EVALUATION OF IMMUNOSTIMULATORY PROPERTIES OF INDIGENOUS MEDICINAL PLANT *ACHYRANTHES ASPERA* IN *LABEO ROHITA* & *CLARIAS BATRACHUS* IN LABORATORY & POND CONDITIONS

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JULY, 2022

DEDICATED

TO

MY BELOVED PARENTS

CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"Evaluation of Immunostimulatory Properties of Indigenous Medicinal Plant *Achyranthes aspera* in *Labeo rohita* & *Clarias batrachus* in Laboratory & Pond Conditions"** submitted to Delhi Technological University, Delhi, for the award of Doctor of Philosophy is based on the original research work carried out by me under the supervision of **Prof. JaiGopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi, India and **Prof. R. Chakrabarti**, Head, Department of Zoology, University of Delhi, Delhi, India. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other university or institution for the award of any degree or diploma.

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I, Neelesh Kumar, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of **Prof. JaiGopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi and **Prof. R. Chakrabarti**, Head, Department of Zoology, University of Delhi, Delhi, India for a period of July 2017 to July 2022 at the Department of Biotechnology, Delhi Technological University, Delhi and Department of Zoology, University of Delhi, Delhi, India. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have devotedly acknowledged, given credit and refereed to the research workers wherever their work has been cited in the text and the body of thesis. I further certify that I have not wilfully lifted up some other's work, paragraph, text, data, results etc. reported in the journal, books, reports, dissertations, thesis etc., or available at websites and included them in Ph.D. thesis and cited as own my work.

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ABSTRACT

Aquaculture, the culture of aquatic organisms (*viz*., fish, mollusks, crabs, plants etc.) contributes immensely in world's food basket. Aquacrops are excellent source of nutrients such as protein, lipid, amino acids, fatty acids, vitamins and minerals etc. for human consumption. Along with the food security and nutrition, aquaculture provides job opportunities in both developed and developing countries. The aquaculture industry employs more than 41 million people globally, the majority of them engaged in fish production in underdeveloped nations. Various bacteria, fungi, viruses and parasites cause diseases at larval stages of fish. Each year, larval mortality cause economic loss to aquaculture sector. Various antibiotics, vaccines and immunostimulants are used to prevent infections in fishes. The use of antibiotics and chemicals are unsustainable because they can cause negative impact on consumers and are threat to aquatic ecosystem. Immunostimulants are a group of natural and synthetic compounds that increase the non-specific cellular and humoral defense responses. Immunostimulation through medicinal plants is a natural, safe, sustainable and eco-friendly approach to enhance the fish immunity. The present investigation evaluates the immunostimulatory and disease resistant properties of *Achyranthes aspera* (prickly chaff flower) leaves and seeds incorporated diets on the performance of rohu *Labeo rohita* and magur *Clarias batrachus*.

The experiments were conducted in aquaria under laboratory conditions and in hapas under field conditions. Fishes were fed with test diets containing

leaves and seeds of *A. aspera* and control diet without any supplementation of plant ingredients. Fishes were either challenged with virulent *Aeromonas hydrophila* or with UV-B radiation or immunized with c-RBC after feeding with test diets. The survival rate, growth of fish, non-specific immune enzymes such as myeloperoxidase, nitric oxide synthase, lysozyme and hemagglutination titer levels were significantly (P<0.05) higher in 0.5% seeds supplemented diet fed fishes compared to the fishes fed with other diets. The oxidative stress indicators such as thiobarbituric acid reactive substances, carbonyl protein levels were significantly (P<0.05) lower in the enriched diets fed fishes. The anti-oxidant enzyme, superoxide dismutase was significantly (P<0.05) higher in 0.5% seeds supplemented diet fed fishes. Various immunological and stress related genes, *viz*. l*ysozyme-C*, *lysozyme-G*, interleukin (*IL-10*), toll-like receptor-4 (*TLR-4*), tumor necrosis factor-α (*TNF-α*), inducible nitric oxide synthase (*iNOS*), nuclear factor-kB (*NF-kB*), bcl-2-associated X protein (*BAX*), cytochrome complex (*cytochrome-c*), superoxide dismutase-c (*SOD-C*), *caspase-3, caspase-9*, B-cell lymphoma 2 (*BCL-2*) were evaluated. The presence of various amino acids, fatty acids, vitamins and minerals in seeds and leaves of *A. aspera* enhanced the growth of fishes and improved their physiological conditions. The plant is growing in the tropical climatic conditions and therefore, easily available. The application of *A. aspera* as a immunostimulant of fishes is cost-effective.

CONTENTS

Chapter 1

Introduction

Aquaculture, the culture of aquatic organisms (*viz*., fish, mollusks, crabs, plants etc.) contributes immensely in world's food basket. Aquacrops are excellent source of nutrients. Along with the food security and nutrition, aquaculture provides job opportunities in both developed and developing countries. The aquaculture industry employs more than 41 million people globally, the majority of them engaged in fish production in underdeveloped nations (Finegold, 2009). Protein, lipid, amino acids, fatty acids, vitamins and minerals are all abundant in aquaculture crops. In 2020, global aquaculture and fisheries production was around 177.8 million tonnes (capture: 90.3 million tonnes, culture: 87.5 million tonnes) and total fish exports valued 150.5 billion US$. Farmed aquatic animals consist of inland finfish: 49.12 million tonnes, marine and coastal finfish: 8.34 million tonnes, crustaceans: 11.24 million tonnes, mollusks: 17.74 million tonnes and other aquatic animals: 1.06 million tonnes. It has been estimated that in 2020, the aquacultural production from Asian countries around 91.6% of the world's aquatic animals and algae. The annual growth rate of world aquaculture was 6.1% during 2000-2010. The annual growth rate declined to 3.3% during 2015-2020 (The State of World Fisheries and Aquaculture Report, FAO, 2022).

India contributes around 6.56% of global fish production. The total fish production was 14.164 million metric tonnes (MMT) during 2019-2020 including 3.727 million metric tonnes from marine and 10.434 million metric tonnes from inland sectors. The annual growth rate was 4.35% during 2019-2020. During 2019-2020, total fisheries export was 1289651 tonnes valued Rs.46662.85 crore including 1149341 million tonnes from marine products (worth 5556.42

million US$). The total seed production of the country was 521706.1 lakh in the year 2020-2021 (Handbook of Fisheries Statistics India, 2020).

In 2017, the freshwater fish contributed 66% of total fish production of the world (FAO, 2018). In India, carps contribute around 82% of total fish production. The three Indian major carps, rohu *Labeo rohita*, catla *Catla catla* and mrigal *Cirrhinus mrigala* are economically important species and they contribute around 87% of the total freshwater aquaculture production of the country (Bais, 2018). Catfish are also economically important group. They are suitable for culture in warm climates and easily cultivable at local grocers. The occurrence of infectious diseases is a major challenge to the aquaculture industry as it is affecting sustainable aquaculture. Due to their immature immune systems, young group of fishes like fry and fingerlings are more vulnerable to infectious disease (Swain *et al.,* 2002). Appropriate nutrition and feeding regimens are essential to enhance the survival rate of larvae (Lee, 2003). Larval mortality is one of the main issue and it costs aqua farmers a lot of money every year. Fish mortality is caused by different diseases, including vibriosis, furunculosis, red mouth disease etc. Various bacteria, fungi, viruses and parasites cause infections in fish. *Aeromonas hydrophila* is a Gram-negative bacterium present in aquatic environment and cause motile aeromonas septicemia (MAS) in fish (Odeyemi *et al.*, 2012).

