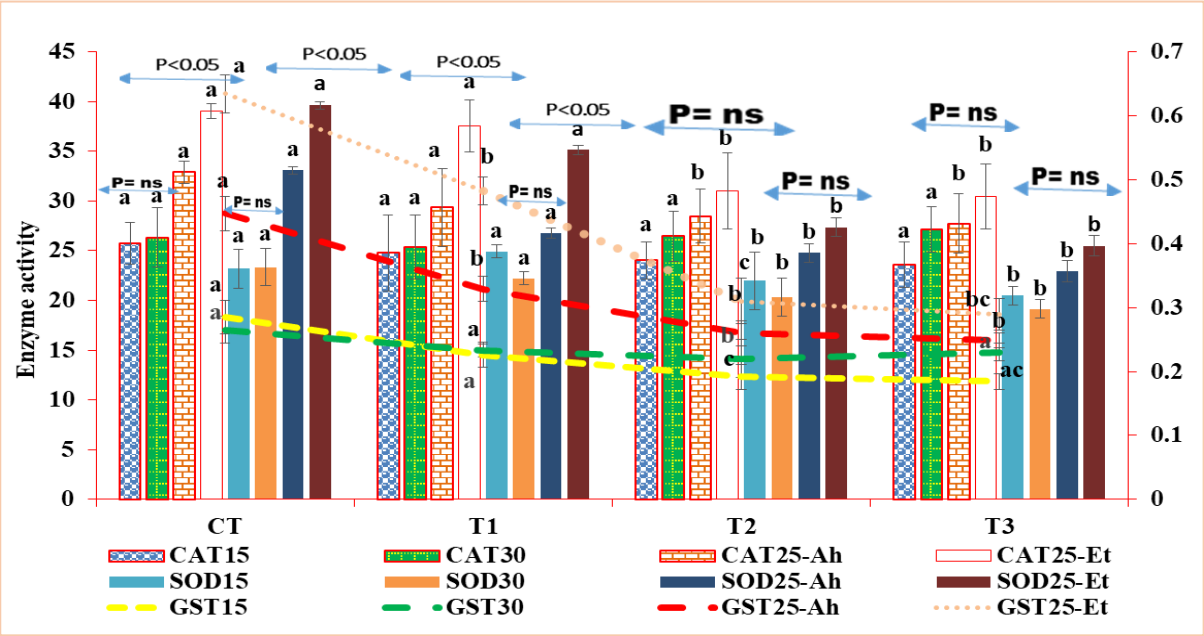

Fig. 82. Showing variation in catalase (CAT), superoxide dismutase (SOD) and glutathione-s-transferase (GST) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

Here, Catalase: mmol H_2O_2 decomposed /min/ mg protein at 37 ℃; SOD (superoxide dismutase): µmol/mg protein/min at 37 ℃; GST: Glutathione-S-transferase Units/mg protein.

4.8.3.4. Enzyme of neuro-transmission

In intraperitoneal inoculation experiment, AchE15, AchE30, AchE25-Ah, AchE25-Et exhibited no significance ($p<0.05$). There was no significance ($p>0.05$) change between T3 and T1 and, T3 and T2. Upon infection with Ah, AchE25-Ah and AchE25-Et exhibited no substantial change ($p>0.05$) between CT and T1, and T2 and T3. Within treatments, a considerable ($p<0.05$) rise was reported in CT and T1, and T2 and T3 (Fig. 83). The linear equation for AchE15 is as follows, Y=-0.2802x+3.1063, R^2= 0.86.

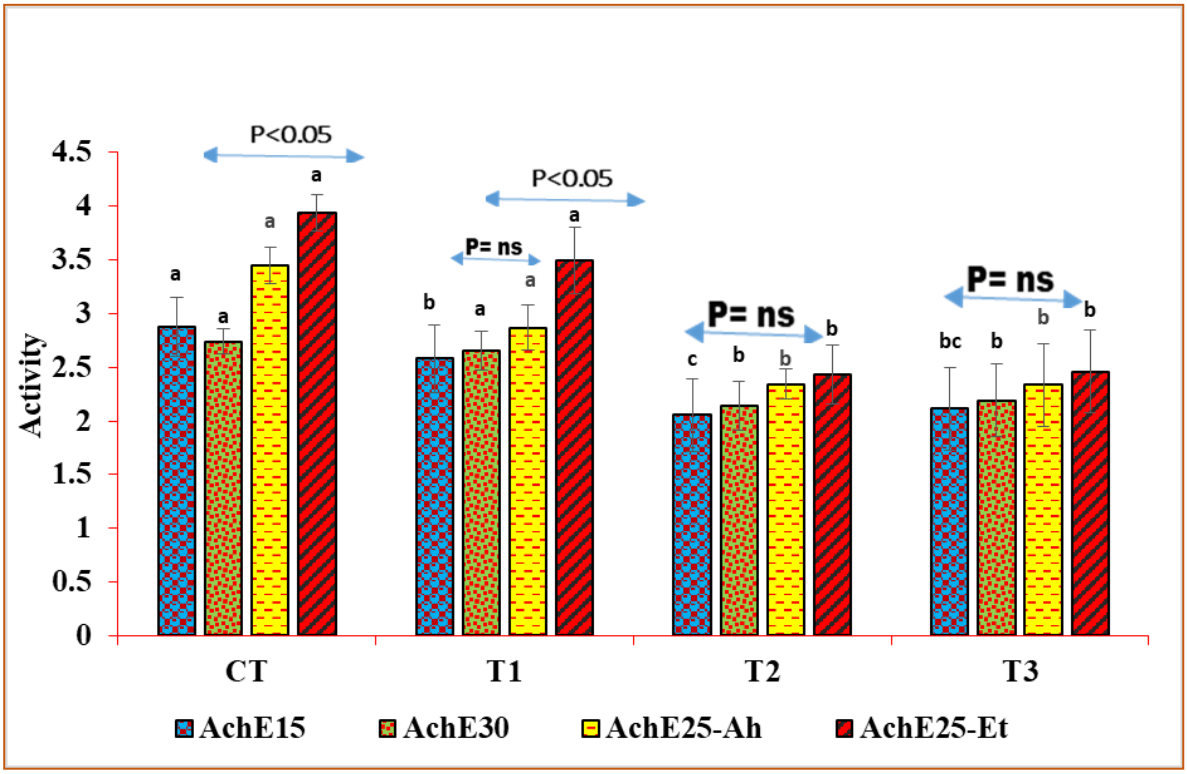

Fig. 83. Showing variation in acetyl choline esterase (AchE) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

4.8.3.5. Triiodothyronine (T3), thyroxine(T4) and cortisol

The T3-15 and T3-30 differed non-considerably ($p<0.05$) between CT and T1, and T2 and T3. Upon infection, the values differed substantially ($p<0.05$) among the treatment, being maximum in T3 but no significance ($p>0.05$) with T2. Upon infection, within the treatment's values decreased considerably ($p<0.05$) in CT and T1 but T2 and T3 exhibited no significance ($p>0.05$) (Fig. 84).

The parameters, CORT-15, CORT-30, T4-15, and T4-30 did not demonstrate any substantial ($p<0.05$) relationship between CT and T1, or T2 and T3. CT indicated a considerable rise ($p<0.05$) with other treatment after infection. The CT exhibited a substantial ($p<0.05$) rise in CORT25-Ah and CORT25-ET after infection as compared with CORT-15 and CORT-30.

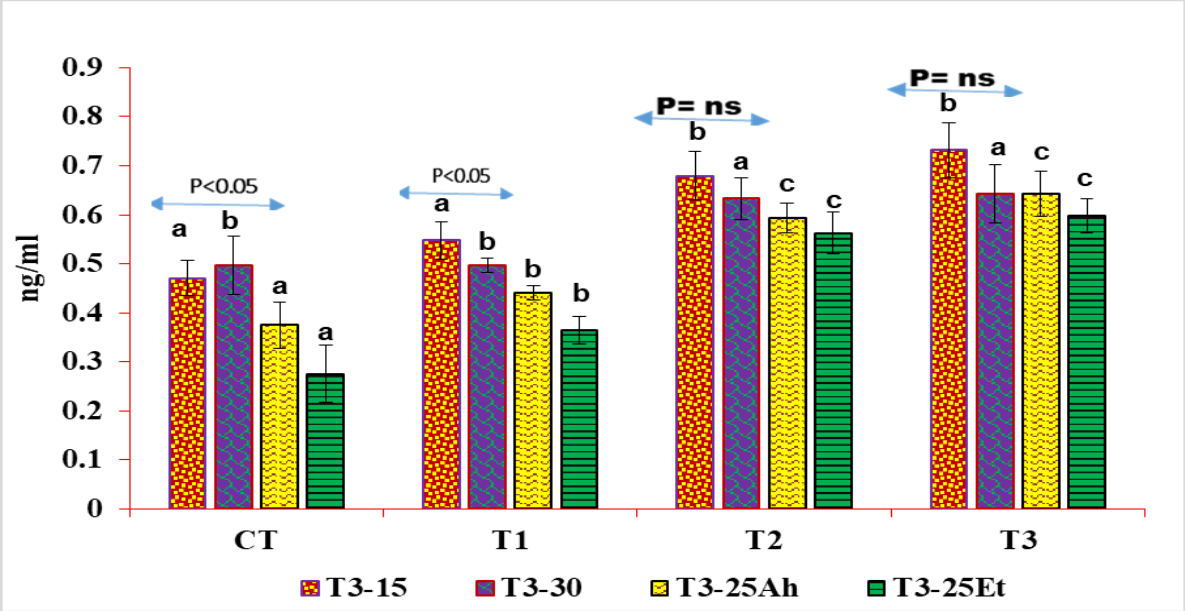

Fig. 84. Showing variation in triiodothyronine (T3) values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

The CORT-15 and CORT-30 had no significance (p>0.05) for CORT25-Ah in T1, but they did have significance (p<0.05) for CORT25-Et. The T2 and T3 on the other hand did not demonstrate any significance (p>0.05). When bacterial pathogens were infected, the value of T4 reduced considerably (p<0.05) between T4-15, T4-30, T4-25-Ah, and T4-25Et in CT and T1 groups/treatments compared to other treatments. There was no substantial variation was reported (p>0.05) in T2 and T3 following infection (Fig. 85).

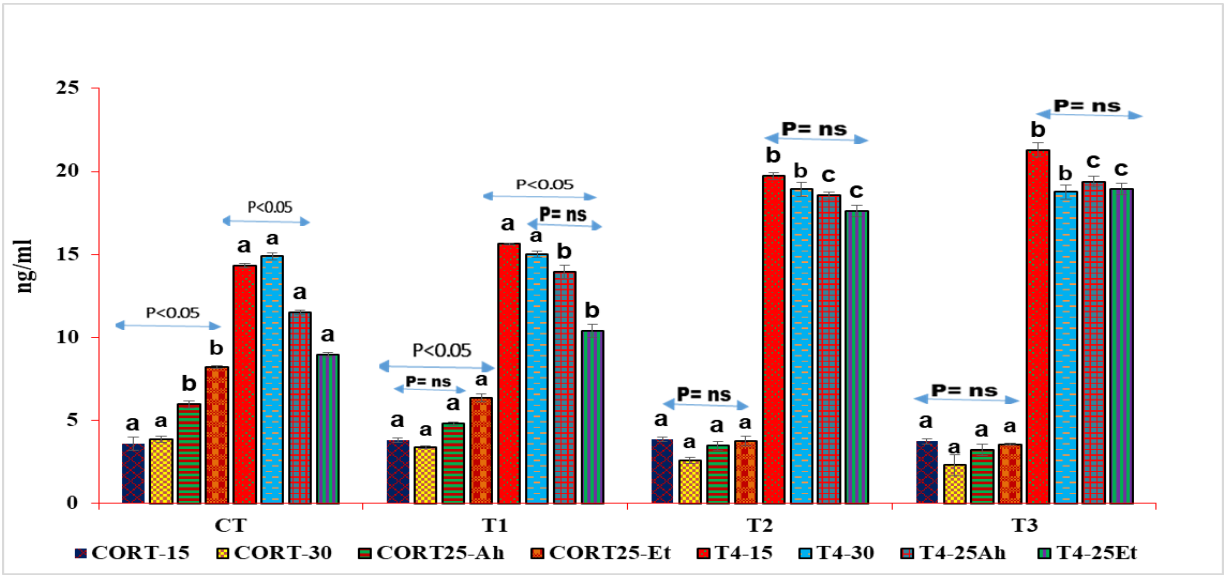

Fig. 85. Showing variation in thyroxine (T4) and cortisol values of *L. rohita* in intraperitoneal inoculation experiment and challenge study

4.8.3.6. Antibody titre

In the intraperitoneal inoculation experiment, the IgM level increased considerably ($p<0.05$) after 15 and 30 days, however there was a modest reduction at 30 days in T1, T2, and T3. In challenge study, a substantially ($p<0.05$) rising pattern was seen. A variety of patterns were seen within the treatments. At 15 days, 30 days, and 25Ah and 25Et in CT, there was no considerable variation was reported ($p>0.05$). In T1, there was no substantial change was seen ($p>0.05$) between 15 and 30 days, but there was a considerable ($p>0.05$) rise between 15 days, 30 days, and 25Ah, but no substantial ($p>0.05$) reduction with 25Et.

In T2, there was a statistical significance was reported ($p<0.05$) between 15 days, 30 days, 25Ah, and 25Et. In T3, there was no substantial change eas seen ($p>0.05$) between 15 and 30 days, but a substantial rise in 25Ah and 25Et was found. The 25Et and 25Ah exhibited a substantial ($p<0.05$) rise when compared to 15 and 30 days, but a non-significant ($p>0.05$) decline was reported when compared to 25Ah (Fig. 86).

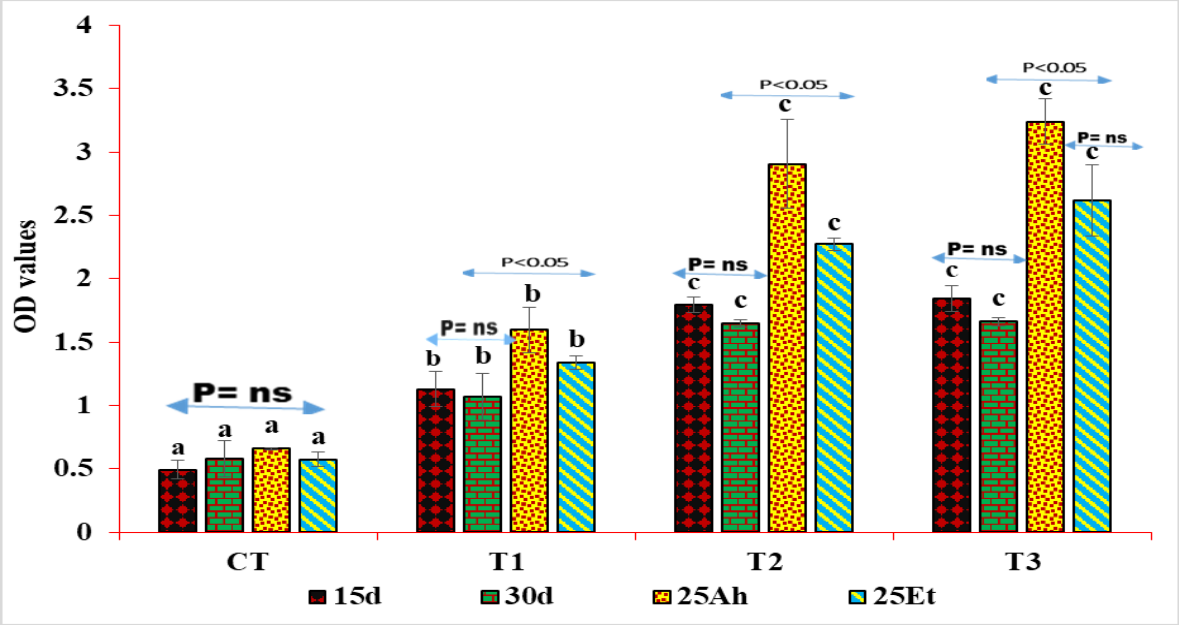

Fig. 86. Showing variation in serum immunoglobulin M (IgM) level of *L. rohita* in intraperitoneal inoculation experiment and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

4.8.3.7. Gene expression

4.8.3.7.1. RNA isolation

For gene expression studies RNA was isolated using Qiagen RNA isolation Kit. Isolated RNA was separated on 1% agarose gel and both 28s and 18s RNA bands were seen (Fig. 87).

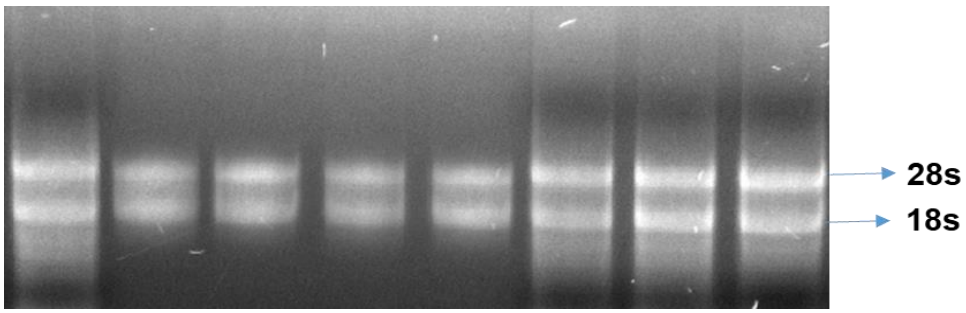

Fig. 87. Showing two distinct RNA bands (28s & 18s) in gel in intraperitoneal inoculation experiment

4.8.3.7.2. Semi-quantitative PCR

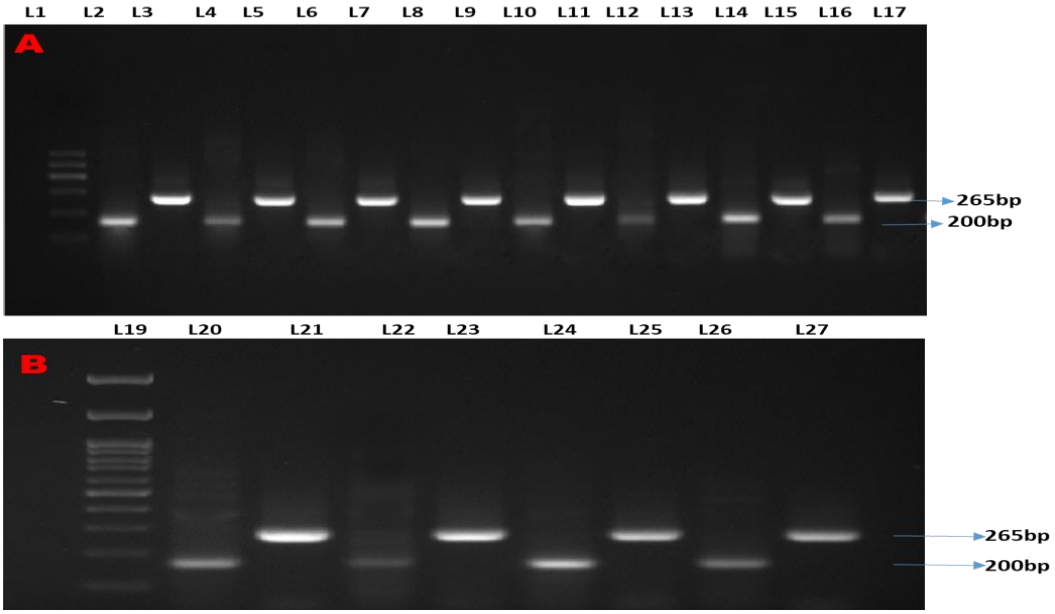

Fig.88a. Showing semi-quantitative PCR picture of ISG15 gene

Legends: L-1, 11 and 19: 1Kb molecular marker; L-2,4,6,8,11,13, 15, 17, 20, 22, 24 and 26: β-actin (housekeeping gene); L-3: CT15; L-5: T1-15; L-7: T2-15; L-9: T3-15; L-12: CT-25Ah; L-14: T1-25Ah; L-16: T2-25Ah; L- 18: T3-25Ah; L-21: CT-25-Et; L-23: T1-25Et; L-25: T2-25Et; L-27: T3-25Et

Two distinct bands at 200 bp and 265 bp are representing β-actin (as housekeeping gene) and ISG15, respectively (Fig. 88a). Two distinct bands at 200 bp and 265 bp are representing β-actin (as housekeeping gene) and Mx, respectively (Fig. 88b). Two distinct bands at 200 bp and 700 bp are representing β-actin (as housekeeping gene) and STAT1, respectively (Fig. 88c).