Antibiotics and vaccinations are used to keep fish free of bacterial, viral, fungal and parasite illnesses (Dügenci *et al.,* 2003). Consumers may be negatively affected by the use of antibiotics and chemicals. It helps in the

development of antibiotic resistant bacteria (Huys *et al.,* 2000) and has residual effect on fish (Jian and Wu, 2003). These are unsustainable and causing a threat to ecological system (Hektoen *et al.,* 1995; Wang *et al.,* 2015). Hence, alternative strategies should be adopted to enhance resistance of fish against diseases (Defoirdt *et al.,* 2011). As a result, various tactics, *viz*. the applications of probiotics, prebiotics and immunostimulants as feed additives are becoming more popular. It is essential to improve the immune system of fish through various methods. This will help in the production of disease free fingerlings and thereby enhance the fisheries and aquaculture production. Fish use both non-specific and specific defence mechanisms to combat various harmful pathogens. The non-specific immune response acts as a first line of defense (Matsukawa *et al*., 2000) and represents a wide range of immune responses (Dalmo *et al.,* 1997). Fish has cellular as well as humoral immune responses (Biller-Takahashi and Urbinati, 2014).

Immunostimulants are a group of natural and synthetic compounds that increase the non-specific cellular and humoral defense responses (Jeyavani *et al*., 2022). Immunostimulation with medicinal plants is a long-term, environmental friendly and cost-effective way to stimulate the immune system of fish (Van Hai, 2015). It has been reported that medicinal herbs are used as chemotherapeutics and feed additives (Wang *et al.,* 2016). Alkaloids, flavonoids, steroids, quinones, saponins, tannins, terpenoids, phenolics and other secondary metabolites are found in immunostimulatory plants (Sivaram *et al.,* 2004; Harikrishnan *et al.,* 2011a). These substances aid in the promotion of growth as well as stimulate the immunological functions. They also act as

stress relievers and antimicrobial agents (Chitmanat *et al.,* 2005; Citarasu, 2010; Chakraborty and Hancz, 2011). Medicinal plants show positive response on growth and survival of various aquatic organisms (Effendi *et al.,* 2022).

In fishes, the whole plant or various parts (leaves, seeds and roots) of the plants are used as immunostimulants in the forms of crude, extract, blended and active chemicals (Vaseeharan and Thaya, 2014). These plants work by stimulating cellular and humoral immune responses, which are evaluated by increased levels of immunological enzymes such as serum lysozyme, respiratory burst activity and myeloperoxidase activity. Medicinal plants have shown the different levels of immune stimulation at different doses through various routes such as injection, immersion and oral administration (Awad and Awaad, 2017; Salem *et al*., 2022). It is essential to determine the optimum dose of Immunostimulant to avoid risk of immunosuppression.

The effects of numerous medicinal plants such as herbs, spices, herbal medicines, seaweeds etc. have been studied in various aquatic organisms (Van Hai, 2015; Reverter *et al*., 2017). Prickly chaff flower *Achyranthes aspera* L. is a traditional medicinal plant (family: Amaranthaceae). This plant has been used to cure a variety of maladies, including inflammation, diabetes, hypertension, pneumonia, diarrhoea, dysentery and asthma in human (Baraik *et al.,* 2014). Several studies have shown that addition of *A. aspera* seeds to fish diets improves their innate immunity and provides resistance to harmful pathogenic bacterium *Aeromonas hydrophila* (Rao and Chakrabarti, 2005; Rao *et al.,* 2006; Chakrabarti and Srivastava, 2012; Chakrabarti *et al.,* 2012).

Various compounds have been isolated from the various extract of *A. aspera* roots (Sharma and Chaudhary 2015; Srivastav *et al.,* 2011). Seven chemicals compound were isolated in *A. aspera* leaves, including 1,4-benzoquinone, hydroquinone, spathulenol, nerol, α-ionone, asarone and eugenol (Rashmi *et al.,* 2007). Enhancing fish immunity is a promising method of preventing fish diseases (Wangkahart *et al.*, 2022). Animals come into contact with a large number of pathogens in aquatic environments. Most of the vaccines are effective only against certain type of pathogens. There is no vaccine which can give protection against all types of pathogens (Ardó *et al.,* 2008). Medicinal plants are substances that have the ability to perform the activities of vaccinations. The isolation, characterization and quantification of bioactive compounds present in herbal plants are also essential to know the mechanism behind positive effects of these plants (Bulfon *et al.,* 2015).

Bony fishes are diverse group of vertebrates and show high variance in their immunological responses (Magadan *et al.,* 2015). Cellular defence consists of macrophages, monocytes and granulocytes. The non-specific immune system's core components include the lysozyme, a vital humoral defence (Magnadottir, 2006). In fish, enzymes including lysozyme, myeloperoxidase and nitric oxide synthase are indicators of non-specific immune responses (Rao and Chakrabarti, 2005; Rao *et al.,* 2006; Chakrabarti and Srivastava, 2012; Chakrabarti *et al.,* 2012). The amount of lipid peroxides, carbonyl protein and anti-oxidant enzymes (like superoxide dismutase) indicate the health of the fish (Mohanty and Samanta, 2016). The health of fish is influenced by several of immune-related genes like *lysozyme C, lysozyme G,*

TLR-4 and other pro-inflammatory and anti-inflammatory cytokines etc. (Sanjabi *et al.,* 2009; Ye *et al.,* 2010; Zou and Secombes, 2016; Zhang *et al.,* 2018).

In this background, the present study has been conducted to see the immunostimulatory properties of seeds and leaves of *Achyranthes aspera* in Indian major carp rohu *Labeo rohita* and Asian catfish magur *Clarias batrachus*. The UV-B protective properties of plant ingredients have also been evaluated in fish.

There are three objectives in the present study:

- ✓ The study of immunostimulatory and disease resistance properties of seeds and leaves of *Achyranthes aspera* in Indian major carp rohu *Labeo rohita* and Asian catfish magur *Clarias batrachus*.
- ✓ Study of expression pattern of immune-related genes *viz*. *TLRs*, antimicrobial gene encoding *iNOS*, *lysozyme C* and *lysozyme G* in rohu *Labeo rohita* and magur *Clarias batrachus*.
- ✓ Study of UV-B protective properties of seeds and leaves of *Achyranthes aspera* in magur *Clarias batrachus*.

Chapter 2

Review of Literature

Fishes are the most successful aquatic organisms and have great importance among various vertebrate groups. Fish immune organs share a lot of similarities with mammalian immune organs. Modern Cyclostomes (myxinoids and lampreys), Chondrichthyes (sharks and rays) and Osteichthyes (sharks and rays) form the diverse groups of creatures known as bony fish (Nelson, 1994). The fish has the ability to use non-specific and stronger immunological responses than higher vertebrates' innate immune responses. There are some similarities and differences between fish and mammals in terms of immune function. Although there are notable differences in body compartmentalization and cell architecture, fish have most of the secondary lymphoid organs that mammals have, with the exception of lymph nodes and bone marrow (Press and Evensen, 1999). Fish have an aglomerular structure called the head kidney that performs hemopoietic activities (Meseguer *et al.,* 1995; Zapata *et al.,* 1996). The major immunological organ in fish, the head kidney, exhibits phagocytic activity, antigen processing, IgM antibody generation and memory immunity through melanomacrophagic centres (Herraez and Zapata, 1986; Tsujii and Seno, 1990).

This chapter mainly contains the following topics as review for the assessment of the study conducted in the area of fish immune system, oxidative stress, the antioxidant system, cytokines, the apoptotic pathway, various fish immunostimulants, including medicinal plants *Achyranthes aspera*.

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2.1. The immune system of fish

The immune system of an organism consists of a set of biological mechanisms that identify foreign agents such as bacteria, viruses, parasitic worms and other pathogens. It gives disease protection capacity to the organisms and helps to distinguish between healthy tissues and foreign particles. Every creature has an immune system, which can be simple or complex (Van Belleghem *et al.,* 2019). All jawed vertebrates have three fundamental immune structures: (i) a highly conserved innate immune system, (ii) consistent expansion of combined innate and adaptive immune systems, and (iii) bilateral interchange between non-specific and specific immune system components. The physical barriers prevent bacteria, viruses and other microbes from infecting living organisms (Boyton and Openshaw, 2002). When pathogens get to cross these barriers, the innate immunity responses immediately (Litman *et al.,* 2005). If infections manage to evade the non-specific immune response, vertebrates have a second line of defense known as the adaptive immune system. Innate immunological responses are the primary activators of this system. It effectively combats the pathogen during infection and also remembers the responses as immunological memory.

2.1.1. Immune cells and organs in fish

Immune responses are triggered by a variety of cell types and release soluble substances. Leucocytes, which include lymphocytes (B and T cells, lymphocytes with big granules), phagocytic cells (neutrophils, eosinophils and mononuclear phagocytes) and auxiliary cells, are the main cells in all immune responses (basophils, platelets and mast cells). Other cells of tissue, such as macrophages and T cells take part in immunological reactions by communicating with leucocytes and reacting to the cytokines produced by leucocytes. The lymphoid system is made up of immune system cells that are organized into tissues and organs (Neyt *et al.,* 2012). They can be found in the form of a distinct encapsulated organ or scattered lymphoid tissue. The lymphoid organs and tissues are divided into two groups: primary and secondary lymphoid organs and tissues, commonly known as central and peripheral lymphoid organs and tissues, respectively (Chen *et al.,* 2022). Leucocytes are produced primarily in central lymphoid organs and carry out their functions in peripheral lymphoid organs and tissues (Bjørgen and Koppang, 2022).

2.1.1.1. The thymus

In jawed vertebrates, the thymus is the main organ of the immune system (Ching *et al.,* 2021). It is thought to have evolved in early fish as a thick epithelium of the pharyngeal portion of the gastro-intestinal tract (Bowden *et al.,* 2005) and it can be found in both Chondrichthyes and Osteichthyes. Fish have three essential lymphoid organs: the thymus, kidney and spleen (Sayed *et al.,* 2022). The thymus is the first organ in freshwater fish to become lymphoid;

before that, the kidney can contain hematopoietic precursors but not lymphocytes. The order of development of important lymphoid organ in marine fish is kidney, spleen and then thymus (Zapata *et al.,* 2006).

2.1.1.2. The head kidney

Mammalian hematopoietic stem cells are important throughout life because they have the potential to differentiate as well as create hematopoietic lineages while maintaining self-renewal capacity. Although fish lack bone marrow, the cephalic section of the kidney (head kidney/pronephros) resembles bone marrow in mammals, at least in terms of hematopoiesis (Zapata, 1979). The trunk kidney is also hematopoietic in nature and carries renal tissue. The Y-shape dispersed kidney is present in fish with body axis (Chesneau, 2018). The lower section of the kidney is located near the vertebral column, which serves primarily as a renal system. The upper component, which looks like two Y arms and is located beneath the gills, is known as the head kidney or pronephros. Corticosteroids and hormones are secreted by the head kidney, which is also an important endocrine organ (Li *et al.,* 2020). It is homologous to adrenal gland of mammals. Thus, head kidney is a central organ for interaction between immune-endocrine system and also for neuroimmuno-endocrine connections. The head kidney of fish seems to be main organ of antibody production (Tian *et al.,* 2009).

2.1.1.3. The spleen

In fish, the spleen is the main secondary lymphoid organ, while in Agnathans hematopoietic tissues are present instead of the spleen (Fänge and Nilsson,

1985; Press and Evensen, 1999). The spleen of fish, like that of other vertebrates, contains red pulp, white pulp, blood vessels and ellipsoids.

2.1.1.4. The gut

The mammalian gut associated lymphoid tissue (GALT) is mainly consisted of accumulation of lymphoid follicles which known as Peyer's patches. The GALT is also made up of effector cells that are scattered throughout the body, including the lamina propria and the intraepithelial lymphocyte compartment. Teleost fish lack lymph nodes and do not exhibit Peyer's patches. Fish must have a mucosal defense against antigens (Muñoz-Atienza *et al.*, 2021) and produce Ig responses (Hamuro *et al.,* 2007; Zhang *et al.,* 2010). The lamina propria and intestinal epithelial cells of the fish gut possess leucocytes profusely.

2.1.1.5. The gills

The fish gill performs multiple functions such as gas exchange, osmoregulation, acid-base balance, excretion of ammonia, circulating metabolites and immune responses (Rombough, 2007). Pathogens may easily develop in water and pathogens can quickly pass through the gills' thin respiratory epithelial cells. Like in salmon, it is believed that infectious anaemia virus initially attacks the gills and after that it expands to other organs (Rimstad and Mjaaland, 2002). The glycocalyx, mucus layer and gill epithelium are the physical barriers for fish gills. The gills are important antibody secreting organ following intestinal immunization (Dos Santos *et al.,* 2001). The flow cytometry data shows that T cells are present in high numbers in gills (Koppang *et al.,* 2010).

2.1.1.6. The liver

The liver is accountable for metabolism of proteins, carbohydrates and lipids as well as responsible for release of bile and detoxification process. With metabolic activities, it is always under estimated that liver is also an immune organ. The relevance of immune responses of fish liver are always understudied but the impact on mRNA expression of immune relevant genes after bacterial challenge is widely studied (Martin *et al.,* 2010; Millán *et al.,* 2011). This suggests that fish liver actively participates in immune defense mechanisms.

2.1.1.7. The integumentary surface

The fish body is constantly in contact with various microorganisms in the aquatic environment. The integumentary surface, which has defense mechanisms against pathogen entrance, is the first physical barrier against different pathogens. Mucus secretion is an important mechanism because it contains different anti-bacterial peptides which act mainly on constituents of bacterial cell wall either directly or indirectly and cause lysis of bacteria. Mucus is a chief barrier and covers mostly external surfaces, mainly the skin. Besides anti-bacterial peptides (Chen *et al.,* 2020), mucus also possess anti-viral agents (Raj *et al.,* 2011) and interlectins (Rajan *et al.,* 2011; Tsutsui *et al.,* 2011). Mucous secretion is considerably increased when fish are stressed in any way and freshwater species have significantly greater mucus secretion than marine species.

2.1.2. The non-specific cellular immunity

The greater and faster secondary immune responses are lacking in insects response to repeated exposure to the same pathogens due to the lack of immunological memory in non-specific immunity (Prakash and Khan, 2022). Interactions occur between cells of the particular immune system and those involved in non-specific immunological responses (Covián *et al.,* 2021). Different types of leukocytes, including monocytes/macrophages, natural killer (NK)-like cells, granulocytes and non-specific cytotoxic cells (NCC) similar to neutrophils, take part in non-specific cellular defence mechanism of fish. Macrophages play an important role in endocytosis and phagocytosis by removing physiological and foreign waste materials present in the circulation (Singh *et al.,* 2022). Macrophage reactions include increased respiratory burst activity, associated oxygen radical generation, increased phagocytosis and synthesis of pro-inflammatory cytokines (Forrester *et al.,* 2018; Golovynska *et al.,* 2022). The majority of research on killer cells in teleosts has focused on NCCs, which were first described in channel catfish. Carp, rainbow trout, tilapia and damselfish have non-specific cytotoxic cell activity (Shen *et al.,* 2002). Granulocytes, which are mostly found in blood and secondary lymphoid organs, are significant mobile phagocytic cells (Özcan *et al.,* 2022). Numerous tasks can be carried out by neutrophils, including phagocytosis, the formation of harmful reactive oxygen species and nitrogen intermediates, degranulation and the release of extracellular neutrophil traps against a variety of pathogens (Lamas and Ellis, 1994; Kemenade *et al.,* 1996; Katzenback and Belosevic, 2009). Additionally, for the first time in catfish, natural killer (NK) - like cells, monocytes/macrophages, neutrophils and NCCs were identified (Shen *et al.,* 2004).

2.2. The non-specific humoral immune responses

Various chemicals found in the mucus, serum and fish eggs inhibit the growth of pathogenic diseases in an indirect manner (Wang *et al.,* 2022a). The majority of these compounds are glycoproteins, the blood and hemolymph of non-chordates include their precursors. They react with compounds that are relatively abundant in nature and do not simply inhibit the development of one bacterium, they are known as non-specific. Pattern recognition receptors (PRRs), anti-microbial peptides (AMPs), lysozyme, interferons, complement, C-reactive protein (CRP), lectin and transferin are examples of chemicals that fall under the category of humoral defensive mechanisms (Ángeles Esteban, 2012; Smith *et al.,* 2019).