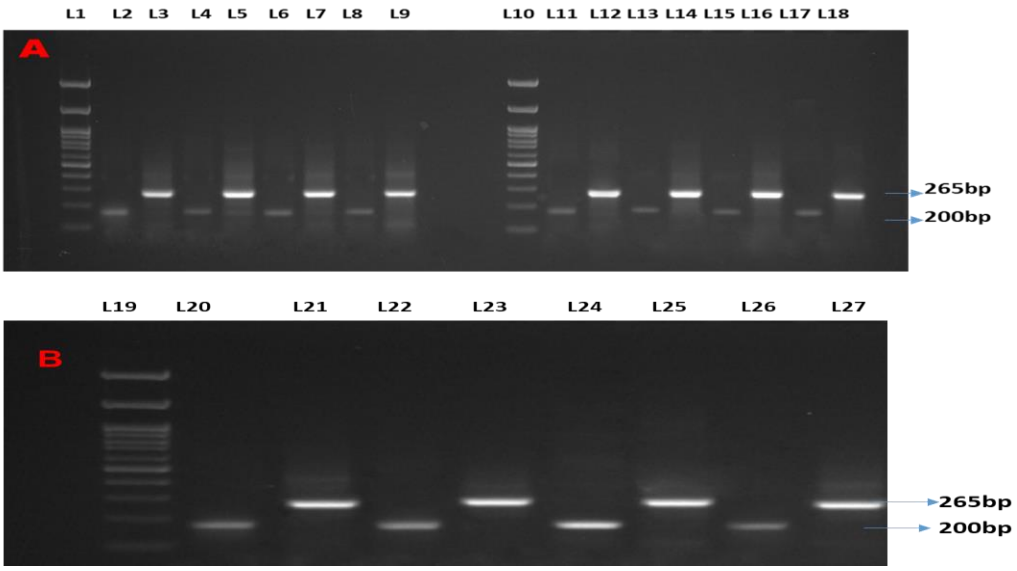

Fig. 88b. S**howing semi-quantitative PCR picture of Mx gene**

Legends: L-1, 11 and 19: 1Kb molecular marker; L-2,4,6,8,11,13, 15, 17, 20, 22, 24 and 26: β-actin (housekeeping gene); L-3: CT15; L-5: T1-15; L-7: T2-15; L-9: T3-15; L-12: CT-25Ah; L-14: T1-25Ah; L-16: T2-25Ah; L- 18: T3-25Ah; L-21: CT-25-Et; L-23: T1-25Et; L-25: T2-25Et; L-27: T3-25Et

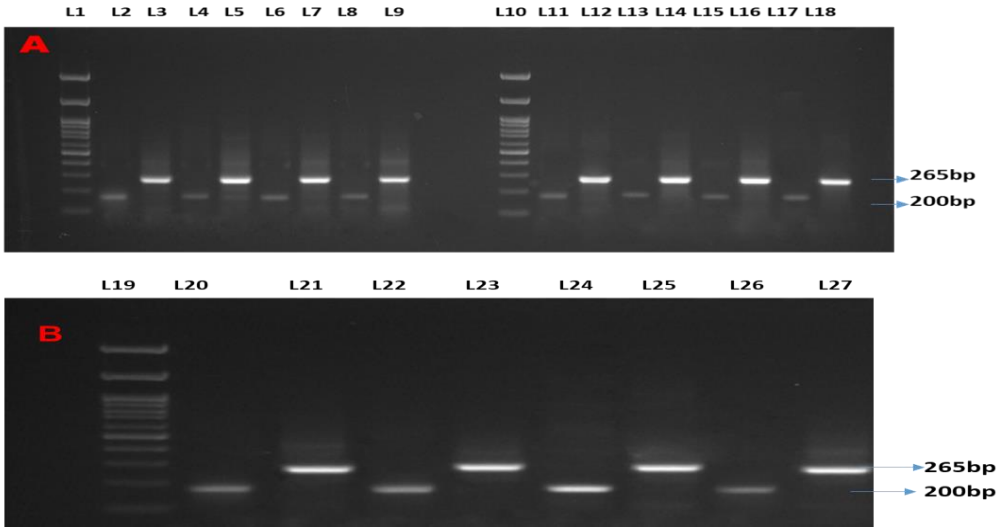

Fig. 88c. Showing semi-quantitative PCR picture of STAT1 gene

Legends: L-1, 11 and 19: 1Kb molecular marker; L-2,4,6,8,11,13, 15, 17, 20, 22, 24 and 26: β-actin (housekeeping gene); L-3: CT15; L-5: T1-15; L-7: T2-15; L-9: T3-15; L-12: CT-25Ah; L-14: T1-25Ah; L-16: T2-25Ah; L- 18: T3-25Ah; L-21: CT-25-Et; L-23: T1-25Et; L-25: T2-25Et; L-27: T3-25Et

In intraperitoneal inoculation experiment, the gene expression of three genes namely, ISG15, Mx and STAT1 showed the expression as follows Mx > ISG15 > STAT1. The ISG15 exhibited no significance ($p>0.05$) between the fold change value of T2-15 and T3-15 but CT15 and T1-15 showed a substantial rise ($p<0.05$). Between treatments, the expression values at 25 days exhibited no significance ($p<0.05$) between CT15-Ah and T1-15Ah and T2-15Ah. Among the treatments, highest expression was seen in T3-25Ah.

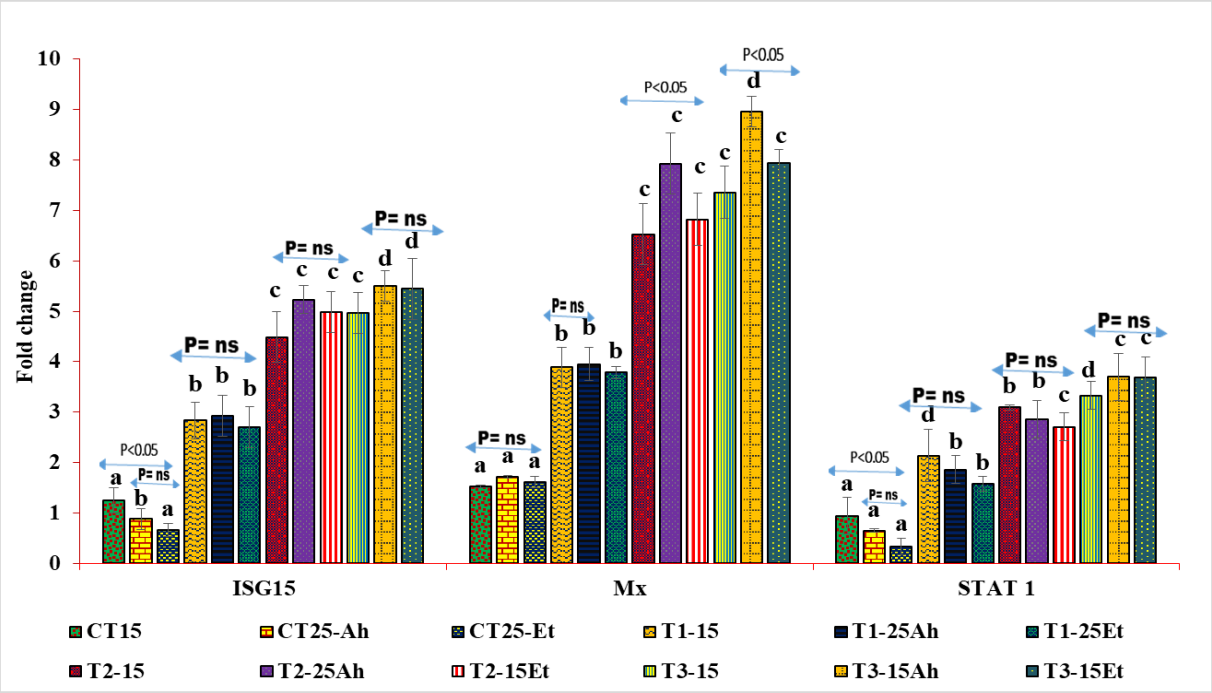

Fig. 89. Expression of three immunogenic genes in intraperitoneal inoculation experiment and challenge study

**The superscript indicates the significance between and among the treatments. Whereas arrow mark indicates the variation within the treatments before challenge and after challenge study.

The Mx expression differed considerably ($p<0.05$) among treatments. There is a substantial change was reported ($p<0.05$) between the treatments. The order of expression was; CT15>T115>T215>T315. The CT15, CT25-Ah, and CT25-Et treatments revealed no significance ($p>0.05$). The CT and T1 showed no substantial increment, however T2 and T3 showed a substantial ($p<0.05$) rise.

The STAT1 was shown to be considerable ($p<0.05$) among CT15, T1-15, T2-15, and T3-15. The CT-25Ah and T1-25Ah differed considerably ($p<0.05$), however there was no remarkable change was reported ($p>0.05$) between T1-25Ah and T2-25Ah, but T3-25Ah differed considerably ($p>0.05$). The same pattern was reported in Et, but there was no considerable difference was seen ($p>0.05$) between T2-25Et and T3-25Et. Following infection with Ah and Et, there was a substantial ($p<0.05$) drop in CT15 and CT25-Ah, but not in CT15 and CT25-Et. Between 15 and 25 days, no considerable drop was found in T1 and T2 ($p>0.05$), however a non-significant ($p>0.05$) increment was reported in T3 (Fig. 89).

5. DISCUSSION

Fish is considered as one of the preferred foods that provide cheaper animal protein. Fish production is continuously increasing, however, it would not be adequate to encounter the surging demand. To bridge the demand-supply gap, culture practises must be strengthened with innovative external inputs such as growth promoters, immunomodulators, prophylaxis, and so on. Every step toward intensification left a huge scope for multiple stressors in the culture environment that led to disease infestation which eventually turn into a huge economical loss (Lieke et al., 2020).

Aquaculture has two main constraints: first, fish feed and second, fish health. As far as fish health is concerned, aquaculture encounters infection due to bacteria, parasites and fungus etc. (Assefa and Abunna, 2018). Conventional prophylaxis through antibiotics and other chemical have proved either harmful or expensive for economically viable aquaculture enterprises. As an alternative to those chemical and conventional therapeutics, herbal extracts/ medicinal plants or plant parts are in demand due to their biodegradability and minimal side effects (Boukhatem et al., 2020).

Medicinal plants and herbs have been reported to possess immunomodulatory, growth promoting and stress mitigating functions (Shukla et al., 2012). Till date, plant products are being used without any *in-vitro* studies based on their ethnomedicinal applications. It's highly warranted to screen and characterize for their potential bioactive principles that are beneficial to aquaculture in terms of growth promoter and immunomodulator.

Thus, the current research is an attempt to appraise the impact of dietary *Terminalia arjuna* bark powder (TABP) in in-door trial and pond conditions and also tried as intraperitoneal inoculation of fractionated *T. arjuna* bark extract.

5.1. Extractions

Plant materials were extracted sequentially in increasing order of polarity with different organic solvents. Previous research (Altemimi et al., 2017) has shown that the sequential extraction method ensures the extraction of active compounds from plant material according to their polarity. In this study, three parts (leaf, bark and fruit) of *T. arjuna* were extracted using non-polar solvent such as hexane, ethyl acetate and chloroform; polar aprotic, acetone; polar protic, ethyl alcohol, methyl alcohol, and distilled water.

5.2. Effects of solvent system and extraction methods on biochemical properties of *T. arjuna* dry powders and its facile extract matrix

In the present experiment, a diverse pattern was found in proximate composition particularly for bark which has minimum moisture content and highest ash content in powdered form (dry). Amalraj and Gopi (2016) also reported that bark contains 34% ash, however, it has got maximum moisture and less ash content on encountering the different extraction methods and selective solvents which ascribed the inverse relationship between moisture and ash content of the selected solvent extracts. The differences for the proximate composition might be attributed to the adsorptive nature of ethanol as compared to other solvent systems and subsequently, resulted in lowering the ash content of the ethanolic bark extract. This finding agrees in part with Ajazuddin (2010), who stated that the acid-insoluble ash value of the prepared formulation revealed a very small amount of the inorganic component that was acid insoluble. It shows that adulteration of raw ingredients by substances like silica and rice husk is extremely rare.

In the present experiment, the maximum yield for both fractions namely, individual and serial was ascertained in ethanolic bark extracts but exhibited no significance ($p>0.05$) between individual and serial fractions of methanolic extracts irrespective of solvent systems. The findings of Akhter et al. (2012), revealed that maximum yield was obtained from ethanolic extract of *T. arjuna* bark, are in accordance to present research. In contrast to previous research (Sultana et al.,

2009), aqueous organic solvents yielded higher extract yields, phenolic contents, and antioxidant activity in plant material than absolute organic solvents.

The mineral profiling also showed the same trend for both the fractions. Mineral profiling of plant material is relying on various parameters, *i.e*. the type of tissue extracted, the stage of the plant used, and the parts of the plant material used for extraction, which is a corollary to Butkute et al. (2018), who summarised that mineral concentration of herbal extracts influenced due to growth of etho-medicinal plant and its phase and physiological aspects.

In the present investigation, it has revealed that overall mineral profiling was better in terms of methanolic fruit extract which has considerable variation with the ethanolic bark extract ($p<0.05$) but exhibited no substantial changes ($p>0.05$) with the ethanolic extract of leaf and fruit, acetone fruit extract and distilled water fruit extracts. Similarly, although acetone is a less polar solvent, its bark extracts have better mineral content as compared to the fruit and leaf extracts which ascribed to a type of solvent, nature of material extracted, and alteration in bonding pattern of polar aprotic solvents which is in harmony to previous studies conducted on other medicinal plants (Ramesh et al., 2010; Kazmi et al., 2015; Felhi et al., 2017; Meghwani et al., 2017; Ngo et al., 2017; Tiwari et al., 2018; Meena et al., 2020b).

5.3. Antibiogram profiling

The solvent extracts of arjuna namely, Br5 (ethanolic extract of arjuna bark), Br6 (Methanolic extract of arjuna bark) L5 (Ethanolic extract of arjuna leaf) and F6 (Methanolic extract of arjuna fruit) encompass a good activity against 17 selected elite bacteria. The Br5 and L5 showed antifungal activity against *Aphanomyces invadans*. Ethanolic extract of Arjuna bark was found to be the most effective solvent extract showing maximum antibacterial activity against most of the bacterial strains particularly, *Edwardsiella tarda* (ETML-3) represented as B1, and *Aeromonas hydrophila* (AH-01) represented as B6 (OTC resistant) and fungus, *A. invadans*. These findings conform to the previous studies which advocated the effectiveness of the antibacterial activity of the bark and leaf of the Arjuna plant (Kumar et al., 2017; Singh et al., 2018). In the current study, ethanolic and methanolic fractions of fruit

also showed the maximum antibacterial activity against *Acinetobacter* (ACI-10, OTC resistant and ACI-02, OTC sensitive) represented as B10 and B16, respectively, which might be different antibiogram patterns of the fruit extract mediated with different phytochemical and metabolites such as flavonoids, phenolics etc., (Latha and Kannabiran, 2006; Singh et al., 2018). Antibiogram profile of 17 bacterial strains against 21 solvent extracts, indicated strain-specific activity of the particular solvent extract. For instance, in most of the cases, ethanolic and methanolic extracts of bark and leaf showed wider efficacy, however, acetone extract of bark also recorded to exhibit the maximum antibiogram against *Stayphylococcus aureaus* (SA-5) represented as B17 which might be attributed to the inherent nature of compounds exhibit in bark extracts of acetone.

MIC value and zone of inhibition were varied and have an inverse relationship. MIC value in our study was lower than the previous studies and the zone of inhibition was higher and even comparable to the standard antibiotics in some cases *i.e.* B1 and AH-1. Varied efficacy of the same bacterial strain against different solvent extracts may likely due to the loss of certain bioactive principles during the extraction procedure (Marasini et al., 2015). Thus, present results substitute the results of previous findings which are showing higher MIC and lower antibiogram profiling. The increased antibiogram values corresponding to increased polarity of the solvent is in accordance to the previous research, however, in current investigation maximum efficacy has recorded in ethanol fractions that are also in harmony to other research (Kumar et al., 2017; Chandel et al., 2019). On contrary, some researchers had reported methanol as the most suitable solvent, however, in the present study the maximum values obtained for ethanol with narrow differences with methanol. As far as the side effects and health hazards are concerned, to the host animals, the ethanol has superiority in terms of compatibility with the normal routine of the tested animal but a bit costlier as compared to the methanol.

The present study revealed that bacteria having more antibiograms are having fewer MIC values and vice versa, this is in accordance with the many researchers who have revealed the inverse relationship between these two parameters. In the present study, association pattern and antibiogram profiling varied with corresponds to the nature of extract, the solvent used for extraction, and the organism tested

which is in corollary to the past studies (Dahiya and Purkayastha, 2012). The antibiogram against OTC resistant bacterial strains depicted a trend in which all were gram-negative and pathogenic to fish. It can be assumed that the different nature of the solvent extracts, coloration and physical appearance attributable to be a particular compound that would hold the responsibility of bio-efficacy. *For instance*, the hexane fractions are having pale yellow, light orange to light greenish coloration, sticky to oily appearances and no-polar nature indicated presence of volatile and oily substances which may not have the antimicrobial activities against the bacteria, fungus and parasites used in the present study but maybe having anti-parasitic and insecticidal activity.

5.4. Anti-parasitic activity

Argulus infestation in the present study was achieved through the co-habitation method for ascertaining medium infestation with 25-30 *A. bengalensis*/fish. More or less similar infestation intensity was also found by Kumar et al. (2012b) in experimentally challenged goldfish (15-20 parasites/fish). During the summer season, Trujillo-Gonzlez et al. (2018) found a considerably greater ($p<0.05$) prevalence of *Argulus* parasite. The co-habitation method successfully infected healthy *C. auratus* with *A. japonicus*, resulting in an average of 20 to 26 juvenile *Argulus*/fish. The present study showed the dose-dependent responses of the moderately infected *L. rohita* juveniles which is in accordance to the studies of Kumar et al. (2012b), who claimed a dose dependent efficacy of piperine solution to eliminate ectoparasite *Argulus* spp. in dose-dependent manner. The *in-vitro* study revealed the best five solvent extracts *i.e.* Br5, Br4, Br6, F6 and F4 and their LC_{50} values of these five extracts at all-time intervals followed the same trend as Br4>F6>F4>Br6>Br5 that might be attributable to their toxicological efficacy against *Argulus* spp., under *in-vitro* conditions which is in agreement with Kumar et al. (2012a), who explained the more efficacy of herbal material in *in-vitro* conditions.

Further, the Therapeutic index (TI) of the solvent extracts was found to be proportionately related with LC_{50} which indicates the potential of the particular solvent extract against *A. bengalensis* infestation that is conformity with the previous study of Kumari et al. (2019), who revealed LC_{50} decreases as the exposure time increases.

However, unlike the study of Kumari et al. (2019), a higher TI value was not recorded in the present study for the most efficient solvent extract. This deviation might be attributed due to two factors *i.e.* EC_{50} and LC_{50}. Comparatively, Br5 out of five solvent extracts (Br5, Br6, F6, F4 and Br4), in all treatments including *in-vitro, in-vivo* (bath and immersion).

Five best solvent extracts showed the highest efficacy indicating lower LC_{50} and TI values, and higher RPS that may likely be archived to the presence of phenolic and other bioactive constituents in those extracts that is in harmony to the previous studies, mentioning highest efficacy by ethanolic extracts, mediated by the presence of terpenoid (Mordue and Nisbet, 2000; Costa et al., 2008; Kumari et al., 2019) against *Argulus* spp. Meena et al. (2021) reported flavonoids and terpenoids in facile solvent matrix extracts, comprising fruit, leaf and bark of *T. arjuna* that corroborates the previous findings. It can be publicized that LC_{50} decreases as the exposure time increases, and AE proportionately increases. The same results were reported in previous studies mentioning a significantly ($p<0.05$) decrease inverse relationship between LC_{50} and time of exposure, while AE increased with exposure of azadirachtin and neem leaf (Kumar et al., 2012a & b; Kumari et al., 2019).

In current investigation, the LC_{50} values of the solvent extracts, particularly, a serial fraction of 100% ethanolic bark extract, for fish were higher than the ED_{50} and LC_{50} values of the solvent extracts for *A. bengalensis*. However, unlike in the present study, Suely et al. (2016) reported the LC_{50} for 80% ethanolic bark extract against freshwater catfish, *Heteropneustes fossilis*. The 96-hour LC_{50} values of the plant extract have reported as to be lowered side as compared to malachite green and cypermethrin (Mishra et al., 2005; Srivastava et al., 1995). The huge deviation in the finding of the present study, from the earlier reports, might be due to differences in extraction methods, mode of treatment, the season of collection of herbal material, water quality parameters, geographical location of the sample collection and storage condition of solvent extracts. The same findings have reported by Chandrawathani et al. (2002). In the current experiment, the LC_{50} values, for bath and immersion treatments followed the same trend for each extract at a different time interval, and bath treatment exhibited the lower LC_{50} value as compared to the immersion treatment. Kumar et al. (2012b) and Kumari et al. (2019), have also highlighted bath

treatment as a more efficient mode of treatment against argulosis in goldfish. TI also followed the same fashion as in the case of LD_{50} for both the treatments whereas RPS and AE followed a similar pattern of efficacy that is supported by Kumar et al. (2012b).

The toxicological study of two factors, LC_{50} and EC_{50} plays a critical role in determining the drug effectiveness and safety concern of the host animals. In the present study, the LC_{50} value for fish was recorded much higher than the LC_{50} value under *in-vitro* & *in-vivo* treatments. The EC_{50} for each solvent extract was either much below or just less than the LC_{50} value which indicates that the solvent which has a vast difference in these two parameters. The LC_{50} and EC_{50} would be safe for the host but may not be much effective as compared to the solvent extract which having less difference between these two and *vice-versa*.

Kumar et al. (2012b), reported median EC_{50} was nearly four times less than the toxic dose and it has no risk for the host. A substantial variation ($p<0.05$) was reported among, LC_{50}, RPS, TI and AE % might be due to temporal difference in the intensity and occurrence of parasites and also the fact that in the *in-vitro* condition the parasite does has weak host parasites interactions. It may likely also possible that host handling during bath treatment may likely provoke the short and acute exposure to the parasite which might be enhancing the efficiency of the solvent extracts. In previous research by Kirby (1996) and Kumar et al. (2012b) it has investigated that *Ocimum gratissimum* showed varied efficacy under in vitro and in vivo conditions against *Argulus* spp.

Similarly, a higher F value (4453.21) with a low P (0.0001) value validates the efficacy of solvent extract for controlling the *A. bengalensis* in *L. rohita* juveniles. Even, Suely et al. (2016), estimated a significantly ($p<0.05$) diverse responses in fishes in relation to concentration and exposure time. It can be suggested that solvent extracts such as Br4-IM and F4-IM and Br4-BA having higher TI values can be considered safe for a host in immersion and bath treatment, respectively on one hand. While, other solvent extracts are having twofold benefits, safe for the host and can be a potential material for drug synthesis against *A. bengalensis* on other hand. Similarly, the results of Khodadadi et al. (2011) *in-vitro* study revealed that

therapeutic index can be used as a tool for evaluating the safety profile of host and efficacy of the drug for further down line applications.

5.5. Assessment of the antioxidant potential

5.5.1. Qualitative assessment of bioactive compounds

The profiling of bioactive constituents revealed a wide array of phytochemicals including saponin, tannins, steroids etc. which are in accordance to Beigi et al. (2018). Tannin and phenolics were found to be more abundant in Br5, Br6, L5, L6, F6, F5, Br4 and L4 than in other solvent extracts. Kumar et al. (2016) made a similar observation, observing lower quantitative fractions of bioactive compounds as well as their weak in vitro responses.

5.5.2. *In-vitro* antioxidant activity

Leaf fractions of acetone and methanol have higher saponin and less quantity in aqueous fractions as compared to Br5, F5 and L5. Similarly, Djilani et al. (2006), could not report saponin in aqueous fractions. The sample F1 contained a high concentration of alkaloids in all solvent extracts, which is consistent with previous research (Mandal et al., 2013; Dhawan and Gupta, 2017). Contrastingly, Benzie et al. (1999), reported a highest content of flavonoid in methyl alcohol bark extract of *T. arjuna*, which could be due to differences in processing or extraction procedures. Br5 had the highest FRAP value in bark, followed by Br6, Br4, and Br7 in fruit extracts, F6 and L6 in leaf extracts. Djilani et al. (2006) discovered that the alcoholic fraction has the greatest antioxidant potential thereby bark extract exhibits better free radical scavenging properties than leaf extracts.

The other variables of antioxidant potential, flavonoid content and phenolic content were reported to be greatest in Br5 followed by Br6, Br4 and Br7 whereas for fruit extract highest was recorded in F5 followed by F6, F4 and F6, and in leaf extract L5 followed by L6, L3 and L7. The Br5 had the highest TFC and TPC contents in any solvent extract, implying that it has strong antioxidant and free radical scavenging properties. The same results were also obtained by Jayathilake et al. (2016), who

found a linear relationship between antioxidant potential and phenolic content of the plant material. The phenolic compounds and flavonoids have reported as prominent bioactive constituents of the antioxidative significance in medicinal herbs (Kumar et al., 2013).

The parameters of antioxidant potential and free radical scavenging exhibited a considerable relationship with Br5, Br6, Br4, and L6, whereas TFC and TPC are positively correlated with L5, F7, F6, F5, and so on. Previous research has found a strong correlation between the extracts FRAP activity and DPPH and ABTS radical scavenging which is mainly attributed to flavonoids and phenols in the plant (Irobalieva et al., 2015; Jayathilake et al., 2016; Sundar and Habibur, 2018).

Antioxidants protect DNA scission under free radical mediated three-dimensional structural changes in DNA. These changes in DNA configuration also have an effect on DNA movement which can be visualised in agarose gel. Although, pBR322 appears with two distinct bands, however, it has three forms (I, II and III). Relaxed form is also known as form I or supercoiled form that transform into other form as quickly as possible. Consequently, due to breakdown of form I, the circular form which is nicked in appearance, evolves form II. An intermediary form that appears between these two forms, is linear form and designated as form III (Li et al., 2011). The plasmid analysis includes the reporting of intensity of transformation of form I to Form II or Form III (Phani Kumar et al., 2013).

The strong antioxidants have the capability to possess better densiometric factors such as relative quantity, relative front, lane and band percentage. The current research recorded highest levels of densiometric parameters in Br5, Br6, and Br4, followed by F5, F4, L3, L2, F2, and so on. The previous studies have also reported the same findings on DNA scission inhibition with polar protic solvent extracts of *T. arjuna* (Amalraj and Gopi, 2016; Phani Kumar et al., 2013). Meena et al. (2021) reported a strong relationship between densiometric parameters and antioxidant potential of solvent extracts of *T. arjuna.* This could be because densiometric parameters, play a significant role in the recovery of the linear form of plasmid DNA during DNA nicking. Other researchers reported similar findings, claiming that supercoiled circular DNA migrates faster than open circular DNA, and

that control untreated gel electrophoretic images had a faint or black appearance, similar to what was seen in the current study (Guha et al., 2011; Darkwah et al., 2018). The prevention of DNA damage is thought to be a function of phenolics and flavonoid groups, which is in agreement with the present investigation (Sabahi et al., 2018).

5.6. Compound characterization

5.6.1. TLC fingerprinting

It is a chromatography technique used to evaluate the behaviour of bioactive compounds by assessing the Retention factor (Rf) value. Compounds with different Rf values disclose details about their polarity, which helps in the selection of a suitable solvent system (Liu et al., 2015). A compound with a high Rf value has low polarity in a less polar solvent setting, while a compound with a low Rf value has high polarity.

The TLC is mainly used to learn about chemical constituents for pharmacological research on new drugs (Ciura et al., 2019). Extract analysis showed significant evidence of the presence of potentially active metabolites in this study. These variations in Rf values help in recognizing their polarity and selecting the appropriate solvent method for column chromatography separation of pure compounds present in different fractions (Koparde et al., 2019).

Variable polarity in a mixture of solvents in different ratios is used to isolate a pure compound from plant extract. As a result, determining the best solvent system for a given plant extract is achieved mainly by comparing the Rf values of bioactive constituents' various solvents systems. In this study, the Rf values for the phenolic constituent, total antioxidant, and flavonoid were 0.93, 0.88, and 0.83, respectively. Similar findings were reported by Solanki et al. (2020), who stated the Rf value of *Withania somnifera, T. arjuna, Bacopa monnieri, Ranunculus sceleratus,* and *Acalypha indica* in relation to phenolic, flavonoids, and alkaloids standard. This linear relationship between solvent spot and mobile phase gives a clue about the presence of compounds.

5.6.2. Liquid chromatography coupled to electrospray-Orbitrap mass spectrometry (LCMS)

In the present study, the *T. arjuna* bark extracts showed the presence of flavonoids and phenolics as a major bioactive principle which is in accordance with the previous studies by Amalaraj and Gopi (2016). However, some of the constituents of these major groups have been reported firstly, such as jasmonic acid, 18-β-Glycyrrhetinic acid, 4-Methoxycinnamic acid and quinine that might be due to the solvent system and extraction method used in isolation of the extracts. Similarly, Meena et al. (2020a) reported that due to deviation in solvent systems and extraction methods, the bioactive compounds varied significantly ($p<0.05$). In leaf extracts apart from flavonoids and glycosides, some of the compounds such as atropine, vitexin, andrographolides and myricetin firstly reported, for which we don't have data to substantiate the same in *T. arjuna*.

However, previous studies showed the presence of vitexin and isovitexin in leaf of the plant, *Ficus deltoidea* (Choo et al., 2012) and andrographolides, which is a triterpenoid group reported to be in *Andrographsis paniculata* (Tajidin et al., 2019). The fruit extracts showed the presence of betulin, 4-Coumaric acid and 4-Methoxycinnamic acid those are firstly reported in *T. arjuna* that is in agreement with Hordyjewska et al. (2019). White birch bark, which contains approximately 34% of the pentacyclic triterpenoid betulin, is one of the above-mentioned exceptions (Laszczyk, 2009). The researchers from Canada were also able to extract 56% of betulin from yellow birch bark extract (Diouf et al., 2009). According to research, active compounds can also be extracted efficiently from birch bark, with up to 70 and 90% of active compounds obtained, respectively (Dehelean et al., 2012a; Kovalenko et al., 2009).

5.7. UV-Visible Spectroscopy

Important and simple techniques used in separating molecules from solutions include UV-Visible spectroscopy. In UV-VIS, the functional groups in molecules are called phenol blues, which allow the recovery of important organic compounds that

are present in solutions, and the further identification of other compounds which are composed of phenols (Mohammed, 2018). The principle of UV-VIS depends on the absorption of ultraviolet light and visible light by a compound that further results in the production of spectra. Spectroscopy is based on the interaction between light and material/compounds that will get activated upon absorption of light and then upon deactivation produce a spectrum (Taniguchi and Lindsey, 2018).

Flavonoids possess conjugated aromatic compounds and thus show strong absorption bands in the UV and visible regions of the spectrum. Phenolic compounds also show intense absorption in the UV region of the spectrum and show bathochromic changes in their spectra in the presence of alkali. While alkaloids take on an orange colouring when they absorb light in short velocity. In the present study, the flavonoid spectral absorption spectrum consists of two maxima: Band I (230-285 nm) and Band I and Band II of I report claims to be very distinctive (300-350nm). Flavonoids bands, as well as their maximum levels at lambda, are the key indicators for determining their flavonoid strength (Divya, 2017).

5.8. FT-IR analysis

In the present study, bands at 3585 and 3589 in the extract Br3 and Br2, respectively, might be due to stretching vibration of alcohols and phenols where O-H (free) is usually sharp (Plyler, 1952). Bands at 3415, 3405, 3400, 3394, 3388 and 3387 were probably due to O-H (H-bonded) stretching of alcohols (Coates, 2000c). The bands 3335, 3325 and 3295 found in the extract Br6, Br5 and Br7 were attributed due to ≡C-H stretching of alkynes. The presence of alkanes and alkyls could be detected by the bands observed at 2937, 2934, 2927, 2926, 2925, 2923, 2920, 2854, 2853, 2852 and 2851. A medium intensity peak was measured at 2309 in the BCHL extract which might be due to P-H phosphine. The presence of aldehydes could be detected because of the bands at 1722 (Br5 extracts), 1721 (Br4 and Br6 extract) and 1720 (Br5 extract). C=O stretching of ketone formed band at 1712 in the Br1 extract. However, the bands at 1722, 1721 and 1720 might be also due to asymmetrical stretching of anhydrides. Aryl ketone present in the extracts Br2 and Br3 formed bands at 1691 and 1693 respectively. Bending vibration (NH 2 scissoring) of 1° amine was detected due to the bands measured at 1613, 1612,

1611 and 1610. All the extracts except for BW contained 2° amide as bands were found at 1523, 1522, 1519, 1518, 1517, 1515 and 1514 due to bending vibration of N-H.

The bands that appeared at 1463, 1454, 1453, 1447, 1446, 1445 and 1444 might be due to bending vibration of alkanes (CH 2 and CH 3 deformation). Again, bands at 1380, 1378, 1377 and 1376 might be due to CH 3 deformation in alkanes. However, bands at 1380, 1378, 1377 and 1376 along with the bands at 1350, 1349, 1346 and 1338 might be also due to O-H bending of alcohols. The presence of carboxylic acid might be the probable reason for occurrence of bands at 1286, 1285, 1271, 1265, 1261, 1239, 1214, 1211, 1208 and 1203. Bands at 1200, 1198, 1194, 1172, 1168, 1109, 1105, 1104, 1092, 1083, 1078, 1051, 1049, 1047, 1041, 1037, 1027 and 1022 might be due to stretching vibration of either C-O of alcohols or C-N of amines. Bending vibration of =C-H of alkenes might be the reason for the formation of bands at 971, 929, 882, 881, 872, 825, 823, 822, 820, 819 and 798 while bending vibration of alkanes formed bands at 722, 721, 718 and 715. Bands at 771, 770, 767 and 765 were probably because of O-H bending of alcohols. The bending vibration of ≡C-H is attributed to the bands at 638, 636, 629, 628 and 625. Stretching of alkyl halides formed bands at 594, 564, 560, 559, 557, 556, 524, 512, 509 and 506.

Bending of alcohols (O-H bend) formed band at 3578, 3429, 3414, 3399, 3395, 3391, 3379, 1380, 1378, 1377, 1373, 772, 771, 770 and 769. The bands that occurred at 1189, 1172, 1171, 1170, 1115, 1080, 1052, 1051 and 1050 might be due to stretching vibration of either C-O of alcohols or C-N of amines. The bands observed at 2925, 2924, 2922, 2921, 2854, 2853, 2853, 2852, 2851, 1463, 1462, 1461, 1454, 1380, 1378, 1377 and 1373 might be attributed by alkanes and alkyls. Stretching vibration of carboxylic acids caused bond formation at 2672, 2664, 1273, 1241, 1235, 1234, 1223 and 1219. Band measured at 2312 in the extract F2C might be due to P-H phosphine. The presence of aldehydes could be detected by the band at 1739. Bands at 1715, 1712 and 1711 indicated the presence of ketones while aryl ketones show the band at 1691 in F5 extract. Bands appearing at 1614 and 1611 were due to bending of 1° amine and amide produced band at 1514 in the extracts F4 and F6. The O-H bending alcohols might also form the bands at 1380, 1378, 1377

and 1373. The bands at 1189, 1172, 1171, 1170, 1115, 1080, 1052, 1051 and 1050 might be formed because of stretching vibration of either C-O alcohols or C-N of amines. The presence of alkenes could be detected by 971, 970, 969, 968, 877, 874, 867, 823 and 805. Bending vibration of alkanes might form bands at 1359, 723, 722, 721 and 718. Alkynes might form bands at 664, 642 and 639 due to the bending vibration of ≡C-H in the extracts FE5 and F2. The bands at 567, 564, 563, 562, 556, 514, 496 and 450 were probably due to the stretching of alkyl halides.