2.2.1. Myeloperoxidase activity

Myeloperoxidase (MPO) is a lysosomal protein and stored in azurophilic granules of the neurtrophils (Rehring *et al.,* 2021; Czerwińska and Owczarczyk-Saczonek, 2022). During the neutrophil's respiratory burst activity, hydrogen peroxide and chloride ions are converted to hypochlorous acid and it uses hydrogen peroxide to oxidise tyrosine to tyrosyl radicals (Heinecke *et al.,* 1993, Dahlgren *et al.,* 2019). Neutrophils employ tyrosyl radicals and hypochlorous acid, both of which are cytotoxic which help to eliminate bacteria and other harmful intruders (Hampton *et al.,* 1998). Myeloperoxidase produced by neutrophils and monocytes during inflammation contributes significantly to the non-specific immune response.

2.2.2. Nitric oxide synthase

Nitric oxide (NO), a signalling molecule made from L-arginine, is produced by the action of an enzyme family known as nitric oxide synthase (NOS) (Förstermann and Sessa, 2012; Fu, 2014). Inducible nitric oxide synthase (iNOS), a defensive mechanism, produced a significant amount of nitric oxide (NO). Fish rely on innate immunological defence mechanisms throughout the early stages of development and this may be used to protect farmed fish against numerous pathogenic invaders (Rombout *et al.,* 2005). Nitric oxide, which also controls immunological processes, has a specific antimicrobial impact (Schairer *et al.,* 2012; Wiegand *et al.,* 2021). Fish phagocytes also produce NO against intracellular infections with the help of iNOS. It has been reported that, NO is secreted by activated macrophages in Japanese flounder *Paralichthys olivaceus* in response to a particularly virulent *Edwardsiella tarda* strain (Ishibe *et al.,* 2009).

2.2.3. Lysozyme

Lysozyme is an important defense molecule for invertebrates as well as for vertebrates (Gálvez-Iriqui *et al.,* 2020). It mostly breaks down the β-1-4 linked glycosidic bonds between N-acetylglucosamine and N-acetymuramic acid found in the peptidoglycan layer of Gram-positive bacteria's cell walls (Salton and Ghuysen, 1959) and thus help in controlling invasion of pathogens. Lysozyme doesn't directly affect Gram-negative bacteria, but it does become active when other enzymes and complement tear the outer cell wall, exposing the germs inside. Fish lysozyme is found in tissues *viz*., skin, gills, gut and eggs where the danger of pathogen assault is high and where leucocytes are

plentiful, such as in the head kidney (Firdaus-Nawi and Zamri-Saad, 2016). In addition to direct antimicrobial effect of lysozyme, it may modify the host immune system's ability to fight against infections (Ragland and Criss, 2017).

2.2.4. Hemagglutination titer

The serum can be used to identify antibodies. Hemagglutination is the name of the test where red blood cells (RBCs) are used as an antigen (Slieman and Leheste, 2020). Fish will produce antibodies against chicken RBC (c-RBC) when immunized with it (Sharma *et al.,* 2021). In the hemagglutination experiment, serially diluted serum from the vaccinated fish is mixed with c-RBC. The c-RBC placed to a test tube/ 96-well U-bottom ELISA plate will agglutinate with the antibodies present in the blood, producing a distinct pattern at the bottom of the tube/ELISA plate. Hemagglutination titer calculated as the reciprocal of the maximum dilution of the sample that exhibits antigen-antibody response in the form of titer. It calculates the relative level of antibody in the serum. The hemagglutination activities of sera of grass carp *Ctenopharygodon idella* and other seven fish species *Megalobrama amlycepnalas, Micropterus salmoides, Carassius auratus, Clarias fuucus, Hypophthalmichthys molitrix, Aristichthys nobilis* and *Monopteurs albus* were evaluated against different animals and human RBCs (Chao and Chengping, 2004).

2.2.5. Tumor necrosis factor (*TNF's*)

TNF's are extensively studied cytokines in fish (Baloch *et al.,* 2022). *TNF-α* is a pro-inflammatory cytokine and one of the first immune genes produced in infected fish at the early stages of the infection. It has an important role in

regulation of inflammation. The first *TNF-α* gene in fish was reported from Japanese flounder *Paralicthys olivaceus* (Hirono *et al.,* 2000, Li *et al.,* 2018) and also have been reported from rainbow trout *Oncorhynchus mykiss* (Laing *et al.,* 2001; Zou *et al.,* 2002), common carp *Cyprinus carpio* (Saeij *et al.,* 2003; Savan and Sakai, 2004), gilthead seabream *Sparus auratus* (Garcia-Castillo *et al.,* 2004) and catfish *Ictalurus punctatus* (Zou *et al.,* 2003a). The *TNF-α* has also been detected in fugu *Takifugu rubripes* and zebrafish *Danio rerio* (Savan *et al.,* 2005). *TNF-α* was administered to trout's primary head kidney leucocytes and monocytes/macrophages *in-vitro*. It was found that *TNF-α* stimulated the expression of a number of immune genes, including those involved in inflammation and antimicrobial responses such as *IL-1, IL-8, COX-2, IL-17C* etc. (Zou *et al.,* 2003; Kim *et al.,* 2009; Zhang *et al*., 2012; Hong *et al.,* 2013). The *NF-kB* pathway was also activated due to stimulatory effects of *TNF-α* (Kim *et al.,* 2009; Bakshi *et al.,* 2022; Ma *et al.,* 2022). The *TNF-α* protein increased the phagocytic activity of fish leucocytes (Zou *et al.,* 2003c; Zou and Secombes, 2016; Sakai *et al.,* 2021). The study showed that supernatants of *TNF-α* treated endothelial cell were potential to promote respiratory burst activity and migration of leucocytes in carps (Forlenza *et al.,* 2009).

2.2.6. Interleukins (*ILs*)

The *IL-1α* and *IL-1β* genes were found in animals. *Interleukin 1* is a cytokine that promotes inflammation. The most well studied cytokine among fish with the *IL-1* gene was *IL-1β* (Huising *et al.,* 2004). The *IL-1β* gene was cloned in bony fishes (Fujiki *et al.,* 2000; Engelsma *et al.,* 2003) and in cartilaginous fishes (Bird *et al.,* 2002). Lipopolysaccharides (LPS) *in-vitro*, bacterial infection and

LPS *in-vivo* all resulted in the production of induced *IL-1* (Zou *et al.,* 2003b). Several experiments were conducted with trout and common carp to understand the roles of *IL-1*. In common carp, *IL-1* served as a potent adjuvant and exhibited all elements of an inflammatory response (Yin and Kwang, 2000; Kono *et al.,* 2002). *IL-10* is an anti-inflammatory cytokine. It reduces inflammatory responses, immune system activation and promotes the growth of memory B cells, T cells and IgM. The *IL-10* was identified in mammals and known as cytokine inhibitory synthesis factor (Fiorentino *et al.*, 1989). When the genomic data was released, the first cytokine *IL-10* gene was identified from common carp, rainbow trout and puffer fish *Fugu rubripes* via in silico cloning (Savan *et al.,* 2003; Zou *et al.,* 2003a; Inoue *et al.,* 2005). The goldfish and common carp were used to study the functional characteristics of *IL-10* (Grayfer *et al.,* 2011; Piazzon *et al.,* 2015). When monocytes of goldfish were activated with heat killed *Aeromonas salmonicida* and incubated with *IL-10*, the expressions of *TNF-α1*, *TNF-α2*, *IL-1β1, IL-10* and *CXCL8/IL-8* genes decreased (Grayfer *et al.,* 2011).