Bending of O-H in alcohols caused bands at 3430, 3426, 3390, 3381, 1377, 1376, 1352, 1340, 772, 766, 765. The occurrence of bands at 2923, 2922, 2919, 2852, 2851, 2850, 1462, 1459, 1450, 1448, 722 and 720 might be due to the bending vibration of alkanes. The presence of phosphine might be detected by the bands at 2311 in the extract LH. The bands at 1719, 1718 and 1711 might be due to C=O stretching of ketones. The extract LE showed a band at 1702 which might be due to C=O stretching of carboxylic acid. Bending of 1° amine formed bonds at 1613 and 1612 while N-H bending of 2° amide formed bonds at 1536, 1514 and 1514. The bands at 1263, 1246, 1223 and 1219 might because of carboxylic acid.

Either C-O stretching of alcohols or C-N stretching amines formed the bands measured at 1199, 1177, 1169, 1099, 1076, 1043, 1035 and 1031. The =C-H bending of alkenes might be formed bands at 971, 873, 869, 855, 834 and 820. The band at 735 in LE might be due o-distributed aromatic compound with C-H bend. The band at 693 in LH might be formed by ≡C-H bending of alkynes. Stretching of alkyl halides might be formed bands at 564, 560, 551, 511 and 509. The illustration of the above analysis was corroborated with previous research conducted on spectroscopy (Coates, 2000a & b; Coates and Sanders, 2000b; Coates and Shelley, 2006; Coates and Reffner, 2000a; Abd El-Kareem et al., 2020).

5.9. Growth performances and survival

The feeding of herbal products in the feed of livestock and aquaculture has been used and continually increasing due to the surging demand for conventional feed ingredients (Talukdar et al., 2010). A good diet for a commercial culture includes a capsule of ingredients that supplies growth promoters, immunostimulants and basic

nutrients to maintain general functions and metabolisms. Average weight gain (AWG) and weight gain percentage (WGP) are the relative functions of feed intake (Gonzales and Law, 2013). The SGR showed the same trend like other growth performances parameters and almost a reverse trend to FCR that is in accordance to the studies of Jayasree et al. (2016) who highlighted the growth-promoting effect of Triphala (*Emblica officinalis*, *T. bellerica* and *T. chebula*) biofortification on *Oreochromis mossambicus*. Corollary to the current investigation, Asadi et al. (2012) and Nugroho et al. (2019) evaluated the effects of leaves of *T. catappa* on the growth performances in fish, *Pangasianodon hypophthalmus* and revealed that fish fed at higher dose (250 g kg^{-1}) resulted in reduced growth.

This might be due to higher concentration of bioactive compounds or the existence of ANFs fish feed and improved FCR due to their beneficial effects on feed conversion. The active components might have enhanced the feed utilization by the assimilation of most nutrients from the diet which has been converted to the flesh. The improvisation in growth performance parameters could be attributed to the credence of active principles of TABP that has been fortified in the diet manifested a growth-promoting effect. Present results are in agreement with Ahilan (2010) who reported that the inclusion of *Phyllanthus niruri* and *Aloe vera* had enhanced the growth performance of goldfish, *Carassius auratus*. And, also, Ahilan et al. (2015) revealed that Ashwagandha, at 10 g kg^{-1} feed could perform better in terms of mean weight gain (2.346 g) as compared to garlic and tulsi.

Among the nutrient utilization parameters, apparent net protein utilisation (ANPU), fat retention (FR), and energy retention (ER) showed significantly higher values in T2 group. Similar feed intake, protein intake, fat intake and energy intake in control and treatment groups. Nucleic acid analyses for all vital organs followed the same trend and decline in the case of T3 treatment. Hua et al. (2019) found that *T. arjuna* bark powder-based feed can promote growth promoting activity and nutrient retention capability in mice without any growth retardation due to anti-nutritional factors. The study found that the tannin content of TABP was 7.8 g kg^{-1} and within tolerable limits as described by Francis et al. (2001).

Terminalia bark is known for its anti-cholesteric property that has been investigated in animal models (Subramaniam et al., 2011; Dwivedi and Chopra, 2014; Kumar et al., 2019). Nucleic acid analyses for all vital organs followed the same trend and decline in the case of T3 treatment that might be attributed to impair functions of protein synthesis machinery by fostering low cranio somatic index (CSI) (Prusty et al., 2011; Jayant et al., 2018). The same result was obtained by Nugroho et al. (2019) who had pointed that fish fed at a higher dose of *T. catappa* (250 g kg^{-1}) resulted in reduced growth might be due to higher concentration of bioactive compounds in fish feed and improved FCR due to beneficial effects of them on feed conversion as explained in the study. The study found a gradual increase of all parameters up to T2 and then a sharp decline in T3 group.

The RNA contents and DNA: RNA ratio has been used as an indicator to evaluate the dietary prominence of fish (Ciji et al., 2013). In current research, the DNA: RNA ratio followed as CT > T3 >T1 >T2 indicates that might be due to impaired RNA synthesis parameters in T3 and improved function in T2 which significantly differ from treatments groups for all the vital organs. DNA:RNA ratio was highest in the intestine and lowest in the liver which is due to de-activation and activation of RNA synthesis in intestine and liver, respectively that coincides with the results of Ciji et al. (2013). The survival (%) was considerably greater ($p<0.05$) in T2 as compared with other groups that is attributed to the improved health status upon feeding of herbal feed at a particular dose which is in accordance to past research (Sahu et al., 2007a & b; Sahu et al., 2008; Al-Thobaitiet al., 2018; Hassan et al., 2018; Gabriel, 2019).

The feed ingredient processing is an intermediary process of feed formulation to increase the feed palatability and scalability for shaping it as quality feed (Hajra et al., 2013). Suely et al. (2016) reported that *T. arjuna* bark extract was found to be toxic while applied at 4.71 mg L^{-1} in *Heteropneustes fossilis* in the form of immersion. Nugroho et al. (2016) investigated the impacts of *T. catappa* L. leaves (TCL) extract on the physio-chemical parameters of water, haematological indices and percentage survival *Betta* sp., and discovered that immersing TCL above 375 ppm is advantageous to augment survival, haematological parameters of *Betta* sp., even reporting that lymphocyte was found to be effective at high dose 625 ppm as compared to low dose. To substantiate the presumptions of being *T. arjuna* bark as a

beneficial feed supplement in current investigation might be due to the improved nutritional prominence of TABP that has happed during the intermediary process of feed formulation.

Similarly, Kumar et al. (2012) suggested TABP has got processed during feed formulation that may lead to reduce the anti-nutritional factor that proper processing reduce the anti-nutritional factors (Tannin) and got improved status of bioactive principle due to application of phytase enzyme., Meena et al. (2020b) have reported that the ethanolic bark extract possesses 22% yield. In the present study TABP at 10 g kg^{-1} feed and 7.9 g kg^{-1} of feed reported as to be effective and optimised, respectively. Accordingly, the effective dose and optimised dose referred to 2.2 g kg^{-1} and 1.73 g kg^{-1} of feed at the time of feed formulation is less than the previous studies. In addition, Meena et al. (2020b) have reported that ethanolic bark extract found to be moderately toxic at 1030 mg L^{-1} concentration in *Artemia salina* model that will be many folds higher in the case of fish, apparently that advocates the inclusion of TABP and its extracts as to be safe for the experimental animal. The antinutritional factor, saponin normally does not interfere with the normal functioning of the feed physiology, and tannin and phytate have wide ranges of their distribution across the plant kingdom (Gemede and Ratta, 2014) and in the present study, these were well within the tolerable range (Francis et al., 2001).

In the present research, the greatest carcass CP content was recorded in the T2 group compared to the other groups, with the lowest value in the T3 group possibly due to inefficient protein retention, as confirmed by the results of Jayant et al. (2018). It is well known facts that *T. arjuna* bark has anti-lipidic and anti-cholesteric activity. It was observed that crude lipid followed an inverse relationship with dietary TABP and minimum fat content was observed at 15 g TABP/kg feed, which is corollary to Subramaniam et al. (2011). The Ash (%) content was reported maximum in CT and then decreased linearly in the order of T1> T2> T3. This decreasing trend might be attributed to the presence of inorganic soluble ash upon enhanced herbal dietary supplementation. Shah et al. (2017) documented that ash contents increased during starvation and stressful environment. Moisture content showed a reverse trend with the crude lipid content. T3, being the highest moisture content, in agreement with the studies is revealing higher moisture content while fed

at a graded level of unconventional feed ingredient interfering feed intake (Arguello-Guevara et al., 2018; Jayant et al., 2018). Conversely, moisture content has been reported to be decreased substantially ($p<0.05$) in treatments fed with different herbal plants compared to CT (Hassan et al., 2018). This deviation in our study from the above study may likely due to the modulatory effects of herbal plant on the biochemical composition of fish species.

However, the growth performances, nutrient utilization and body indices differ significantly ($p<0.05$). When it comes to replacing fish meal with local plant sources and nonconventional feed ingredients, plant-based feed ingredients perform similarly to their counterparts (Shakya, 2017; Hua et al., 2019). The basic factors of maximizing the farmer benefit by reducing the operational cost of the fish production. Firstly, the replacement of the expensive basic feed ingredients with cheaper one, easily available non-conventional feed ingredients are supposed to maximize the profit margin of the fish farmers as evident from the previous studies (Hassan et al., 2016; Jayant et al., 2018; Yadav et al., 2020). Secondly, exploration and screening of herbal feed additives for growth enhancer, immunostimulant and use of formulated quality feed etc (Robb and Crampton, 2013). The present study substantiates earlier results by enhancing the growth performances along with higher survival and nutrient utilization in *L. rohita* fed with TABP at an inclusion level of 10 g kg^{-1} feed.

The growth performances, survival and nucleic acid analyses showed a different trend in all three trials *i.e.* feed, pond and injection that might be due to differential intake of bioactive compounds that are supposed to trigger the growth mechanisms. Irrespective of time duration if we compared the growth performances, we could reveal that maximum growth trigged in injection in first 15 days > pond experiment and then feed experiment. The same results with intraperitoneal injection were also observed when andrographolides were injected into fish (Mussard et al., 2019). The per month WG (%) was recorded higher in outdoor pond feed trail (67%) as compared to indoor feed trial (49%). This might be due to preferences for natural feeds available in the pond and the formation of an algal mat on the wall of hapa that is one of the preferred feeds of carps. Previous research has found that hydrobiological and geoclimatic conditions, as well as stocking density, water body biological productivity, and the provision of supplementary feeds, are all important in

carp culture in pond conditions (Tripathi et al., 2000; Jena et al., 2001; Jena et al., 2002a & b).

5.10. Hematological parameters and humoral responses

A key component of fish immunity is innate (non-specific) as well as specific (humoral and cell-mediated) responses. Mucus and skin act as a line of first defence. The long-term feeding of herbs caused an increase in serum total protein, haemato-biochemical and humoral responses in fish (Hassan et al., 2018). Misra (2004) found that the addition of the *Curcuma longa***,** *Mangifera indica* and *Allium sativum* could enhance the hematological parameters in *L. rohita.*

TABP based feed increased overall protein content. The T2 group (10 g kg^{-1} feed) had higher serum albumin and globulin levels than the other groups. Serum protein and globulins are thought to be linked to a more powerful innate fish response (Misra et al., 2006). Changes in immune function can be revealed by the number of WBCs in the blood (Wedemeyer and McLeay, 1981). It is a good marker of a fish's health because of its role in non-specific defense (Roberts, 1978). The TABP feeding increased WBC, RBC, Hg, HCT, MCV, MCH, MCHC, albumin, globulin and protein, NBT, lysozyme activity. Bactericidal activity increased in T2 group (10 g kg^{-1}) as compared to other groups and then decline in T3 group (15 g kg^{-1}) as compared to T1, T2 groups which might be due to the negative effect of dietary TABP in T3.

Similarly, Suely et al. (2016) investigated that ethanol bark extract negatively affected the hematological parameters when an overdose of the extract was given to *Heteropneustes fossilis*. Immunostimulants can boost nonspecific immunity in the host by increasing phagocyte count and NBT to combat pathogenic microbes (Shoemaker et al., 1997). Following infection with bacterial pathogens, NBT activity reduced non significantly, when compared with 90-day feed trial. Many things could happen in the challenge study, but it's safe to assume that TABP-induced superoxide anions protect fish from bacterial infection. Similar findings were reported by Bahrami et al. (2015), who claimed that dietary *Stachys lavandulifolia* extract could improve blood indices in *Cyprinus carpio*. The erythrocyte count increased after TABP administration, which could be attributed to TABP's immunostimulatory effects.

Previous research (Sahu et al., 2007a) found that rohu fed garlic-containing diets for 60 days had significantly higher erythrocyte counts.

Duncan and Klesius (1996b) discovered that when channel catfish were fed a glucan-based diet, the number of erythrocytes was considerably greater ($p<0.05$) than when they were fed a control diet. The presence of leucocytes is known to help trigger the immune system in rohu following immunostimulation application (Misra, 2004; Misra et al., 2006; Sahu et al, 2007a). The antimicrobial properties of traditional herbs, such as garlic, are supported by an increase in total white blood cells, and other cell types upon feeding of herbs (Iranloye, 2002; Sahu et al., 2007b). The low level of haemoglobin in strained treatments could reflect physiological alternations to a lower demand of oxygen and the substrate for metabolism in oxygen stressed environment thereby leads to fluctuate the pH of the blood (Waagbo et al., 1994; Moyle and Cech, 1982).

According to Gill et al. (1991), it could also be due to haemopoiesis exhaustion under hypoxic conditions. The breakdown of blood cell due to temperature and salt stress could explain the observed decrease in RBCs and haemoglobin (Akhtar et al., 2012b). The addition of TABP to the diet significantly improved RBC count restoration. Furunculosis caused by *Aeromonas salmonicida* has been linked to a drop in RBC count in Atlantic salmon (Foda, 1973). After infection with *A. hydrophila*, the Haemoglobin of *L. rohita* fingerlings was found to be lower (Misra et al., 2006; Alexander, 2011). *A. hydrophila* and *E. tarda* are known to cause severe etiological signs such as dropsy, septicaemia, ulcer, and rotting of fins, all of which result in high collapse of fish population (Karunasagar et al., 1997). According to this research, the addition of TABP to *L. rohita* juveniles increased their survival by allowing them to resist *A. hydrophila* infection. When fish are stressed, the concentration of haemoglobin in their blood increases. Because the increase in haemoglobin concentration was within the expected range, TABP feeding did not cause stress in the fish until T2.

The results showed that outdoor pond feed trial and intraperitoneal inoculation experiment have a different pattern of expression of the parameters as summarized above. The feeding experiment showed a positive impact in T2 group while in the

outdoor pond, feed trial a non-considerable variation observed between T2 and T3 groups. Under intraperitoneal inoculation experiment, *T. arjuna* bark extract was observed to have a positive impact on haematological parameters in T3 (120 µg mL^{-1}). The similar finding has been reported by Sahu et al. (2007a & b). In aquaculture, administrations of immunostimulants by intraperitoneal injection are more effective route than the oral route (Duncan and Klesius, 1996a & b). The effectiveness of the inoculation method is due to the quick absorption of the injected material.

The current study was carried out by injecting the specified dose of extract intraperitoneally into the fish body in order to evaluate the effectiveness as soon as possible. The *T. arjuna* ethanolic bark extract substantially ($p<0.05$) enhanced the haematological parameters including total protein, albumin, globulin etc., in T3 (120 µg mL^{-1}) and T2 (100 µg mL^{-1}). Similarly, findings were recorded by Sahu et al.(2007a) who has investigated the impact of 0.5% extract inoculation of garlic, turmeric and mango in rohu. Even, the highest levels of total protein and globulin in rohu serum were found after 14 days of feeding with 0.5 percent prickly chaff-flower, with levels gradually declining at 21 and 28 days (Rao et al., 2006). Shalaby et al. (2006) discovered a rise in plasma protein in fish *L. rohita* and *O. niloticus* fed different concentrations of turmeric, mango kernel, and garlic powder for 60 days, followed by a decrease following the challenge study.

Previous research found that ethanolic extracts of 0.5 percent turmeric, mango kernel, and garlic increased biochemical parameters like serum protein, albumin, and globulin in rats. This discovery demonstrated that these substances stimulate the production of antibodies in fish, which is reflected in increased serum protein, albumin, and globulin levels, allowing the fish to fight against infection. Natural fish food organisms are assumed to be essential factor for better overall performances and development of fish particularly, Indian major carps in pond conditions.

Immunostimulants have been reported to augment humoral responses by enhancing the efficiency and number of cell type for lysozyme synthesis per cell in fish (Edahiro et al., 1990). The amount and type of stressors to which the fish are subjected have a significant impact on changes in lysozyme activity. After ingesting immunostimulants, several fish species had higher lysozyme levels (Lapatra et al, 1998; Paulsen, 2003). Fall and Dong (2011) found that feeding 0.5 percent garlic to

hybrid tilapia for 2 or 4 weeks resulted in a similar increase in lysozyme activity (*Oreochromis niloticus* x *Oreochromis aureus*). Several studies have discovered that phagocytic cells from various fish species have increased bactericidal activity (Jorgensen et al., 1993; Sahu et al., 2007a & b).

The NBT is a measure of an oxygen-reliant protective machinery in vertebrate phagocytic cells that yields responsive oxygen intermediates with great antimicrobial responses (Itou et al., 1997). Sharp and Secombes (1993) discovered that increased NBT is linked to increased phagocyte bacterial pathogen killing activity, and thus improved immunity. In this study, the reduction of NBT by intracellular superoxide radicals produced by leucocytes was used to determine phagocyte respiratory burst activity. The T3 exhibited a decrease in respiratory burst action, whereas the T2 group showed an increase. According to Laith et al. (2017), *Excoecaria agallocha* at 50 mg kg^{-1} enhanced the non-specific and humoral immune response in *O. niloticus* including a respiratory burst. Montero et al. (2001) discovered that when juvenile gilthead seabream were subjected to repetitive stresses, their respiratory burst activity decreased. According to Akhtar et al. (2011b) *L. rohita* reared at higher temperatures also had lower respiratory burst activity.

Lower doses of herbal stimulants resulted in better survival against pathogenic challenges in fish, according to Jian and Wu (2003) and Sahu et al. (2007a). When *Penaeus monodon* were fed an *Artemia*-rich herbal diet containing a blend of five plants resulted in the improvement of their health status (Citarasu et al., 2002). In current investigation, dietary TABP promotes non-specific and protection against microbial pathogens in rohu fingerlings. Dietary TABP in fish may have increased nonspecific immune components including lysozyme activity resulting in long-term protection. Furthermore, additional research into the molecular mechanism of immunomodulation, as well as the dose optimisation and suitable method for administration of extract of TABP for fish health improvement, is essentially required.

5.11. Serum enzymes

Serum enzymes can be used as diagnostic tools to confirm organ-specific metabolic stresses. Enzymes such as lipase, amylase, SGPT, SGOT, GGT, LDH

creatinine, CKNac decreased in T2 and then again increased in T3 that reflects the positive impact of TABP bioactive ingredients on metabolic functions in T2 and again negative impact in T3. This might be due to an overdose of TABP in T3 that might have altered the functions of vital organs negatively which resulted in cell damage, necrosis etc. Similar findings were obtained by Mlozi et al. (2020) who had reported that root extract of *Tephrosia vogelii* at 2000 mg kg^{-1} exhibited a negative impact on the liver of albino rats in terms of necrosis and vacuolation of liver cells on the 14^{th} day of post-feeding with root extract.

Transaminases (ALT and AST) are tissue damage markers found in vital organs including liver (Rajyasree and Neeraja, 1989, Oluah, 2000). The increased ALT and AST activity suggests that in response to stress, aspartate and alanine are mobilised for glucose production via gluconeogenesis. When transaminase activity increases during stress, elevated feeding of keto acids into the trichloro acetic acid cycle occurs, according to Chatterjee et al. (2006). The T3 group had the most ALT and AST activity in the current study. These findings are in contrast to those of Tejpal et al. (2009) and Alexander (2011), who discovered increased serum enzyme activity in response to stress or cellular injury.

AST is found in the most of the vital organs and muscles of cardiac protection, blood stream etc., while ALT is found mostly in the liver. Phosphophatase activity is critical in pathological situations (Reddy and Rao, 1990). The ALP activity has reported to be greater in T2 as compared with other groups in indoor feed trial. Phosphatase activity is increasing, indicating that energy reserves used for fish growth and survival are being depleted faster. At an alkaline pH, the brush border enzyme (ALP) mediates membrane transport by splitting phosphorus esterases (Goldfisher et al., 1964). The ALP is also involved in glycogen transport, glycogen membrane (Gupta and Rao, 1974), protein synthesis (Pilo et al., 1972), and enzyme synthesis, as well as secretory activity (Pilo et al., 1972). As a result, any change in ALP activity can have a wide range of consequences for an animal. Pradhan (2012) discovered an increase in ALP activity in rohu fed various doses of chlorella for 90 days following challenge with *A. hydrophila.* The current discovery implies that the changes in enzyme profiles are similar to those reported by Pradhan (2012).

In fish, the enzyme LDH has been used to demonstrate injury to the cells of vital organs (Ramesh et al., 1993). Increased LDH activity in T3 compared to other groups may be due to pyruvate, the final output of glycolysis, entering the oxygen deficient pathway and increasing LDH action (Calbreath, 1992). Stresses that increase LDH activity include thermal, starvation, crowding, nitrite, and endosulfan stress (Grigo, 1975; Vijayaraghavan and Rao, 1986; Das et al., 2004; Tejpal et al., 2009; Akhtar et al., 2010). Thermal stress and salt stress targets the gills and respiratory tract of fish, respectively Kumar, 2008; Talbot et al., 1992; Avella et al., 1993), resulting in hypoxia and increased LDH level in vital organs mainly in liver (Das et al., 2004). Fish fed at 10 g kg^{-1} TABP maintained the same level of LDH activity as the control group, indicating that TABP supplementation may play a role in metabolic stress management. Similarly, T2 showed a decrease in CKNac, GGT, and other metabolic enzymes, which could be due to TABP's positive effect, but there is no prior evidence to support our findings.

5.12. Enzyme of oxidative damage

The majority of the living cells has capacity to combat with oxidative stress by enhancing their antioxidative efficiency (Hermes-Lima, 2004). The SOD and CAT are considered as two important anti-oxidative enzymes which thought to reduce oxidative stress by increasing the availability of antioxidants (Portner, 2002). The key enzymes investigated in this study were SOD, catalase, and GST. The SODs are metalloenzymes which initiate the transformation of the ROS anion to H_2O_2 and oxygen, lowering the levels of reactive oxygen species (Villafranca et al., 1974). The H_2O_2 is then detoxified by CAT into oxygen and H_2O (Aebi et al., 1984).

In the present study for all three enzymes studied, a substantial ($p<0.05$) decrease till T2 group and then a considerable rise ($p<0.05$) in T3 group indicate two things. Firstly, the T3 group encountered more oxidative stress and therefore these enzymes expressed more in T3. Secondly, more antioxidant potential witnessed with the higher dose of TABP in T3 and T2 group imposes the least stress among the treatments. The findings are corollary to the results of Akhtar et al. (2012a). Similarly, the GST also followed the same trend as like other two enzymes. The same trends

were also reported by Spanou et al. (2011), who pointed initiation of SOD action advocates an antioxidant ability, the inhibition of CAT and xanthine oxidase (XO) specifies a pro-oxidant stroke.

5.13. Enzyme of neurotransmission (acetylcholine esterase)

The AChE is an enzyme that initiates the chemical breakdown of acetylcholine due to water activity, the chemical that transmits nerve impulses. Generally, the pseudo cholinesterase hydrolyzes acetylcholine, that inhibits AChE, and thus frees AChE from its inhibitory effect. AChE inhibition raises acetylcholine levels, which causes behavioural changes. Acetylcholine increases normal physiological action and electrical reactions. In this study, *L. rohita* was given a higher dose of TABP (15 g kg^{-1}), which resulted in an increase in AChE activity. Our findings are consistent with those of Akhtar et al. (2012a), who reported a decline in AChE activity in rohu bared to 33 °C compared to their counterparts at ambient temperature (26 °C).

A decrease in AChE activity has been reported by several authors in fish exposed to various stressors (Sancho et al., 1997; Dembele et al., 2000; Akhtar et al., 2010; Kumar et al., 2011). Reduced release of pseudo-cholinesterase, the enzyme responsible for acetyl choline hydrolysis, may lead to increased cholinesterase accumulation at the synaptic region, inhibiting AChE activity. The inhibition of AChE activity in T2 of the current study was found to be increased with TABP (15 g kg^{-1}) supplementation in the diet. Dietary tryptophan supplementation reduced inhibition of AChE activity in Indian major carps under crowding and nitrite stress has been reported (Tejpal, 2007; Ciji, 2012). Herbal extracts have been shown to have anti-AchE inhibitory activity (Mathew and Subramanian, 2014; Balkrishna et al., 2019b). Furthermore, Brzezinski and Ludwicki (1973) proposed that inhibiting AChE raises acetylcholine levels.

This condition can cause an increase in catecholamines, which can affect glycogenolysis and glycogen synthesis enzyme activity. As a result, an increase in catecholamine levels could result in hyperglycemia. In this study, there was no

considerable variation in the activity of AchE between the treatments during the 90-day indoor feeding trial, the 60-day outdoor pond, and the 30-day intraperitoneal inoculation experiments, however, and also within the groups upon infection, except for control, which was supported by the fact that glucose levels were also maintained, removing hyperglycemic conditions.

5.14. Digestive enzyme activity

Previous research on fish larvae found a link between digestive enzyme activity and digestive tract maturation (Ribeiro et al., 1999; Yang et al., 2018). Adult feeding habits and diet, on the other hand, have an effect on the roles of digestive enzymes (Chakravorty and Sinha, 1982). The vast majority of current digestive enzyme research focuses on the progress of digestive tract and the ontogenesis of digestive enzymes in larval fish, with a particular attention on marine carnivorous fish. This is due to the fact that creating an effective artificial diet necessitates a thorough understanding of larval growth as well as the expression of key digestive enzymes. Both larval and adult rohu digestive enzyme activities have been investigated (Chakrabarti et al., 2006a; Debnath et al., 2007).

According to the current study's findings, amylase activity in the indoor feed exhibited no significance between the T2 and T1, or the CT and T3 groups. Similarly, protease activity elevated in the T2 as compared with other groups, and digestive alkaline phosphates (ALPU) followed the same pattern. When infected, the trend was similar; however, the decrease in *E. tarda* infection was greater than in *A. hydrophila* infection. In summary, the indoor feed trial revealed no significant difference in APLU enzyme activity between T2 and T1, or CT and T3. Some studies, such as Lopez-lopez et al. (2005) found no relationship between activity of amylase and dietary protein.

However, TABP supplementation in the diet could significantly increase the activity of the enzyme in *L. rohita* juveniles. The lack of difference between the T2 and T3 groups in the outdoor pond feed trial was most likely due to the availability of digestive enzymes and protease in natural feed available in the pond environment. At 15 days after intraperitoneal inoculation, there was a slight increase in protease,

ALPU, and lipase activity in the T3 as compared with T2 group. At 30 days, all treatments except CT showed a slight decline, which could be attributed to a short-term increase in enzyme activities following intraperitoneal inoculation.

The previous research also showed that herbal extract and medicinal plants were reported to show a positive impact on digestive enzyme activity in *L. rohita* (Sahu et al., 2007a & b). Recently, some studies conducted on other species also showed that after optimum dose there was a decline in enzyme activity in accordance to the present study (Doan et al., 2020; Heydari et al., 2020; Huang et al., 2020; Xu et al., 2020).

Fish growth rate is influenced by efficiency of digestive system, oxygen abundance and digestive capacity, oxygen availability, and the physio-metabolic ability, essential for protein synthesis across the tissues (Blier et al., 1997). The ability of large nutrients in the digestive tract of an animal to be broken down into small absorbable subunits is highly dependent on the enzymes available (Wakin and Grewal, 2021).

In the current study, we discovered that protease behaviour in *L. rohita* juveniles was strongly related to weight gain percentage (R^2 = 0. 95). The protease activity of the TABP-enriched treatments was significantly greater ($p<0.05$) as compared with CT. This could be as a result of consuming more protein and assimilation. Lipolytic enzymes are essential in biological systems for lipid digestion (Walton and Cowey, 1982). Dietary TABP had no effect on amylase activity in the indoor feed, outdoor pond feed, or intraperitoneal inoculation trials, and also had no effect on lipase activity in the indoor feed and outdoor pond feed trials.

5.15. Triiodothyronine (T3), thyroxine(T4), cortisol and glucose

Cortisol and glucose are used to identify different stress. Hyper stress and stress above the threshold lead to enhance the level of blood glucose owing to glycogenolysis and the formation of glucose from amino acids in another causes

such as extrahepatic tissue to supply energy for maintaining physio-metabolic activities (Thau et al., 2021).

The structure of cortisol has been proved sa to be highly conserved across the species (Hontela, 2005). Until now, two main effects of cortisol action have been discovered: osmorecepticism and keeping energy levels stable. Physiologic changes in the hypothalamus-pituitary-pituitary axis (Hontela, 2005). Indoor feed trial showed decreased cortisol value in T2 group, while CT showed higher values of cortisol and glucose, which implies that TABP inhibition exists over a dose range. At a higher dose, the TABP negatively affected the development in terms of growth and feed performances as observed in the T3 of the indoor feed trial. There was no considerable variation was reported ($p>0.05$) between T2 and T3 groups of outdoor pond feed trial; however, the natural food component reduced the possible dietary impact of higher dose of dietary TABP.

The catecholamine level elevates during all types of stress, which has a significant effect on glycogen and glucose metabolism with an increase in blood glucose level. At the optimum dose, TABP is found to have significantly reduced the stress-induced blood glucose elevation followed by a stress-reducing role with graded level of dosages (Tejpal et al., 2009; Kumar, 2008; Alexander, 2011; Neeraj et al., 2011). Under stress, the level of cortisol was found to be elevated in the *L. rohita* (Kumar, 2008) and the tilapia (Fiess, 2007), respectively. Bhattacharjee et al. (2019) reported the stress-reducing effect of *T. arjuna* bark extract as food additives in rats.

The present study showed that at a dose of 15 g kg^{-1} of TABP, there was a substantial ($p<0.05$) decrease in both T3 and T4. Similarly, decreases in the T3 and T4 levels were observed in the fish in stressful conditions (Klaren et al., 2007). With regard to a study on silver-seabream fish, T4 level significantly($p<0.05$) reduced (as well as other thyroid hormones) following stress for seven days, while no such changes were observed in T3 level (Deane and Woo, 2007). A variety of hormones identified in fish such as thyroid, growth, development, reproduction, play an important role in fish physiology (Peter, 2007). It has been inferred that the group

whose growth rates were reduced, has the lowest T4 (T3 group) and highest T4 level in the group exhibited the highest growth (T2).

5.16. Gene expression

In the present study, three genes namely, Mx, STAT1 and ISG15 have been used for testing, the efficacy of herbal extracts in terms of gene modulation. The Mx is an antiviral protein belonging to the family of dynamins that includes amino-terminal with G-domain an, interactive central area, and leucine zipper as an effector area of GED or GTPase (Melen et al., 1992). Interferon is known to induce anti proliferative, antiviral and immunomodulatory proteins (Lee and Ashar, 2018). Out of three interferon mediated protein, the Mx proteins are specially inhibits the protein synthesis of viruses including influenza and stomatitis (Verhelst et al., 2013). The cellular functions of Mx protein are still unclear, however, it is proved that all proteins are having GTP binding site, of which COOH-terminal leucine zipper domain, is an important structural element (Melen et al., 1992).

The STAT1 (Signal Transducer and Activator of Transcription), is an essential signal transduction protein that is intricate in the interferon pathway. The STAT1 plays a significant role in the non-specific defense system (Tso et al., 2013). The ISG15 is an interferon stimulating antiviral gene. Although ISG15 was thought to be an integral part of classically antiviral immunity, it has newly appeared as a regulator of genome steadiness, with main roles in the DNA nicking inhibition to modulate p53 signalling and error-free DNA replication (Sandy et al., 2020). The herbal material or plants are mainly known to enhance non-specific immune systems, however, it has been also reported that they trigger the specific immune system which in turn the up-regulation of the immune genes thereby protects against pathogens (Awad and Awaad, 2017; Trinh et al., 2020). Similarly, Nhu et al. (2019) evaluated the effects of five herbal extracts; garlic, neem, asthma-plant, bhumi amla and ginger in *P. hypophthamus* fingerlings which could enhance the specific immune parameters such as various types of cytokines including mhc class II cytokines. The previous study also showed a very common spice garlic used as a food supplement, has been recorded to enhance specific immune systems *i.e.* rainbow trout (Nya and Austin,

2009), hybrid tilapia (Fall and Dong, 2011), Asian seabass (Talpur and Ikhwanuddin, 2012), and caspian roach (Ghehdarijani et al., 2016).

The gene expression showed an elevated level of a respective gene upon infection except for control that might be due to immunomodulatory effects of treatment at varying doses (Nahak and Sahu, 2014; Hoseinifar et al., 2015; Yogeshwari et al., 2015; Trejo-Flores et al., 2018; Kaur et al. 2020; Salomon et al., 2020). In the indoor feed trial followed by challenge study, three genes namely, Mx, ISG15 and STAT1, were expressed is the maximum expression of Mx. Most importantly, Mx was reported to express in the control treatment and further infection with bacterial pathogens also which is in agreement to Zavyalov et al. (2020). Whereas the other two genes, ISG15 and STAT1 exhibited no considerable ($p>0.05$) expression in the CT followed by infection, might be due to the intrinsic nature of availability of Mx in normal fish species but may not be the case for ISG15 and STAT1.

The same results were reported by Roy et al. (2016) and Das et al. (2019) who had suggested that Mx acts as a natural gatekeeper of fish immune systems that protect them in early life stages and further get activated when encounters the same pathogens. Also, the same results were found by other researchers mentioning the expression of STAT1 is induced by Poly I:C and upon infection its expression was found to enhance by many folds as compared to control (Tso et al., 2013). While upon infection, the expression of three genes was observed but Mx showed its highest expression in the natural condition of fish that is an indication that Mx did not activate directly due to virus rather it is expressed with the activation of IFNS while the other two genes expressed only when they encounter pathogens. The same results were reported by Schiavano et al. (2016).

In indoor feed trial, Mx showed maximum expression as compared to the other two genes in all treatments**,** it might be due to activation of IFNs due to herbal bioactive principles in the case of Mx, while it could be a week or not appropriate factor to hit the activation of IFNs in other two genes particularly when fish was challenged with bacterial pathogens. The same observation was reported by previous researchers (Kim et al., 2017; Trinh et al., 2020). In higher vertebrates, the

application of medicament in the form of injection is considered more effective and short-term relief.

A comparable multiple-herb approach recognized as “Fufang” is a vital constituent in conventional Chinese medicament and is applied to attain improved therapeutic outcomes, and decrease side effects and herbal toxicity (Huang et al., 2016; Hu et al., 2016). In the intraperitoneal inoculation experiment, the pattern of all expression of genes was the same except ISG 15 which was expressed more as compared to the feed trial that might be effects of particular bioactive compounds present in the dose of extract dose (0-120 µg mL^{-1}). The same results were obtained by Balkrishna et al. (2019a). The previous study also showed that injectable herbal extract/bioactive principles showed more gene expression for a short duration (Alice, 2017; Balla et al., 2019; Shen et al., 2019).

5.17. Antibody titre (IgM) and microProteins (MPRs)

Immunoglobulins or antibodies are considered as an integral component of a specific immune system and composed of heterodimeric glycoproteins belonging to the broad Ig superfamily (Mashoof and Criscitiello, 2016). In bony fishes, 3 classes of heavy chains immunoglobulins were identified as compared to their elasmobranch counterparts; IgM, IgD, and IgT/Z, however, each class does not express in the fish species (Rombout et al., 2014). It has been established that IgM levels vary among the species as per the size and age (Klesius, 1990; Magnadottir et al., 1999). Therefore, it is considered as an effective immune parameter to assess the efficacy of herbal products/medicinal plants / herbal extracts. The application of herbal-based immunomodulators in aquaculture is a new beginning (Bairwa et al., 2012).

The oral method is considered as most promising for immunostimulation. The mechanism of action of immunomodulation is yet obscure, although some reports are available. Generally, immunomodulators present in feed trigger non-specific immune systems whereas antigenic substances *i.e.* bactrian or vaccines induce prolonged protection mediated by antibody production and acquired immunity (Galindo-Villegas and Hosokawa, 2004). In the indoor feed trial, at 30 days, there were no substantial variations were reported between the treatments, thereafter, at 60 days and 90 days,

considerable changes were reported ($p<0.05$) between the groups. A remarkable increase in antibody titre/IgM level was observed in T2 ($p<0.05$) and thereafter a decrease was recorded in T3 might be due to beneficial and immunomodulatory properties of herbal extracts or medicinal plants, and overdose might have reacted negatively in T3.