2.2.7. Toll-like receptors

Toll-like receptors (*TLRs*) are a sub-division of pattern recognition receptors (PRRs). These are the first and best studied innate immune receptors for infection detection. TLRs are transmembrane proteins that detect conserved sequences on pathogens to activate immune system effector molecules (Kawasaki and Kawai, 2014). The *TLR* signaling pathway via activation of *NF-kB* gene, induces proinflammatory cytokines such as interleukin, type I interferon molecules, tumor necrosis factor molecules that provide direct

defense response as well as alert adaptive immune cells against pathogens (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2010). *TLR-4* is a central protein of receptor complexes in mammals that react to bacterial lipopolysaccharides (LPS) found in Gram-negative bacteria's outer membrane. However, compared to mammals, fish have a different identification and sensitivity to LPS (Sepulcre *et al.,* 2007; Rebl *et al.,* 2010). *TLR-4* gene is not present in all fish species. The *TLR-4* gene was cloned and characterized in cyprinidae family, like zebrafish, common carp, rare minnow *Gobiocypris rarus*, grass carp *Ctenopharyngodon idella*, mrigal *Cirrhinus mrigala* and in Ictaluridae family, like channel catfish (Sullivan *et al.,* 2009; Su *et al.,* 2009; Kongchum *et al.,* 2010; Huang *et al.,* 2012; Basu *et al.,* 2013; Quiniou *et al.,* 2013).

2.3. Oxidative stress

Oxidative stress is a condition where following reactions can be easily observed such as increased level of free radicals production, reactive oxygen species (ROS) and several reactions which damage the cells (Halliwell, 2007; He *et al.,* 2017). The equilibrium between ROS production and elimination by various antioxidants maintains a steady level of ROS concentration (Schieber and Chandel, 2014). Oxidative stress in cells can result from the unchecked synthesis of ROS and their buildup. Damage to macromolecules like lipids, proteins and DNA molecules is caused by free radicals such as superoxide anions, hydroxyl, hydrogen peroxide, peroxynitrite and nitric oxide (Martemucci *et al.,* 2022). Small molecules with an unpaired electron and a natural ability to diffuse are called free radicals. Proteins, lipids and carbohydrates are just a few of the biological components that free radicals interact with and denature.

Aberrant mitochondria are well known sites of ROS production and targets for ROS action (Marchi *et al.,* 2012; Li *et al.,* 2013). Small levels of ROS perform several useful processes like phagocytosis, apoptosis and necrosis. ROS also play a significant role in cell signalling (Spooner and Yilmaz, 2011).

2.3.1. Impact of reactive oxygen species on lipids

The presence of a large number of unsaturated fatty acids in the lipid components of cellular membranes makes them vulnerable to oxidation by reactive oxygen species (ROS). Lipid peroxidation and lipid hydroperoxide (LOOH) are produced when ROS interacts with the membrane's lipids. Aldehydes including malonaldehyde, isoprotans, hydrocarbons and 4-hydroxynonenal (4-HNE) can be produced by further decomposing this lipid hydroperoxide. Thus, lipid peroxidation is a chain process started by radicals that further compromises the integrity of cellular membranes by causing free radicals to self-replicate in the membrane (Beevi *et al.,* 2010). The primary degrading byproducts of lipid peroxidation are malondialdehyde and hydrocarbons (Oleszko *et al.,* 2015). Thiobarbituric acid reactive substances (TBARS) were used to test the lipid peroxidation's byproducts. TBARS is a well-known indicator of oxidative stress.

2.3.2. Effect of reactive oxygen species on protein

Reactive oxygen species assault proteins, making them susceptible. The exact region of the side chains of amino acids where ROS interact with proteins changes the protein structure, fragments peptide chains, modifies electric charges and accelerates the breakdown of cross-linked processes (Levine *et*

al., 2000). According to Garrison (1987), reactive carbonyl groups are formed when active oxygen combines with the side chains of amino acids and cleaves the polypeptide chain (Kaur *et al.,* 2011). There are numerous ways that proteins can oxidise and among these reactions, carbonylation is a commonly recognized indicator of oxidative stress (Lushchak, 2007). Proteins carbonyls can also form through secondary reactions of free radicals with other cellular components like carbohydrates, lipids and nucleic acids (Cecarini *et al.,* 2007).

2.4. Superoxide dismutase

Free radicals can be stabilized or inactivated by antioxidant enzymes before they damage various cell components. Enzymes work by either decreasing the energy of free radicals or by creating their instability through an electron transfer process (Blokhina *et al.,* 2003; Noori, 2012). In addition, these enzymes have capacity to stop the oxidative chain reactions and thus play significant role in reducing the harmful effect of free radicals. Numerous studies have proven the advantages of antioxidant enzymes.

Superoxide dismutase (SOD) belongs to a group of enzymes that degrades superoxide anions and also functions as an antioxidant enzyme to remove harmful consequences of oxidative stress. For optimal catalytic activity, the enzyme also needs a variety of micronutrients, including iron, copper, selenium, zinc and manganese (Krishnamurthy and Wadhwani, 2012; Ozougwu, 2016). The superoxide anion breaks down into oxygen and hydrogen peroxide and the SOD is a crucial component of the body's defensive mechanism against free radical damage (Rao *et al.,* 2011). Superoxide ion

($O_2^{\bullet-}$), a highly reactive species, was dissimulated into the less reactive species hydrogen peroxide through the action of SOD (H_2O_2). Through the further breakdown of the hydrogen peroxide (H_2O_2) into H_2O and O_2, antioxidant enzymes like, catalase or glutathione peroxidase can be used (Sandalio *et al.,* 1997; Teixeira *et al.,* 1998; Cao *et al.,* 2022).

2.5. Extrinsic and intrinsic apoptosis pathways

Apoptosis, or programmed cell death, is regarded as an essential part of a number of processes, including healthy cell turnover, embryonic development, immune system function, chemically induced cell death etc. (Elmore, 2007). A complex chain of chemical reactions that depends on energy makes up the apoptosis mechanism (Palai and Mishra, 2015). The two primary apoptotic mechanisms are the extrinsic and intrinsic pathways, sometimes known as the mitochondrial pathway and the death receptor pathway (death receptors), respectively. Both the pathways converge on the same execution pathway which starts with the cleavage of *caspase 3* and consequences the following changes: DNA fragmentation, apoptotic bodies' formation, degradation of proteins (cytoskeletal and nuclear), cross-linking of proteins, ligands expression for phagocytic cell receptors and ultimately uptake by phagocytic cells (Elmore, 2007). There are numerous approaches to support cell survival during the host-pathogen conflict. Some pathogens can trigger host cell apoptosis and in this situation, the pathogen cannot live since the host cell can eliminate the pathogen with little negative effects (Hongmei, 2012; Behar and Briken, 2019). The exotoxin of the important fish pathogen *Photobacterium damselae* induced the apoptosis in macrophages and neutrophils collected from peritoneal cavity

of sea bass *Dicentrarchus labrax* through extrinsic and intrinsic pathways (Costa-Ramos *et al.,* 2011). *Edwardsiella tarda* is a significant Gram-negative intracellular bacterium found in fish. The interaction of *E. tarda* with two cell lines: *Epithelioma papulosum cyprinid* and flounder gill *Paralichthys dentatus* cell line FG-9307 revealed that bacterium interacted with fish cells in different ways and two different pathways involved in cell hosts to mediate apoptosis (Wang *et al.,* 2013).

2.6. Immunostimulants for fish

The application of immunostimulants in fish diets has gain momentum as more production-grade diets are building up with various substances that promise to increase the immune response of fish (Lin *et al.,* 2011; Kiron, 2012; Ganguly *et al.,* 2013). Immunostimulants are defined in a variety of ways. According to Bricknell and Dalmo (2005), an immunostimulant is a natural or synthetic chemical that modulates the immune system by boosting the hosts' resistance to diseases brought on by diverse infections. Additionally, immunostimulants can be divided into various groups based on their places of origin, delivery systems and mechanisms of action. Various substances such as β-glucans (Kuhlwein *et al.,* 2014; Dawood *et al.,* 2015a; Dawood *et al.,* 2015b; Dawood and Koshio, 2016), threonine (Sharf and Khan, 2022), phytogenic mixture (Mohammady *et al.,* 2022), lactoferrin (Sakai *et al.,* 1993; Miyauchi *et al.,* 1998; Kumari *et al.,* 2003; Esteban *et al.,* 2005; Yokoyama *et al.,* 2006; Welker *et al.,* 2007), levamisole (Findlay and Munday, 2000; Li *et al.,* 2006; Kumari and Sahoo, 2006b), lipopolysaccharides (LPS), carbohydrate (Nya and Austin, 2010; Skalli *et al.,* 2013; Kadowaki *et al.,* 2013; Wang *et al.,* 2022b),

peptidoglycans (Zhou *et al.,* 2006), soybean lecithin (Tan *et al.,* 2022), chitin, chitosan (Esteban *et al.,* 2000, 2001; Gopalakannan and Arul, 2006; Yan *et al.,* 2017), fucoidan (Immanuel *et al.,* 2012; Kitikiew *et al.,* 2013; El-Boshy *et al.,* 2014; Isnansetyo *et al.,* 2016) and vitamins combinations (Yildirim-Aksoy *et al.,* 2008; Lim *et al.,* 2010; Sivagurunathan *et al.,* 2022; Xu *et al.,* 2022) can stimulate the immune system of fish. Fish also exhibit immunostimulatory characteristics from a variety of medicinal plants and their compounds.

2.6.1. Medicinal plants as immunostimulants

Aquarists are opposed to the use of chemotherapy since it has detrimental impacts on both the environment and human health (Harikrishnan *et al.,* 2011a). Thus, the introduction of natural, safe, economical and environmentally friendly additives that can increase aquatic animals' resistance to disease has gained pace in the feed manufacturing industry. As a result, current research has concentrated on finding immunostimulants that are risk-free, all-natural, efficacious and affordable. The use of medicinal plants is a promising and an alternative method for controlling fish diseases. Using medicinal plants has been shown to have a variety of biological impacts on fish, including growth promotion, hunger stimulation, immunostimulation, anti-stress and anti-microbial properties (Giri *et al.,* 2015a; Singh *et al.,* 2019; Sharma *et al.,* 2021; Dandi *et al.,* 2022). The use of medicinal herbs as feed additives can have a favourable effect, which may be attributed to the presence of numerous secondary metabolites (Sivaram *et al.,* 2004; Harikrishnan *et al.,* 2011b). Due to their widespread availability and low cost, medicinal plants and their derivatives are widely employed in the aquaculture industry for enhanced

growth and invasion defence. They have also been used with probiotic or animal product (Bahi *et al.,* 2017; Awad *et al.,* 2022; Mohammady *et al.,* 2022). Various plants and their products which have shown the immunostimulatory properties in fish are enlisted here (Table 1).

Table 1. Application of various medicinal plants and their products as immunostimulants of fishes.

Medicinal plants and their products	**Fish species**	**Dose and length of administration**	**Observations/ Parameters**	**References**
Cinnamomum zeylanicum (Chinamon)	*Oreochromis niloticus* (Nile tilapia)	0 and 10 g/kg (60 days)	Antioxidant and immune indicators, haemato-biochemical assay	Hamed *et al.* (2022)
Aloe vera (aloevera)	*Sparus aurata* (seabream)	0, 0.5, 2.5 and 5% (60 days)	Oxidative stress parameters, hepatic gene expression	Amri *et al.* (2022)
Pandanus tectorius (screw pine)	*Cyprinus carpio* (common carp)	0, 5, 10, 20 and 30 g/kg (8 weeks)	Serum parameter, gene expression profile, enzymatic antioxidant levels	Cheng *et al.* (2022)
Elaeagnus angustifolia (Russian olive)	*Cyprinus carpio* (common carp)	0, 1, 2 and 3% leaves extract (8 weeks)	Hematological parameters	Hoseini *et al.* (2021)
Artemisia absinthium (common wormwood)	*Cyprinus carpio* (common carp)	0, 0.5, 1 and 1.5% aqueous extract (60 days)	Biochemical, immunological and antioxidant parameters	Yousefi *et al.* (2021)
Coffea Arabica (coffee silver skin)	*Oreochromis niloticus* (Nile tilapia)	0, 10, 20, 40 and 80 g/kg (8 weeks)	Innate immunological assays, alternative complement pathway activity	Van Doan *et al.* (2021)
Psidium guajava (guava leaf)	*Cyprinus carpio* (common carp)	0, 100, 150, 200 and 250 leaves mg/kg (8 weeks)	Antioxidant and immunological responses, stress and immune genes	Giri *et al.* (2020)
Nigella sativa (black seed)	*Labeo rohita* (rohu)	0, 1 and 2.5% (28 days)	Oxidative stress and antioxidant enzymes, histological preparations	Latif *et al.* (2020)
Citrus limon (lemon peels)	*Labeo rohita* (rohu)	0, 1, 2.5 and 5 g/kg (60 days)	Blood, serum hemato-biochemical, antioxidant enzyme, immune genes	Harikrishnan *et al.* (2020)
Psidium guavajava (guava leaves)	*Cyprinus carpio* (common carp)	0, 0.25, 0.5 and 1% (8 weeks)	Enhanced serum lysozyme activity	Hoseinifar *et al.* (2019a)

Medicinal plants and their products	**Fish species**	**Dose and length of administration**	**Observations/ Parameters**	**References**
Camellia sinensis (Assam tea)	*Oreochromis niloticus* (Nile tilapia)	0, 1, 2, 4 and 8 g/kg (4 and 8 weeks)	Mucosal immune response and serum immune responses	Van Doan *et al.* (2019)
Agaricus bisporus (mushroom powder)	*Cyprinus carpio* (common carp)	0, 0.5, 1 and 2%(8 weeks)	Antioxidant enzymes, mucosal immunity related genes	Khaleghi *et al.* (2019)
Ziziphus jujube (jujube fruit extract)	*Cyprinus carpio* (common carp)	0, 0.25, 0.5 and 1% (8 weeks)	Serum, total immunoglobulin level increased	Hoseinifar *et al.* (2018a)
Citrus sinensis (orange peels)	*Oreochromis niloticus* (Nile tilapia)	0, 5, 10 and 29 g/kg (8 weeks)	Mucosal immune response, serum immune responses etc.	Van Doan *et al.* (2018)
Gracilaria gracilis (red algae)	*Danio rerio* (zebrafish)	0.25, 0.5 and 1% (4 and 8 weeks)	Mucosal immune parameters, immune related gene study	Hoseinifar *et al.* (2018b)
Eriobotrya japonica (loquat)	*Cyprinus carpio* (common carp)	0, 0.25, 0.5 and 1% leaf extract (7 weeks)	Alternative haemolytic complement activity	Hoseinifar *et al.* (2017a)
Mespilus germanica (medlar)	*Cyprinus carpio* (common carp)	0, 0.25, 0.50 and 1% leaf (49 days)	Non-specific immune parameters, immune and antioxidant related genes	Hoseinifar *et al.* (2017b)
Myrtus communis L. (myrtle)	*Oncorhynchus mykiss* (rainbow trout)	0, 0.5, 1 and 1.5% of myrtle powder (60 days)	Hematological, serum biochemical, non-specific immunity	Taee *et al.* (2017)
Ferula assafoetida (ferula)	*Cyprinus carpio* (common carp)	0, 0.5, 1 and 2% (8 weeks)	Serum lysozyme, immunoglobulin level increased	Safari *et al.* (2016)
Citrus limon (limon peel)	*Oreochromis mossambicus* (Mozambique tilapia)	0, 0.5, 0.75 and 1% (60 days)	NBT positive cell count, WBC count increased	Baba *et al.* (2016)
Musa acuminata (banana peel flour)	*Labeo rohita* (rohu)	0, 1, 3, 5 and 7% (60 days)	Phagocytic activity and SOD level increased	Giri *et al.* (2016)
Astragalus membranaceus and *Lycium barbarum*	*Oreochromis niloticus* (Nile tilapia) and *Cyprinus carpio*	0.2, 2% (8 weeks)	Total immunoglobulin, bactericidal acitivity etc. increased	Mo *et al.* (2016)
Citrus depressa (lemon leaf)	*Lates calcarifer* (Asian seabass)	1, 3, 5% (56 days)	Respiratory burst activity, phagocytic, lysozyme etc. increased	Shiu *et al.* (2016)