This is the first report that illustrated the immunomodulatory effects of TABP or bark extracts in fish, however, its immunomodulatory effects in other animal models being studied (Nazish et al., 2017). The immunomodulatory effects (antibody titre) of herbal product/ medicinal plants have been reported in other fish species *i.e.* tilapia (Jayathirtha and Mishra, 2004; Bairwa et al., 2012), carps (Pratheepa et al., 2010); *Channa punctatus* (Verma et al., 2012); Shrimps (Fu et al., 2007; Subramanian et al., 2013).

In intraperitoneal inoculation, the experiment also an elevated level was observed but was less than that indoor feed trial which might be due to the availability of natural food that has reduced the uptake of artificial feed. Whereas, under the intraperitoneal inoculation experiment, a substantial rise was observed ($p<0.05$) which is in contrast to a previous study (Dotta et al., 2014). This deviation in the current investigation may likely be due to dose optimization and efficacy of the extracts. Upon infection, T2 responded well in the indoor feed and outdoor pond feed trials but in the intraperitoneal inoculation experiment, T2 and T3 both performed better as compared to other groups. The bibliographic study showed that Total Ig level was regulated by the inclusion or inoculation of herbal extracts to fish (Mo et al., 2016; Safari et al., 2017; Hoseinifar et al., 2018; Jahanjoo et al., 2018). Overall, our study also revealed that in all three-tier trials; indoor feed, outdoor pond feed and intraperitoneal inoculation, the herbal powder, and *T. arjuna* extracts was found to statistically regulate the IgM level in *L. rohita.*

MicroProteins are small size globulins that contain only a single protein domain and probably protein-protein interactions but lack other functional moieties of total protein and are reported to influence biological processes in both plants and animals (Bhati et al., 2018). The Features of MPRS were characterized by primarily identified MPR, named as INHIBITOR of DNA BINDING (id) in animals. In the

current research, serum micro-Protein level was recorded to be highest at 90 days in T2 treatment of indoor feed trial, T2 and T3 in outdoor pond fee trial, and in T3 under intraperitoneal inoculation experiment. Our study showed that TABP and *T. arjuna* extract found to regulate the MPR level in fish. However, we do not have facts to substantiate the results.

5.18. Gut microbiome

The fish microbiota is impacted by a number of factors and the interaction of these microbiotas with fish physiology is still not clear. The fish encounters many pathogens from culture environment and when the population exceeds fish leads to disease infestation. Bacteria derived from the fish microbiome and enzymes produced by quorum quenching have reported an innovative strategy for manipulating the gut microbiota. At present, a new concept “forward microbiomic” is in discussion nowadays that mainly focuses on manipulating the microbiome to improve health status and ensure better growth performances. So, it is imperative to study, the gut microbiome to establish a relationship between microbiota and fish well-being. There are two possibilities, either majority of organisms are beneficial to fish then, it is termed as “normobiosis” or a majority are pathogenic, lead to “dysbiosis” state that later turns into disease condition of fish (Talwar et al., 2018).

The microbiota has key roles in producing vitamins, nutrient mobilization, maintenance of the integrity of intestinal tissues and regulation of non-specific immunity that is subjected to modulate or altered by manipulation in diets and culture environments (Tarnecki et al., 2020). Herbal materials and medicinal plants perhaps induce or alter the beneficial microbial community to produce post biotic which have active pharmacological effects (An et al., 2019). In the indoor feed trial, the community structure of gut microbes varied and observed a variation in T2 as compared to other treatments/groups. The Aeromonadaceae was found more in CT then decreased in T2 and then again increased in T3 and the reverse trend was found for Bacillaceae. This might be beneficial effects of TABP on gut microbiome which is in parallel to the study of Perez-Burillo et al. (2020).

In the outdoor pond feed trial, the trend was the same except the area of aeromonadaceae was intense as compared to the feed trial that might be the presence of pathogenic or rudimentary bacteria in an uncontrolled natural environment (Cabral, 2010; Novoslavskij et al., 2016). The similar findings were ascertained by Felipe et al. (2020) who had pointed that numerical abundance of firmicutes in tilapia gut was recorded maximum fed with the microalgae-based diet may likely be due to improvement in digestion and nutrient form microalga could influence the microbiota.

Similarly, Dimitroglou et al. (2009) has reported a high number of species in treated groups as compared to control one. Clustering revealed three sub-groups, those fall under two main clusters. CT and T3 fall in the same cluster and T2 and T1 present separate groups under cluster two. The histological changes in the present study showed better intestinal health in terms of villi number and orientation. The same results were also obtained by Souza et al. (2020).

The results of gut metagenomics of the previous study are in accordance with our other results that revealed better growth, survival and other parameters in T2 as compared with other treatments. From the present study, it can be concluded that feed indoor trial probably promotes “normobiosis” in T2 as compared with the other groups. Although, outdoor pond feed trial also follows “normobiosis”, the intensity was not much strong as compared with the indoor feed trial. In the outdoor pond trial, the two clusters having two treatments in each cluster representing CT and T1 in the same cluster and T2 and T3 in the same cluster is also in agreement with our results of growth data, biochemical and other parameters.

5.19. Histoarchitectural changes

5.19.1. Indoor feed trial

5.19.1.1. Gut histomorphology

The ethno-medicinal herbs and their associated products are known for their ability to enhance specific and nonspecific immune systems, development and growth, and disease protection (Shalaby, 2006). Herbal extracts, particularly

essential oils, stimulate the small intestinal mucosa, pancreas, and liver to secrete digestive juices and enzymes (Williams et al., 2001, Lee, 2003). Histological examination of the digestive system is a reliable marker of fish feed and feeding status (Hall and Bellwood, 1995; Green and McCornick, 1999; Caballero et al., 2003). As a result, monitoring the histological structure of the intestine is the preferred method for determining the dietary effect of nutrient mixtures containing herbal products (Raskovic et al., 2011).

Mucosa (columnar/glandular epithelium) and lamina propria (connecting tissue) make up the histological structure of the selected intestinal section (middle and hind gut) Submucosa (stratum compactum and stratum granulosum), mucosal layer, and serosa are the histological components of the selected intestinal section (mid and hind gut). As seen in the control fish, all of these cellular structures were present (Purushothaman et al., 2016).

According to Umer et al. (2009) plant protein inhibited growth by causing pathological changes in the distal part of the intestinal tract, such as shortening of intestinal villi, loss of supranuclear vacuolization of enterocytes, widening of villi and lamina propria, and infiltration of inflammatory cells. Similar observations were made in the current study. The treatment, T3 includes enterocyte shortening, lamina propria inflammation in the form of massive granular cells, and granulocytes completely replacing the connective tissue. On the other hand, the serosa was extremely thin. Many eosinophilic granular cells were found in the muscular, particularly the circular muscles, when compared to the control. According to Obaroh et al. (2020), fish fed a lower dose of plant saponin had inflammatory cells Furthermore, Kakawi et al. (2021) discovered that plant material could promote growth without affecting gut histology, resulting in more space for goblet cells and other absorptive muscles.

Fransis et al. (2001) showed that carp has a better tolerance to the anti-nutritive ingredients and tolerance is species-specific. Whereas T2 group showed numerous mucosal folds as compared to T1 and control groups. Interestingly, the height of the enterocyte of T2 is significantly ($p<0.05$) larger than the T1, T3 and CT. Furthermore, these enterocytes are gastric glands, which is a vital part of the digestion system. T2 had the least amount of inflammation in the cellular epithelial

layer when compared to T1 and T3. It is well documented that enterocytes/villus height is a useful histological parameter that can be applied for testing the digestibility and efficiency of particular plant materials of various origins (Amer et al., 2019). Rainbow trout cultured in cages fed the lowest lipid diet showed a rise in enterocyte height and a decline in the number of mucosal cells, according to Savic et al. (2012).

A similar observation was made in the T2 and T3 groups in the current study. This could be due to the availability of digestive lipid which might have lowered due to the inherent anti-lipidemic property of the *T. arjuna*. In, T3, the proliferation of the inner mucosa containing secretory cells and slight shortening of the enterocytes have been observed. This indicates that T3 with 1.5% addition of TABP caused more inflammation than the T2 group, though, the number of mucosal folds was the same as compared to T_1 and control. Pathological/ inflammatory responses and excess mucus cell proliferation has been observed in T3 which is in accordance to Obaroh et al. (2020), showing similar observations at higher percentage inclusion of *Moringa oleifera* saponia extract in *Clarias gariepinus*. In addition, intense expressions of eosinopholic granular cells have been recorded. This indicates the dietary stress and inflammatory responses in the intestine.

In treatment T1, though, the height of enterocytes was shorter than T2 and T3 groups, but no leucocytes infiltration was recorded. While, morphologically not many changes were observed in T3, but considerable ($p<0.05$) increase in mucus production was reported. Similar observations were also recorded by Raskovic (2016), where feeding with supplementary feed in common carp showed few histopathological changes including leukocyte infiltration and increased mucus production at a higher dose. Obaroh (2020) observed that the intestine of *Clarias gariepines* showed several leucocytes infiltration when *Moringa oleifera* saponin-rich crude extract was fed. Magrone et al. (2016) showed inflammatory cells in the lamina propria upon feeding polyphenol enriched feed to sea bass. Therefore, it is inferred from the current study that the intestinal absorptive layer showed more absorption towardsT2 with increased height of enterocytes gastric gland and mucus cell. While, T3 group showed inflammation in cell type *i.e.* expansion of eosinophilic granular cells, infiltration of leucocytes in the lamina propria and proliferation of mucus cells.

5.19.1.1.1. Histo-pathology of intestine infected with *A. hydrophila*

The present study showed complete necrosis, extremely shortening of enterocytes, lamina epithelial cells (columnar and granular epithelium), lamina propria (connective tissue) and sub-mucosal layer in the CT. Sub-mucosal layer, vacuolated and detached from the muscular layer. The serosa and muscular layers are being separated. Even the cells get necrotized and vacuolated. While in T1 group less necrosis was observed as compared to CT. Shortening of enterocytes, lamina epithelial cells and lamina propria have also been observed. Interesting to note that in T1 group, inflammatory cellular responses were observed in the sub- mucosal and mucosal layer, which has not been seen in CT. In addition, large vacuoles and necrosis were recorded in the muscularis and serosa. However, the serosa layer was extremely thin with a larger number of leucocytes inflammatory cell types in CT. In T2 intact enterocytes and lamina epithelium with inflammatory cell proliferation with mild necrosis were recorded.

Lamina propia which contains connective tissues showed severe necrosis and a large number of eosinophilic granular cells expression and leucocytes migration. Interestingly, the eosinophilic granular cell formed a granuloma like structure to prevent bacterial spreading. Localized, enterocytes with huge inflammatory cellular responses have been recorded. Muscular layer and serosa remained intact with a moderate level of migration of inflammatory cell (leucocytes) type). In the T3 group, similar pathological signs including the moderate level of enterocytes, lamina epithelial, lamina propria and severe necrosis were recorded. The necrosis even deeper into the muscularis layer as compared to T2. In addition to the expression of eosinophilic granular cells, eosinophilic centres have been recorded into lamina epithelial/ lamina propria. These centres have been recorded for the first time in the *Aeromonas* infection studies. These leucocytes are densely distributed in the serosa layer as well as in the lamina propria, even up to the serosa layer. The rupture/ necrosis in this serosa and muscular layer was also observed in treatment T3. Among all, it is clearly shown that T2 showed better intestinal responses against the *A. hydrophila* showing with limited pathological responses and better resistance to infection that led to intact structures of cell types as compared to T3, T1 and CT, respectively.

The pathological severity has been recorded as T2 < T3< T1 and <CT. This indicates that T2, provides better protection against bacterial pathogens than, T3, T1 and CT groups. Similar observations have been recorded by many authors (Abdelhamed et al., 2016 & 2017). Abdelhamed et al. (2017) discovered that 5 h after injecting *A. hydrophila* into channel catfish, about 75 percent of the surface of the whole mucosal layer was necrotized, with prolonged necrosis of the complete mucosa with enterocytes and homogenous constituent within the lumen at 24 and 48 h post-infection (hpi). After 24 h in the kidney, about 75% of the posterior kidney demonstrated pathological responses. This indicates that the intestine, rather than the kidney, may be a more effective target organ for *A hydrophila*.

5.19.1.1.2. Histo-pathology of intestine infected by *E. Tarda*

E. tarda has been identified as one of the most pathogenic bacteria in a variety of fish species. Furthermore, *E. tarda* caused edwardsiellosis is a generalized septicaemia that is often associated with poor water quality. In the present study, it has been recorded that in CT, moderate necrosis in the intestinal mucosal layer particularly in the lamina epithelium with hypertrophic goblet cells and hypertrophy of enterocytes in the mucosal epithelial layer with numerous vacuoles/voids which is an indication of cell death. The connective tissue also showed necrosis with enormous leucocytes proliferation and aggregation. Both muscular layer and serosa did not show any major pathological signs through these minor inflammatory cell migrations. The intensity of intestinal pathogens in T1, showed minor necrosis in the lamina epithelial layer and lamina propria. However, infiltration of the inflammatory cells/ leucocytes was enormous but less than CT. The separation between lamina propria and muscular layer was noticeable with numerous eosinophilic granular cells. But the enterocytes were filled into inflammatory cell types.

In T2, numerous mucosal folds with moderate necrosis in the lamina epithelial layer and lamina propria were observed. Extensive infiltration of leucocytes and expression of eosinophilic granular cells were also recorded. Though the connective tissues of lamina propria showed necrosis, the structure remained intact and the expression of eosinophilic granular cells is extensive in the muscular layer. However,

thinning of muscular layer and serosa have been observed. In addition, inflammation in the serosa was also observed. This indicates that though these are pathological responses, the intestine mucosal fold remained intact.

Whereas in T3, severe pathological sign showing complete detachment of lamina propria from lamina epithelial layer, expression of eosinophilic granular cells in the lamina propria and extensive migration of leucocytes on the necrotized tissue, where the serosa is completely lost and migration of leucocytes in the muscular layer. This indicates the combined impact of the virulence of bacteria as well as the negative impact of high-dose TABP. Many studies reported to have pathological changes in the liver, kidney, spleen, and gill as the target organ in *O. niloti*cus, *C. gariepinus* and in channel catfish (Ruiz et al., 2020). However, the present study has provided an insight into the histological signs in the intestine with a comparative pathological response among C1, T1, T2, and T3 groups.

5.19.1.2. Histomorphology of liver

Histology is a traditional powerful technique used for monitoring the effects of various feeds on fish digestive systems. The effect could include the pathological changes on the liver and intestine due to over or under-feeding (Shan et al., 2016). Liver and intestine are an important organ for digestive and absorption of food nutrition, thus monitoring of these organs are necessary. Melanomacrophages centres (MMCs) are considered as a histological indicator of immune function in fish and other poikilotherms (Steinel and Bolnick, 2017). The liver controls many metabolic processes, including protein, carbohydrate, and lipid synthesis. Furthermore, the liver is involved in the detoxification of both endogenous waste products and toxins, drugs, and pesticides derived from outside sources. Fish secrete bile at a much slower rate than mammals, which contributes to their susceptibility to chemical damage due to the slower flow of metabolites from the liver (Gingerich, 1982).

In the present study, liver pathology such as necrosis, host Inflammation, fibrosis with fat deposits and altered structure in the normal architecture of hepatocytes are considered as a biomarker for liver toxicity and function upon TABP

feeding. The liver of CT group had a normal organization of polygonal hepatocytes, normal bile duct and central vein. The liver section of T3 showed fibrosis with fat deposits and altered the normal architecture of the hepatocytes. A comparable observation has also been recorded by Obaroh et al. (2020) on *Moringa oleifeva* saponin extract on the liver of *Clarias gariepinus* at a high dose of extract.

Further, Alotunde et al. (2011) showed similar results of disarrangement of hepatic cell and necrosis in the liver after feeding of *Moringa oleifera* seeds powder to Nile tilapia. Sheikhlar et al. (2011) also recorded a distraction of the normal architecture of the liver at a high concentration of the plant extract applied. Ahmed et al. (2020) showed that *Moringa Oleifera* leaf extract repairs the oxidative misbalance in Nile tilapia that is an indication of improved liver function. Kakawi et al. (2021) revealed the protective impacts of raw and processed *Mucuna pruriens* based feed on common carp fingerlings and pointed that in response to graded level extract, the liver function gets improved without any side effects on cell types and structure.

Similar observations were also recorded in our study that the necrosis of liver hepatocytes and destruction of normal architecture was observed to be highest in T3 and other treatments showed slight inflammatory responses but cell structure was found to be intact. Host immune responses were recorded in T1 and T2 groups showing mature melanomacrophage centres, and pigmentation while the necrosis and destruction of the normal architecture structure start with high magnitude in T3 group.

A sudden increase in live cell type damage is due to high dose and there is a vast margin between T2 (10 g kg^{-1}) and T3 (15 g kg^{-1}) that reflected in histological changes those also corroborated with the results of serum parameters, biochemical parameters and growth performances of the fish. Even the optimised dose was 7.9 g kg^{-1} that has a vast difference from the maximum dose used in the study. This clearly shows that in T3 liver hepatocytes show comparatively focal necrosis could be due to excess/overdose of the herbal material that the TABP contains. Similarly, Forcados et al. (2021) evaluated the long-term effects of *A. ind*ica and artesunate on Wistar rats. The results demonstrated vascular congestion and necrosis in the liver and kidney and thereby assumed to impose hepato and nephron-toxic effects.

5.19.1.2.1. Clinical signs

The present study showed clinical signs of haemorrhages in the abdomen and base of the pectoral fin on 3rd day post-infection (dpi). While scale rupture and belly swelling were observed on 5th and 6th dpi. Fish mortality started on 7th dpi. Abdelhamed et al. (2017) shown that most of the internal organs including the intestine, gill, kidney and liver lesions start between 24 to 48 h. This infers that after 2 dpi, clinical signs in terms of haemorrhages and other signs start developing as in accordance in the previous studies which recorded in other fish species, Nile tilapia, crucian carps, rainbow trout and channel catfish species (Candan 1990; Baumgartner et al., 2017).

5.19.1.2.2. Histopathology of liver challenge with *A. hydrophila*

Histology of liver in the CT group challenged with *A. hydrophila* showed severe multifocal necrosis, inflammatory cellular aggregation and narrowing of sinusoidal channel. In T1, the liver showed moderate necrosis and inflammatory cellular response. Moderate level of expression of pigment which could be host immune responses which were recorded maximum in T2. Fish liver of T2 challenged with *A. hydrophila* showed prominent melanin pigmentation as (MMC) expression indicating the host inflammatory responses are more prominent in hepatocytes of T2 challenged with *A. hydrophila* indicating the healthy liver cell and same has been corroborated with the higher percentage of survival, growth performances, serum parameters and immune responses of the present study.

While, T3 group showed necrotic hepatocytes with more inflammatory cell aggregation. These inflammatory cells are conspicuous at the necrotic hepatocytes, less major MMC was observed as compared to the T2 group. This could have been due to the excess amount of the antioxidant suppressing the host inflammatory responses. However, the necrotic hepatocytes were less as compared to T1. This is underlining the use of the chemical against the bacterial pathogen is lower in T1 and higher in T2. Higher dose resulted in immunosuppressive but less hepatocytes necrosis than the T1, where, the host immune system though expressed but failed to confirm the host hepatocytes neurosis/death.

Mahmoud et al. (2014) has recorded that the liver of Nile tilapia treated with *curcuma longa* exhibited mild degeneration to almost normal hepatic cords. The findings of current investigation are in accordance with the study of Palanikani et al. (2020) who have highlighted the protective effects of *Andrographis paniculata* in dose-dependent manner against *A. hydrophila* in *L. rohita*. Similarly, Sahu (2004) has revealed in her Ph.D. dissertation that plant extract provides a non-specific and enhanced humoral response that will be protecting against opportunistic pathogens. Further, many authors have highlighted the antioxidant potential of the herbal product, medicinal plants including *T. arjuna*, possessing hepato-protective properties (Soni and Singh, 2019).

5.19.1.2.3. Histo-pathology of liver challenged with *E. tarda*

A comparison of liver histology between CT and T1, T2, T3 groups revealed that *E. tarda* infection caused more necrotic / tissue damage than *A. hydrophila* infection, indicating that *E. tarda* is more pathogenic than *A. hydrophila*, which is consistent with previous studies (Kusnadi et al., 2019).

Liver of control group (CT) fish challenged with *E. tarda* showed severe hepatocytes necrosis and complete architecture has altered/ damaged with the presence of hypertrophoid hepatocytes and karyorrhexis. However, the inflammatory response was less in the form of inflammatory cellular migration. In T1, less alteration in a cell was witnessed with the better shape of the hepatocytes as compared to CT and multifocal necrosis and karyorrhexis and the presence of less inflammatory responses and expression of pigmentation could not be observed. In T2, extremely host inflammatory responses in terms of inflammatory cellular aggregation to the infected site and formation of granuloma were observed. The host inflammatory cells may likely restrict the bacterial proliferation which was extremely visible.

However, the MMC, the host inflammation was absent in the observed section, could be due to the preliminary responses as the granuloma formation and further combined responses could not be observed. While T3 group showed detachment of hepatocytes from the basement membrane and necrosis of hepatocytes.

Interestingly, no-host inflammatory responses in terms of cellular migration aggregation or pigment formation have been observed. This indicates that immunosuppression due to excess herbal products fed to fish could be one of the reasons. Furthermore, the hepatocytes structure showed altered architecture as hypertrophoid and karyorrheaxis in T3 group. Melanocytes necrosis was very conspicuous which is in accordance with Hirai et al. (2015). Furthermore, in T3, focal necrotizing hepatocytes in liver have been observed that is in agreement with Mojzesz et al. (2020). Similar, observations have been recorded due to toxic substances like algal toxins or herbal extracts could have an impact on the hepatotoxicity due to *E. tarda* infection (Oh et al., 2020).

5.19.1.3. Kidney histomorphology

The kidney is considered as a diagnostic vital organ for assessing the toxicological impacts of any material. Kidney of fish is mainly designated to function as a unit for urea retention and body fluid regulations in freshwater fish (Hyodo et al., 2014). It has been reported that kidneys of the fish are sensitive to any changes those are taking place in water (Hussain et al., 2019). Furthermore, toxicity due to herbal material or chemical inoculation as an oral or intraperitoneal inoculation plays an important role in providing credence to toxicity upon long-term and excessive use (Imo et al., 2021). The important cell types of the fish kidney are brush border lumens and pigment cell and melanomacrophages centres (MMCs). Fish MMCs are anatomically parallel to the higher vertebrate's germinal (mammalian) centre (GC), leading to the theory that the MMC plays a role in the specific immune system (Stosik et al., 2019).

Feeding upon TABP resulted in alteration in cell types of kidney expression of melanin pigmentation that ultimately led to the formation of matured MMC were observed. In CT presence of various sized tubular lumens (irregular shaped) with normal hematopoietic tissue were observed. In T1, the kidney showing irregular brush border structure in different tubular lumens with an expression of melanin pigmentation further resulting in matured MMC that might be beneficial and immune enhance the function of the TABP. And in T2 unaltered cell types with slight aggregation of MMCs were observed while in T3 the infiltration of melanocytes,

narrowing of tubular lumens with aggregation of MMCs were observed that might be due to excess dose that is affecting the cell types after T2 and the severe impact was observed in T3 like hematopoietic toxicity that is in accordance to the previous studies (Shahid et al., 2021).

The results of other hematological parameters such as haemoglobin and RBCs also showed a significant reduction in T3 and T2 revealed the best result for these parameters that is an indication that the kidney being a haematopoietic organ gets negatively affected due to excess dose of TABP. The findings of the current study are in accordance to Ayotunde et al. (2011) by feeding aqueous extracts of *Moringa oleifera* to *Oreochromis niloticus* and *Origanum onites* essential oil to rainbow trout (Yigit et al., 2017).

5.19.1.3.1. Histopathology of kidney challenged with *A. hydrophila*

The complete necrosis of proximal & distal tubules, hematopoietic tissue necrosis and narrowing of brush borders and haemorrhages was observed in CT upon infection with *A. hydrophila* that is parallel to the past research (AlYahya et al., 2018; Rosidah et al., 2020). T1 group, showing expression of melanomacrophages centres with extensive inflammatory cell aggregation and necrosis.

In T2 group, distal tubular lumen with slightly altered structure with macrophage aggregation and less necrosis in proximal & distal tubules, vacuole degeneration of hematopoietic tissue, less hematopoietic tissue necrosis, and in T3 group the intensity of necrosis increases and monocytes, MMC proliferation, inflammatory cells massive infiltration were observed.

These results are in accordance to the previous studies (Latif et al., 2021) who has pointed out that black seed did not alter the structure of vital organ till 2.5% inclusion. The extracts also had a significant impact on plasticity to recover the anatomy and swimming pattern of infected fish, according to Palanikani et al. (2020). These findings are consistent with our findings for serum kidney function parameters such as CKNac, GGT, and creatinine.

5.19.1.3.2. Histo-pathology of kidney challenged with *E. tarda*

In CT treatment following infection with *E. tarda* revealed granuloma/edema type macrophage aggregation at the tubular lumen, luminar epeithelium and hematopoietic tissue necrosis and haemorrhages that is in parallel to the recent research (Hoque et al., 2020; Ayunin et al., 2020). In T1 group, proximal & distal tubular necrosis with macrophage aggregation, expression of MMC and less hematopoietic tissue necrosis was reported as compared to CT. Similarly, in T2 mild distal tubular necrosis but the overall tubular architecture was maintained, normal lumen structure, expression of melanomacrophage pigmentation. In T3 moderate to severe necrosis in distal & proximal tubules and melanomacrophage pigmentation. The same results have been obtained in a previous study by Aznan et al. (2018).

5.19.1.4. Gill histomorphology

Due to the large surface area, the gills of fish are considered efficient bio-monitoring tools (Sweidan et al., 2015). The essential role of gills is respiration, osmoregulation and acid-base regulation (Fernandes et al., 2007). Gills are the early warning signals about the unfavourable environment (Benli et al., 2008). Due to exposure to contaminants, the gill epithelium gets damaged or alteration in structure is observed in dose and time of exposure dependent manner (Evans et al., 2005; Gomes et al., 2012). And also, used as an early signal to assess the health condition of fish (Sorour, 2001).

Mucus cells of gills act as the primary structure of gills to protect fish against toxic substances (Bernet et al., 1999). In current investigation, CT showing normal architecture with primary lamellae, secondary lamellae, mucus cell, pillar cell, erythrocyte within a capillary lumen.

In T1, normal secondary lamellae with erythrocytes aggregation at the tip of the lamellae which is in agreement with the study of Kaur et al. (2020) who reported the congestion in cell types that led to more blood flow rate under some biotic and abiotic stresses. In T2, secondary lamellae thickening, erythrocytes and pillar cell

proliferation and fusion at the base of the secondary lamellae might be due to inflammatory response.

In T3, a fusion of the secondary lamellae, erythrocytes, mononuclear cell proliferation might be an overdose of the TABP that causes toxic effects on the morphology of the gill. The same results have been reported earlier also (Chahardehi et al., 2020). However, for herbal toxicity, this organ has not fully been well-thought-out so far.

5.19.1.4.1. Histo-pathology of gill challenged with *A. hydrophila*

The gill of CT treatment showed Telangiectasis lesions characterized by focal necrosis, blood-filled distension via lamellar thrombosis representing edema cell types which are in parallel to the studies of Manoj et al. (2010). In T1 mild edema with thinning of cartilaginous matrix and hyperplasia and hypertrophy of goblet cells within the tip of the lamellae. In T2, detachment of secondary lamellae from the cartilaginous matrix to provide enough space to recover the infected portion, hypertrophoid erythrocytes and normal chondrocyte's structure, comparatively healthy secondary lamellae with less inflammatory responses as compared to CT and T1. While, in T3, a massive fusion of secondary lamellae with discontinuation of primary lamellae with hyperplasia in chondrocytes might be the toxicological reaction of TABP at a higher dose (15 g kg^{-1}).

Histopathological modifications/alterations in the tissues, such as contracted and thin secondary gill lamellae, slight to modest penetration of inflammatory cells in the primary and secondary gill lamellae, were reported in previous studies at sub-lethal concentrations of *Azardirachta indica* leaf extract (Geetha et al., 2019). Histology of gill tissues discovered damage of secondary filaments at the base due to aflatoxin inclusion levels of 20 and 40 ppb, according to Mohapatra et al. (2011).

Similarly, Verma (1997) found swollen, oedematous, and atrophic epithelial cells in secondary lamellae of *Channa punctatus* during acute aflatoxin toxicity. Furthermore, Jantrarotai et al. (1990) observed gill lamellae folding towards filament in channel catfish injected intraperitoneally aflatoxin with 12 mg kg^{-1} body weight. The

results of gill histology are corollary to the results of some of the serum parameters such as triglycerides and also with enzyme of oxidative damage *i.e.* SOD, catalase and GST. Thus, the study concluded that the feeding trial followed by a challenge study could not provide much regenerative capacity in T1 but it has a highly positive impact in T2 while T3 showed mixed performances as it was found to be better than CT and T1 but inferior to T2.

5.19.1.4.2. Histo-pathology of gill challenged with *E. tarda*

In fish, histopathology of the gills *E. tarda* infection caused kidney tissue damage, including edoema, necrosis, and congestion. Edema is caused by secondary lamella gill tissue damage. Inflammation of the secondary lamella occurs in response to disease occurrence and exposure to variable environmental fluctuations. A gill response to the presence of hazardous constituents *i.e.* chemicals or microbial infection, is the cause for swelling of secondary lamella composed of mucus. Because the gill structures were semipermeable, liquid in the form of mucus was able to arrive inside the gill tissue, causing inflammation in the secondary lamella (El-shayad et al., 2010). Infection with *E. tarda* bacteria causes inflammation and gill tissue damage. In the current study, CT revealed edema and fusion of secondary lamellae tips, excessive hypertrophoid mucus cells, and lamellae fragmentation, which is consistent with Ayunin et al (2020).

In T1, massive mucosal cell hyperplasia and complete devastation of primary lamellae including diffusion of gill lamellae and telangiectasia. In T2, intact secondary lamellae with massive infiltration of leucocytes in the cartilaginous matrix and mild telangiectasia. In T3, severe interlamellar fusion and massive infiltration of mononuclear inflammatory cells. Similar pathological signs have been recorded in channel catfish by Pirarat et al. (2006) and Abraham et al. (2015). Thus, the study highlighted that T2 performed better in terms of protecting *E. tarda*.

6. SUMMARY

A paradigm shift in research from basic to applied sciences has created opportunities for cutting-edge research in aquaculture fields such as nutrition, disease management, and nutrigenomics. Due to its consumer preference and adaptability with other carps in six species polyculture, Indian major carps, particularly *L. rohita*, are the mainstay in Indian aquaculture. The current fish production would not be enough to encounter the needs of an ever-growing Indian population in the coming years. There is a need to improve the culture as well as the health management practices. Intensification of culture practices introduces an unwelcome risk of disease infestation. Aquaculture suffers from disease infection caused by microbial pathogens such as bacteria, parasites, fungi, and so on. Chemicals and antibiotics are commonly used as prophylactic measures to prevent or reduce disease outbreaks. In terms of loss of natural beneficial microbiota and unmeasurable antimicrobial resistance, the negative effects of antibiotics and noxious chemicals are irreversible (AMR).

The current study was conducted in this regard to evaluate the facile extract matrix for its antimicrobial and antioxidant properties, elucidation of compound characterization for its antimicrobial and antioxidant properties, dietary effects of *T. arjuna* bark powder (TABP) under in-door feed trial, feed trial in pond conditions, and intraperitoneal inoculation of the effective extract on growth. The present study can be summarized as follows

In-vitro antimicrobial activity, anti-oxidant potential and DNA nicking inhibition, and compound elucidation

- The presence of kanamycin and tetracycline in ethanol bark extract was first reported.
- Ellagic acid, 18-Glycyrrhetinic acid, Azelaic acid, and Caffeic acid were discovered throughout the matrix of the extract.
- The discovery of naringenin in acetone bark extract, orientin and phloretin in leaf, and andrographolide in fruit is of pharmaceutical importance

- A dose of 0.5 mg mL^{-1} of ethanolic bark extract (Br5) was found to be effective against bacterial isolates used in the study.
- The ethanolic and methanolic bark extracts demonstrated maximum activity against *A. invadans* at 1.0 mg mL^{-1}; however, methanolic bark extract demonstrated less efficacy as compared with ethanolic bark extract against *A. invadans*.
- Based on the *in-vitro* screening, the three most effective solvent extracts against *A. bengalensis* were ethanolic bark extract, methanolic bark extract and acetone fruit extract. The LC_{50} values of extracts for fish and *A. bengalensis* were found to be 4:1.
- The maximum ash content was observed in arjuna bark powder 28.95±0.001% (serial) and 28.19±0.008% (individual). After fractionation, the Zn content of fruit extracts increased and recorded highest to be in methanolic extract (45.29 mg L^{-1}).
- Two metabolites, gallic acid and ellagic acid were found across the solvent extracts particularly polar solvent extracts of *T. arjuna.* The estimated concentration on which solvent extracts could reveal DNA nicking inhibition activity was 0.48 mg.
- The presence of phenolic constitutes in TLC plate can be confirmed with visualisation of red colour spot upon bleaching with purple coloured DPPH reagents and anisaldehyde sulphuric acid and vanillin sulphuric acid. The Rf value for the spot was estimated as to be 0.83.

Indoor feed trial

- The highest WGP (148.41±0.854) and SGR (1.51±0.854% per day) and PER (0.52±0.027) were observed in *L. rohita* fingerlings fed with 10 g kg^{-1} TABP among dietary treatments while FCR (1.63±0.081) followed a reverse trend for T2 as compared to other groups. The overall order for growth performances was as followed, T2>T1>CT>T3. PER, ANPU and FTI exhibited no significance ($p<0.05$) among the treatments.
- The polynomial regression optimised the dose of TABP as to be 7.9 g kg^{-1} of feed. The maximum value for moisture and lowest values was recorded in T3

as compared to other treatments. The maximum DNA: RNA ratio was recorded in the intestine and lowest in the liver.

- In the feed experiment at 90 days, lysozyme activity, bactericidal activity, NBT, albumin, total protein, globulin, RBC, WBC, haemoglobin, T3, T4, IgM level, MPR, and other hemato-immunological parameters increased in T2 as compared with CT and T1. In the feed trial at 90 days, digestive enzymes such as lipase, amylase, protease and alkaline phosphatase were found statically regulated between the treatments. The greatest value of albumin and globulin at 90 days, was observed in T2 followed by T1>T3 and CT.
- At the end of 90 days indoor feed trial the serum parameters such as triglycerides, lactate dehydrogenase, cholesterol, glucose, bilirubin total, serum pyruvic transaminase, serum glutamic oxaloacetic transaminase, creatine kinase, creatine, gamma glutamyl tansferase, alkaline phosphatase, and cortisol showed a significant decreased in T2 as compared($p<0.05$) to other groups. Whereas, albumin, total protein and globulin increased significantly in T2 as compared to other groups. Upon infection the trend was almost same for all the parameters, however, no significant change was observed in T2 when the parameters compared within the treatment, before and after challenged study.
- The enzyme of oxidative stress *i.e.* SOD, catalase and GST decreased significantly ($p<0.05$) in T2 and then enhanced substantially ($p<0.05$) in T3 as compared with other groups. The challenge study followed the same trend with higher values and between two bacterial pathogens the values were higher for *E. tarda*. The enzyme of neurotransmission AchE decreased till T2 but the highest values were recorded in T3 as compared with other groups. Upon infection, a no substantial increment was reported in all groups.
- Digestive enzymes, amylase, protease, lipase, alkaline phosphatase at 90 days showed significantly ($p<0.05$) greater values in T2. Upon infection, the values increased significantly ($p<0.05$) except for T2.
- In the feed trial, out of three genes, Mx showed remarkably ($p<0.05$) greatest expression in all groups followed by ISG15 and STAT1. Upon infection with bacterial pathogens, the trend was the same but the expression of ISG15 increased in all treatments as compared to the feed trial.