Medicinal plants and their products	**Fish species**	**Dose and length of administration**	**Observations/ Parameters**	**References**
Lawsonia inermis (henna leaf)	*Cyprinus carpio* (common carp)	6, 60, 600 mg/kg (10 days)	Bactericidal and phagocytic activity increased	Soltanian and Fereidouni (2016)
Zingiber officinale (ginger)	*Labeo rohita* (rohu)	0, 0.2, 0.4, 0.6, 0.8 and 1% (60 days)	Serum lysozyme and total Immunoglobulin level increased	Sukumaran *et al.* (2016)
Psidium guavajava (guava leaves)	*Labeo rohita* (rohu)	0, 0.1, 0.5, 1 and 1.5% (60 days)	Lysozyme, leukocyte phagocytic activities increased	Giri *et al.* (2015b)
Phoenix dactylifera (palm)	*Cyprinus carpio* (common carp)	Control and 200 ml/kg fruit extract (8 weeks)	Total Ig level, lysozyme and protease activity increased	Hoseinifar *et al.* (2015)
Allium cepa (onion powder)	*Huso huso* (beluga)	0, 0.5 and 1% (8 weeks)	Lysozyme, SOD and respiratory burst activities increased	Akrami *et al.* (2015)
Mentha piperita (peppermint extract)	*Rutilus frisii kutum* (aspian white fish)	0, 1, 2 and 3% (8 weeks)	Serum lysozyme and IgM level increased	Adel *et al.* (2015)
Aloe vera (aloe vera)	*Oreochromis niloticus* (GIFT tilapia)	0, 0.5, 1, 2 and 4% (60 days)	Serum lysozyme activity increased	Gabriel *et al.* (2015)
Rehmannia glutinosa (root powder)	*Cyprinus carpio* (common carp)	Control, dried, prepared and extract root powder (80 days)	Lysozyme and leukocyte phagocytic activities increased	Wang *et al.* (2015)
Origanum vulgare (oregano flour)	*Oncorhynchus mykiss* (rainbow trout)	1% (8 weeks)	Increased level of phagocytic, lysozyme, respiratory burst activity etc.	Pourmoghim *et al.* (2015)
Citrus sinensis (sweet orange peel)	*Oreochromis mossambicus* (Mozambique tilapia)	1, 3, 5 g/kg (60 days)	Myeloperoxidase, total protein, lysozyme, globulin increased	Acar *et al.* (2015)
Mentha piperita (peppermint extract)	*Lates calcarifer* (Asian seabass)	1, 2, 3, 4 and 5 g/kg (4 weeks)	Hematocrit, haemoglobin serum protein and globulins level increased	Talpur (2014)
Euphorbia hirta (cats hair)	*Cyprinus carpio* (common carp)	0, 5, 10, 20, 25 and 50 g/kg leaf extract (50 days)	NBT positive cells and antibody titer increased	Pratheepa and Sukumaran (2014)
Ixora coccinea (jungle geranium crude and extracts)	*Carassius auratus* (gold fish)	ethyl acetate extract and fraction eluted from H 40 @ 400 mg/kg diet (30 days)	Bacteriocidal and phagocytic activities increased	Anusha *et al.* (2014)

Medicinal plants and their products	**Fish species**	**Dose and length of administration**	**Observations/ Parameters**	**References**
Muscari comosum (tassel bulbs)	*Sparus aurata* (seabream)	0.5, 2 mg/kg (28 days)	Lysozyme, total protein, stimulation of respiratory burst etc.	Baba *et al.* (2014)
Sauropus androgynus (katuk leaf)	*Epinephelus coioides* (orange-spotted grouper)	1, 2.5, 5 g/kg (30 days)	Superoxide dismutase, stimulation of phagocytic, lysozyme etc.	Samad *et al.* (2014)
Azadirachta indica (neem)	*Lates calcarifer* (Asian seabass)	0, 1, 2, 3, 4 and 5g/kg leaves powder (30 days)	Serum bactericidal activity and superoxide anions increased	Talpur and Ikhwanuddin (2013)
Nigella sativa (black cumin)	*Oncorhynchus mykiss* (rainbow trout)	1, 2, 3% (2 weeks)	Stimulation of lysozyme, IgM, bactericidal, complement etc.	Awad *et al.* (2013)
Sophora flavescens (ku shen, Chinese medicinal herb)	*Oreochromis niloticus* (GIFT strain)	0.025, 0.050, 0.100, 0.200, 0.400% and control (30 days)	Stimulation of antiprotease, complement, lysozyme etc.	Wu *et al.* (2013)
Suaeda maritime (herbaceous seepweed)	*Paralichthys olivaceus* (olive flounder)	0.01, 0.1 and 1% (4 weeks)	Stimuation of lysozyme, respiratory burst, nitrogen species etc.	Harikrishnan *et al.* (2012a)
Pueraria thunbergiana (kudzu vine)	*Epinephelus bruneus* (kelp grouper)	0, 0.1, 1 and 2% (4 weeks)	Haemoglobin, lysozyme and superoxide anion production increased	Harikrishnan *et al.* (2012b)
Rheum officinale (rhubarb)	*Megalobrama amblycephala* (Wuchang bream)	0.1% anthraquinone extract and control diet (10 weeks)	Catalase, lysozyme increased and cortisol level decreased	Liu *et al. (*2012)
Nasturtium nasturtium (watercress)	*Oncorhynchus mykiss* (rainbow trout)	0.1, 1% leaf extract (21 days)	Increased level of lysozyme, complement, peroxidase etc.	Asadi *et al.* (2012)
Eriobotrya japonica (loquat)	*Epinephelus bruneus* (kelp grouper)	0, 0.1, 1 and 2% leaf extract (4 weeks)	Serum agglutinating antibody titre, superoxide anion, increased	Kim *et al.* (2011)
Prunella vulgaris (self-heal leaf)	*Paralichthys olivaceus* (olive flounder)	0.01, 0.1 and 1% (4 weeks)	Stimulation of respiratory burst, complement etc.	Harikrishnan *et al.* (2011b)
Cynodon dactylon (bermuda grass leaf)	*Catla catla* (catla)	0.05, 0.5 and 5% (60 days)	Stimulation of lysozyme, antiprotease, etc.	Kaleeswaran *et al.* (2011)

Medicinal plants and their products	**Fish species**	**Dose and length of administration**	**Observations/ Parameters**	**References**
Basella alba (binomial leaf)	*Oreochromis niloticus* (Nile tilapia)	1% (35 days)	Stimulation of lysozyme, phagocytic, immunoglobulin level etc.	Charkraborty and Hancz (2011)
Lupinus perennis (lupin), *Mangifera indica* (mango) and *Urtica dioica* (stinging nettle)	*Oncorhynchus mykiss* (rainbow trout)	1g/100 g of each diet (14 days)	Complement, lysozyme activity etc. increased, antiprotease, serum bactericidal activity etc increased	Awad and Austin (2010)
Nyctanthes arbortristis (night jasmine seed extract)	*Oreochromis niloticus* (tilapia)	0.01, 0.1 and 1% (3 weeks)	Stimulation of alternative complement, lysozyme etc.	Kirubakaran *et al.* (2010)
Cratoxylum formosum (pink mempat)	*Oreochromis niloticus* (tilapia)	0, 0.5, 1 and 1.5% (30 days)	Lysozyme, phagocytic and respiratory burst activity	Rattanachaikunsopon and Phumkhachorn (2010)
Toona sinensis (red toon leaf)	*Oreochromis mossambicus* (Mozambique tilapia)	4, 8 µg/g (7 days)	Respiratory burst, lysozyme activity increased	Wu *et al.* (2010)
Withania somnifera (Indian ginseng)	*Labeo rohita* (rohu)	1, 2 and 3 g/kg (42 days)	Phagocytic, respiratory burst, lysozyme, IgM etc. increased	Sharma *et al.* (2010)
Camellia sinensis (green tea leaf)	*Oreochromis niloticus* (Nile tilapia	0.125, 0.25, 0.5, 1 and 2% (12 weeks)	Stimulation of respiratory burst and bactericidal activity	Abdel-Tawwab *et al.* (2010)
Tinospora cordifolia (guduchi leaf)	*Oreochromis mossambicus* (Mozambique tilapia)	6, 60, 600 mg/kg (10 days)	Nitrogen species, lysozyme etc. increased	Alexander *et al.* (2010)