- The gut microbiota showed a diverse pattern for their abundance with graded level of TABP, however, the overall community structure remains unchanged. The CT and T3 formed one cluster, and T1 and T2 another cluster. The most prominent family is aeromonadaceae and can be represented as CT>T3>T1 and T2. The family of beneficial gut microbes, bacillaceae was observed prominently in T2 as compared with the other groups.
- Histology of intestine showed higher mucosal fold and height, comparatively more goblet cells and digestive glands expression can be ordered as T2>T1>T3 and CT. Upon infection with bacterial pathogens, *E. tarda*, histopathology of intestine showed a moderate level as follows, T3>CT>T1>T2>. While in the case of *A. hydrophila*, histopathology of intestine showed severe pathology as follows, CT>T1>T3>T2.
- Histology of liver showed of inflammatory responses that can be represented as (T3>T2>T1>CT). Upon infection with *A. hydrophila*, histopathology of the liver showed severe multifocal necrosis and inflammatory cells aggregations. The T2 showed comparatively healthy hepatocytes and pathological signs were as T3> CT T1> T2. While in the case of *E. tarda*, histopathology of the liver showed severe pathology as compared to *A. hydrophila* indicating the pathogenicity of *E. tarda* is higher and it could follow the trend as, T3>CT>T1>T2.
- Histology of gill showed extensive inflammatory responses as T3>T2>T1>CT. Upon infection with *E. tarda*, histopathology of the gill showed extensive fusion and telangiectasia in the gill. The pathological severity was as follows, CT>T1>T3>T2. In case of *A. hydrophila*, histopathology of gill showed extensive fusion and edema in the gill. The pathological severity was as follows CT>T1>T3>T2.
- Inflammatory responses in the kidney were as follows, T3>T2>T1>CT. Upon infection with *E. tarda*, histopathology of the Kidney showed extensive inflammatory responses as T2≥T1>T3>CT. In the case of *A. hydrophila*, histopathology of the kidney showed extensive inflammatory responses as T3>T2>T1>CT with severity of pathology and tubular lumen structure showed as CT>T1> T3≥ T2.

Outdoor pond feed trial

- Maximum average weight gain was observed in T2 (31.8 ± 0.66) followed by T1 (22.17± 0.48) > T3 and CT. FCR, FER, PER, SGR, ANPU etc., which varied substantially ($p<0.05$) among treatments. The HSI and GaSI varied considerably ($p<0.05$) in response to dietary TABP while CSI was insignificant ($p>0.05$). Parameters such as FI, FR, CSI and EI exhibited no significance ($p>0.05$) among the groups.
- The optimized dose of TABP was estimated to be 8.5 g kg^{-1} in the outdoor pond feed trial. The carcass composition of fish exhibited no significance ($p<0.05$) among the groups, however, all parameters increased in T2 except moisture which showed maximum value in T3. The values of RNA content varied significantly ($p<0.05$) among the treatments. Irrespective of treatments and across the vital organs the RNA values showed a trend as follows: RNA-L > RNA-B > RNA-K > RNA-I > RNA-M.
- At the end of outdoor pond feed trial at 60 days, the values of lysozyme activity, bactericidal activity, NBT, albumin, total protein, globulin, RBC, WBC, haemoglobin, T3, T4, IgM level, MPR and other hemato-immunological parameters increased significantly in T2 but it was not significantly different with T3, as compared to other groups. Upon infection, the values decreased significantly ($p<0.05$) in CT but differ non-significantly in T2 and T3.
- The digestive enzymes, protease, amylase, alkaline phosphatase and lipase at 60 increased considerably ($p<0.05$) in T2 and T3 as compared with other treatments. Upon infection (70 days), the values decreased substantially ($p<0.05$) within the treatments except for amylase70-Ah and amylase70-Et.
- The serum parameters such as triglycerides, lactate dehydrogenase, cholesterol, glucose, bilirubin total, serum pyruvic transaminase, serum glutamic oxaloacetic transaminase, creatine kinase, creatine, gamma glutamyl tansferase, alkaline phosphatase, cortisol, decreased significantly in T2 but it has no significance with T3 as compared to other groups and the same pattern was followed in challenge study (70 days).
- The enzyme of oxidative stress *i.e.* SOD, Catalase and GST decreased substantially ($p<0.05$) in T2 and T3 as compared with other treatments. The challenge study followed the same trend with higher values as compared to

the pond feed trial, and between two bacterial pathogens, the values were higher for *E. tarda*. The enzyme of neurotransmission AchE60 exhibited no significance among the treatments, and upon infection with bacterial pathogens, the same trend was followed.

- Gut metagenomics study showed the presence of aeromonadaceae in all treatments (45-65% area). The CT and T1, and T2 and T3 showed the same pattern of distribution, the numerical abundance of gut microbiota in response to dietary TABP.

Intraperitoneal inoculation experiment

- The growth performances and nutrient utilization increased considerably ($p<0.05$) in the first 15 days with a graded level of *T. arjuna* ethanolic bark extract and then a slight reduction was observed in all the parameters.
- The values of moisture content and ash content showed a significant inverse relationship in T3 with maximum moisture content and lowest ash content but T2 and T3 exhibited no significance between them.
- Nucleic acid analyses showed a significant increase in T3 in the first 15 days and thereafter a slight decline was observed in the values. DNA/ RNA also differed significantly ($p<0.05$) between T2 and T3 groups and the trend was as followed, RDL>RDK>RDB>RDI>RDM.
- In intraperitoneal inoculation experiment, the values of lysozyme activity, bactericidal activity, NBT, Albumin, total protein, globulin, RBC, WBC, haemoglobin, T3, T4, IgM level, MPR, and other hemato-immunological parameters considerably ($p<0.05$) enhanced in T3 but it did not differ significantly with T2. Upon infection, the values of all parameters decreased substantially ($p<0.05$) in CT and T1 as compared to other treatments. Upon infection, the values declined ($p<0.05$) considerably in CT for both Ah and Et but in T1, there is significant decrease was observed for Et only. However, T2 and T3 did not show any significant decrease.
- The SGOT15, SGOT30, SGOT25-Ah and SGOT25-Et varied considerably ($p<0.05$) between the groups except SGOT30 which exhibited no significance ($p>0.05$) in CT and T3. Upon infection with bacterial pathogens, the values varied substantially ($p<0.05$) among the groups. Within treatments, the values

increased considerably ($p<0.05$) in CT and T1 but in T3 and T2, a non-significant increase was observed.

- The serum parameters such as triglycerides, lactate dehydrogenase, cholesterol, glucose, bilirubin total, serum pyruvic transaminase, serum glutamic oxaloacetic transaminase, creatine kinase, creatine, gamma glutamyl tansferase, alkaline phosphatase, cortisol, showed no significant difference among the groups at 15 and 30 days. Upon infection, among the groups, the values varied considerably ($p<0.05$). Within treatments, the values are increasing substantially ($p<0.05$) in CT and T1 but in T2 and T3, no significance was observed.
- The enzyme of neuro-transmission, AchE15, AchE30, AchE25-Ah and AchE25-Et varied considerably ($p<0.05$) among the groups. Upon infection also the enzyme varied substantially ($p<0.05$) between and within the treatments.
- The enzymes of oxidative stress, CAT15, CAT30 exhibited no significance ($p<0.05$), however, SOD15, SOD30, GST15 and GST30 showed significant differences among the treatments. Upon infection, enzymes showed significance between and within the treatments.
- In intraperitoneal inoculation experiment, the Mx showed maximum expression followed by ISG15 and STAT1. Upon infection, the expression of Mx and STAT1 showed an elevated level of fold change in T1, T3 and T3 while in case of STAT1 the enhanced level of fold change was observed in T2 and T3 only.

7. CONCLUSION

Terminalia arjuna is well established ethno-medicinal plants which is known for its antimicrobial properties, antioxidant potential, and various health beneficial effects. In the present study three parts, leaf, bark and fruit were extracted with 7 solvents; hexane, ethyl acetate, chloroform, acetone, ethanol, methanol and distilled water, based on their polarity and thus, a total of 21 solvent extract were obtained. The solvent extracts were screened for their efficacy against prevalent fish pathogens including, bacteria, fungus and parasite.

An *in-vitro* study on antimicrobial properties of solvent extract of *Terminalia arjuna* showed that polar solvent extracts exhibited better antibacterial activity. Among 21 solvent extracts, ethanolic bark extract was found to be most effective against gram-positive, gram-negative, and OTC resistance bacterial isolates. The effective dose of ethanolic bark extract was found to be 0.5 $mgmL^{-1}$ and it has shown maximum efficacy against *Edwardsiella tarda*. Similarly, the ethanolic bark and methanolic bark extracts exhibited antifungal activity against *Aphanomyces invadans* at 1 $mgmL^{-1}$. The solvent extract were also screened against *Argulus bengalensis*, a malignant fish ecto-parasite and results showed that ethanolic and methanolic bark extracts, and acetone fruit extracts, showed lower therapeutic index, a measure for efficacy of drugs, as compared with other solvent extracts, thereby leads to a better efficacy against *A. bengalensis.* The compound elucidation showed the presence of some of the antimicrobial compounds such as Azelaic acid, Caffeic acid, Syringic acid, 18-β-Glycyrrhetinic, and one natural antibiotic, Oxytetracycline in higher concentration in ethanolic bark extracts as compared to other solvent extracts which might be attributed to the better antimicrobial activity of ethanolic bark extracts

Thus, present study suggests that ethanolic bark extract has wide spectrum antimicrobial activity which can be used further for designing broad spectrum antimicrobials for aquaculture applications.

The solvent extract were studied for their possible antioxidant activity and DNA nicking inhibition activities. The present study ascertained better phenolic compounds and flavonoids as compared with previous studies. The qualitative test for bioactive compounds showed the strong presence of flavonoids in ethanolic bark extract and

methanolic bark extracts, and in-vitro quantitative test also showed the same trend. The ethanolic bark extract showed better DNA nicking inhibition activity at 0.48 mg concentration as compared to other solvent extracts in pBR322 plasmid model under induced oxidative stress conditions. The compound characterization and elucidation by FT-IR, UV-VIS and HRLCMS showed the presence of strong antioxidant confirmed the antioxidant potential of bark extracts.

The compound characterization showed first reports on Naringenin in Acetone bark extract, Kanamycin, Oxytetratetracycline, Quinine and Azelaic acid in Ethanol bark extract, Orientin and Phloretin in ethanolic leaf extracts, and Andrographolide in fruit and bark extracts. In addition, Gallic acid, and Ellagic acid were the common antioxidants found across the solvent extracts with highest concentration in ethanolic bark extract followed by methanolic bark extract as compared with other solvent extracts. As present study targets the ethanolic bark extract only, however, other extracts also had a wide array of bioactive compounds which might be useful for pharmaceutical applications. This needs further study for their industrial utilization in designing the bio-pesticides and drugs for aquaculture.

Based on the in-vitro screening of antimicrobial activity and antioxidant potential of *Terminalia arjuna* solvent extracts, the feeding trial was conducted to evaluate the effect of *Terminalia arjuna* bark powder (TABP) based feed on growth and survival in *Labeo rohita*. The indoor feed trial and outdoor pond feed trial performed better in terms of growth and survival at 1.0 % inclusion(10gkg^{-1}) of TABP in fish feed. At same inclusion level the growth performances was better in pond trial that is most likely to be due to availability of natural food in pond conditions as compared with indoor feed trial. Similarly, gutmicrobiome study showed a better comminuty structure and proved the hypothesis of “normobiosis”, which is an indication that it might have acted as prebiotic in the gut of treated fish. The histoarchitectural changes in intestinal absorptive area and microvilli, and improvisation in cell types of other vital organs might be attributed to the physiological properties of the feed. The enhanced activity of digestive enzymes show the physiological capability of TABP to trigger the digestion of feed constituents and an elevate level of T3, and T4 that is an indication that TABP feed has positive impact on fish growth without compromising the health status of the fish and histology of vital organs. Similarly, intraperitoneal inoculation experiment was showed

that an inoculation of 8-12 µg per fish could enhance the short-term growth (15 days) and extended survival in *L. rohita*. It may likely be possible that TABP could have triggered the gene interactions at cellular level that might be confirmed with elevated level of albumin, and total protein which are used as marker for assessing the nutritional status of an animal in addition to immune responses. Thus, it can be concluded that TABP based feed and fractionated *Terminalia arjuna* ethanol bark extract can be used as growth enhancer, and further for the preparation of medicated fish feed.

The TABP based feed and ethanolic bark extract could enhance relative percentage survival, hemato-immunological indices, immunoglobulin level, microProtein level when fish was challenged with, *Aeromonas hydrophila* and *Edwardsiella tarda* which is a clue that TABP based feed and ethanolic bark extract can have better utilization in developing disease resistance against bacterial fish pathogens and as non-specific immunity enhancer. In addition, an elevated expression of immunogenic genes, Mx, STAT1 and ISG15, is an indication of enhancing specific immunity in general and Mx shows that it can also have an anti-viral defense mechanism against viral pathogens in particular.

In conclusion, present study recommends that *Terminlia arjuna* bark powder (TABP) based feed and its ethanolic bark fraction can be utilized as feed supplement for growth enhancement and immunomodulation in *Labeo rohita*.

8. FUTURE PROSPECTS

1. A comprehensive study is essentially required to assess whether methanolic fruit extract could be used to treat zinc deficiency and nervous disorders in fish species, as well as human health.
2. Firstly, the compounds showed excellent in-vitro activity, which can be investigated further for pharmaceutical applications in aquaculture and human health benefits.
3. Intraperitoneal inoculation of ethanolic bark extract provides evidence that the extract is immunogenic, which may be useful in developing vaccines for use in aquaculture, but more clinical trials and standardisation are needed.
4. Additional study is essential to validate and unravel the prebiotic effects of *Terminalia arjuna* bark powder using various models.
5. The current study focused on *Terminalia arjuna* bark powder and its extract based on preliminary screening of phytochemical profiling, antimicrobial activity, and antioxidant potential; however, compound characterisation and elucidation revealed that it has excellent compounds in other solvent extracts that need to be investigated further in the future to evaluate the synergistic effects on antioxidant potential.
6. It is highly mandatory to evaluate the synergistic effects of commercial antibiotics (resistance to bacterial isolates) in combination with fractionated ethanolic bark extract to recommend its suitability for future use in combating AMR under the FAO's *ONE HEALTH ONE APPROACH* programme for future applications in sustainable aquaculture.
7. The first reported and amplified partial sequences of STAT1 that require further investigation for its complete sequence to study other immunomodulatory pathways.
8. The research focuses on the nutrigenomics of *T. arjuna* bark powder and extract, which can be combined with proteomics research to allow for the synthesis of the peptide for future aquaculture applications.

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10. PUBLICATIONS

Research articles

1. Meena, D. K., Sahoo, A. K., Chowdhury, H., Swain, H. S., Sahu, N. P., Behera, B. K., Srivastava, P. P. and Das, B. K. (2020a). Effects of extraction methods and solvent systems on extract yield, proximate composition and mineral profiling of *Terminalia arjuna* (Arjuna) dry powders and solvent extracts. *Journal of Innovations in Pharmaceutical and Biological Sciences*. l. 7(1): 22-31.
2. Meena, D. K., Sahoo, A. K., Swain, H. S., Borah, S., Srivastava, P. P., Sahu, N. P., Das, B. K. (2020b). Prospects and perspectives of virtual in-vitro toxicity studies on herbal extracts of *Terminalia arjuna* with enhanced stratagem in *Artemia salina* model: a panacea to explicit the credence of solvent system in brine shrimp lethality bioassay. *Emirates Journal of Food and Agriculture*. 32(1): 25-37.
3. Meena, D. K., Sahoo, A. K., Srivastava, P. P., Sahu, N. P., Swain, H. S., Behera, B. K., Borah, S., Das, B. K. (2020c). Protective effects of selected solvent extracts of *Terminalia arjuna* against environment mediated parasitic *Argulus bengalensis* infection in *Labeo rohita. International Aquatic Research*.12: 267-278.
4. Meena, D. K., Sahoo, A. K., Srivastava, P. P., Jadhav, M., Gandhi, M., Swain, H. S., Borah, S. and Das, B. K. (2021). On valorisation of solvent extracts of *Terminalia arjuna* (arjuna) upon DNA scission and free radical scavenging improves coupling responses and cognitive functions under in vitro conditions. Scientific Reports. 11(1):10656. DOI: 10.1038/s41598-021-88710-w.
5. Meena, D.K., Sahu, N. P., Srivastava, P.P., Jadhav, M., R, Prasad., Malick, R.C., Mohanty, D., Sahoo, A.K.,Behera B.K., Das, B.K. (2021).Effective valorisation of facile extract matrix of *Terminalia arjuna* (Roxb) against elite microbes of aquaculture industry-a credence to bioactive principles: Can it be a sustainability paradigm in designing broad spectrum antimicrobials?. Industrial Crops and Products. 171,113905.

6. Meena, D.K., Sahoo, A.K., Jayant,M., Sahu, N. P., Srivastava, P.P., Jadhav, Swain, H.*S*., Behera B.K., Satvik, K., Das, B.K. (2021). Bioconversion of *Terminalia arjuna* bark powder into a herbal feed for *Labeo rohita*: Can it be a sustainability paradigm for Green Fish production?. Animal Feed Science and Technology. 115132

Abstract presented

1. Meena, D. K., Sahoo, A. K., Swain, H. S. and Das, B. K. (2019). Landscaping of antimicrobial properties of *Terminalia arjuna* as broad-spectrum antimicrobial of fisheries importance. 107th Indian Science Congress held at GKVK, Bangalore from 3-7th January, 2020 pp; 203.
2. Meena, D. K., Sahoo, A. K., Swain, H. S., Mohanty, D., Sarkar, U. K. and Das, B. K. Ethno-medicinal importance of *Terminalia arjuna* (arjuna) against potential fish pathogens, during 19-21 June, 2019 at Chennai Tamil Nadu.
3. Meena, D.K., Das, B.K., Sahoo, A. K., Behera, B.K.,Srivastava, P.P.,Sahu, N.P.,Satvik, K.,Panda, S., Mohanty, D., Sadhukhan, D.(2021). State of art on ethno-pharmacological properties of facile extract matrix of *Terminalia arjuna* against recalcitrant fish pathogens. International Conference on "Integrated Approaches towards Sustainable Management of Environment for Safe Food, Nutrition and Improved Health" University of Kalyani, WB during 15-17th December, 2021.
4. Meena, D.K., Sahoo, A. K., Panda, S.P., Sadhukhan, D., Kumari, M., Samantaray, S., Behera, B.K., Das, B.K. (2021). Utilization of *Terminalia arjuna* bark powder based feed as natural immunomodulator in Fish. International Conference on "Integrated Approaches towards Sustainable Management of Environment for Safe Food, Nutrition and Improved Health" University of Kalyani, WB during 15-17th December, 2021.
5. Meena, D.K., Sahoo, A. K., Jana, A Maity, A., Pradhan, S., Chowdhury, H., Srivastava, P.P., Sahu, N.P., Behera, B.K., Das, B.K. (2021). Elucidation of antioxidant compound in *Terminalia arjuna* (arjuna) acetone leaf extract: A prospective antimicrobial. International Conference on "Integrated Approaches towards Sustainable Management of Environment for Safe Food, Nutrition and Improved Health" University of Kalyani, WB during 15-17th December, 2021.

Sequence submission

Submitted a sequence on STAT1 gene

Meena, D. K., Sahu, N. P., Srivastava, P. P., Sahoo, A. K., Swain, H. S., Borah, S., Behera, B. K. and Das, B. K.