2.7. Use of *Achyranthes aspera* as a fish immunostimulant

Achyranthes aspera (Linn.) is a herb belongs to the Amaranthaceae family. In India, the plant is frequently seen as a weed along highways (Saini, 2015). It is found also in Baluchistan, Ceylon, Africa, Australia, Tropical Asia and America. The plant is known in different names like, in Sanskrit-Apamarga/Aghata/ Kharamanjari, English-Chaff-flower, devil‘s horse whip, Hawai chaff flower, prickly chaff flower etc. (Varuna *et al.,* 2010). The plant is around 0.2-2.0 m in

height with thick, long and cylindrical primary roots. Secondary and tertiary roots are also present. It has yellowish-brown, branching, cylindrical, upright and sturdy stems. The leaves are petiolate, alternating, widely rhombic or elliptic-ovate, hairy on the top side and woolly white on the underside. Numerous single red or white blooms with a width of 3-7 mm are abundant in the inflorescences, which are 8-30 cm long. The seeds are subcylindric, black, rounded at the base and shiny.

2.7.1. Phytochemical studies

The phytochemical study of *Achyranthes aspera* shows the presence of numerous bioactive chemicals with significant medical value. The entire plant has been isolated for the ecdysterone (Banerji *et al.,* 1971). The whole plant has been found to contain the water-soluble alkaloid betaine (Kapoor and Singh, 1966). It has water-soluble alkaloid that shows a number of pharmacological properties (Neogi *et al.,* 1970). The diverse plant parts, including the roots, leaves, shoots and seeds have yielded numerous chemical substances that are isolated and identified.

2.7.2. Roots

The ecdysterone is present in the methanolic extract of the roots of *A. aspera* (Banerji and Chadha, 1970). Through silica-gel column chromatography, the ecdysterone has also been identified in root extracts (Ikan *et al.,* 1971). The glycosidic fraction of the roots has been used to isolate the oleanolic acid (Khastgir *et al.,* 1958). The ethanolic extracts of the roots of *A. aspera* were used to isolate and identify a novel aliphatic acid, n-hexacos-14-enoic acid

(Sharma *et al.,* 2009). Certain other compounds are also isolated and identified from ethanolic extracts of roots such as n-hexacos-17-enoic acid, 22-dien-3-β-ol, n-hexacos-11-enoic acid, trans-13-docasenoic acid, strigmasta-5 etc. A phytosterol, 22-dien-3-β-ol has been obtained from petroleum ether: benzene (75:25) elute as a crystalline mass (Sharma *et al.,* 2009). The addition of root extract (0.5%) to the diets shows immunostimulatory properties in rohu (Rao *et al.,* 2004).

2.7.3. Shoots

Dihydroxy ketones was isolated from the *A. aspera* roots such as 36, 37-dihydroxyhenpentacontan-4-one and triacontanol (Batta and Rangaswami, 1973). Several long-chain substances like, 17-pentatriacontanol (Gariballa *et al.,* 1983) and 16-hydroxy-26-methylheptacosan-2-one and 27-cyclohexyl heptacosan-7-ol (Misra *et al.,* 1993) were isolated from the shoots.

2.7.4. Leaves

The GC-MS analysis shows the presence of various chemicals in the volatile oil of *A. aspera* leaves. Seven chemicals like, hydroquinone, spathulenol, asarone, nerol-ionone, p-benzoquinone and eugenol contribute 63.05% of the oil (Rameshwar, 2007). The main ingredient in this volatile oil is hydroquinone (57.7%). The steroids, terpenoids, alkaloids, flavonoids, saponins and flavonoids are found in the leaves (Umamaheswari *et al.,* 2012).

2.7.5. Seeds

Several phyto-constituents like, D-glucuronic acid (saponin A), β-D-galactopyranosyl ester of D-glucuronic acid (saponin B), amino acids,

hentriacontane, oleanolic acid (Hariharan and Rangaswami, 1970; Rastogi and Mehrotra, 1990), 10-octacosanone, 10-tricosanone and 4-tritriacontanone (Rastogi and Mehrotra, 1990; Ali, 1993) are found in the seeds. Three oleonolic acid glycosides including α-L-rhamn-opyranosyl-(1→4)-(β-D-glucopyranosyluronic acid)-(1→3)-oleanolic acid-28-O-β-D-glucopyranoside, α-L-rhamnopyranosyl-(1→4)-(β-D-glucopyranosyluron ic acid)-(1→3)-oleanolic acid and α-L-rhamnopyranosyl-(1→4)-(β-D-glucopyranosyluronic acid)-(1→3)-oleanolic acid-28-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside are found in the seeds (Rashmi *et al.,* 2007). The seeds produce a brand-new cyclic chain aliphatic fatty acid and sapogenin. The oleanolic acid has been identified from the seeds (Chauhan *et al.,* 2002; Chakrabarti *et al.,* 2012).

The supplementation of *A. aspera* seeds @ 0.5% to the diets showed immunostimulatory properties in rohu *Labeo rohita* (Chakrabarti and Srivastava, 2012; Rao *et al.,* 2006), catla *Catla catla* (Chakrabarti *et al.,* 2014; Rao and Chakrabarti, 2005), magur *Clarias batrachus* (Sharma *et al.,* 2021) and common carp *Cyprinus carpio* (Chakrabarti *et al.,* 2012). The enhanced growth was recorded in *A. aspera* seeds supplemented diets fed snow trout *Schizothorax richardsonii* and UV-B irradiated catla larvae (Singh *et al.,* 2013a; Singh *et al.,* 2013b; Ngasainao *et al.,* 2017).

Chapter 3

Materials and Methods

3.1. Fishes and their culture systems

Indian major carp rohu *Labeo rohita* and Asian walking catfish magur *Clarias batrachus* were used as test species. Rohu (Fig.1a) were purchased from Chatterrjee Brothers' Fish Farm, Mogra, West Bengal. Magur (Fig.1b) were procured from Ghosh Fish Seeds Farm, Naihati, West Bengal. Both farms used induced breeding technique for the breeding of fishes.

Fig.1(a). *Labeo rohita* (rohu)

Kingdom - Animalia
Phylum - Chordata
Class - Actinopterygii
Order - Cypriniformes
Family - Cyprinidae
Genus - *Labeo*
Species - *rohita*

Fig.1(b). *Clarias batrachus* (magur)

Kingdom - Animalia
Phylum - Chordata
Class - Actinopterygii
Order - Siluriformes
Family - Clariidae
Genus - *Clarias*
Species - *batrachus*

Fish were either cultured in aquarium maintained in the wet laboratory facility or in the hapas kept in a pond. Each aquarium (60 l) was equipped with an air pump for the maintenance of dissolved oxygen level in the fish culture unit and a filter (Sera, Germany). The use of filter reduced the ammonia and nitrite levels in the fish culture unit (Fig.2). The hapas (2.0 mx1.5 mx1.5 m) were set inside a pond (54.5 mx30.5 mx2.25 m) situated at Lahali fish farm, Rohtak, Central Institute of Fisheries Education (CIFE), Indian Council of

Agricultural Research (ICAR), Haryana. Hapas were made up of nylon net and the top of hapa was covered with net to avoid the escape of fish (Fig.3).

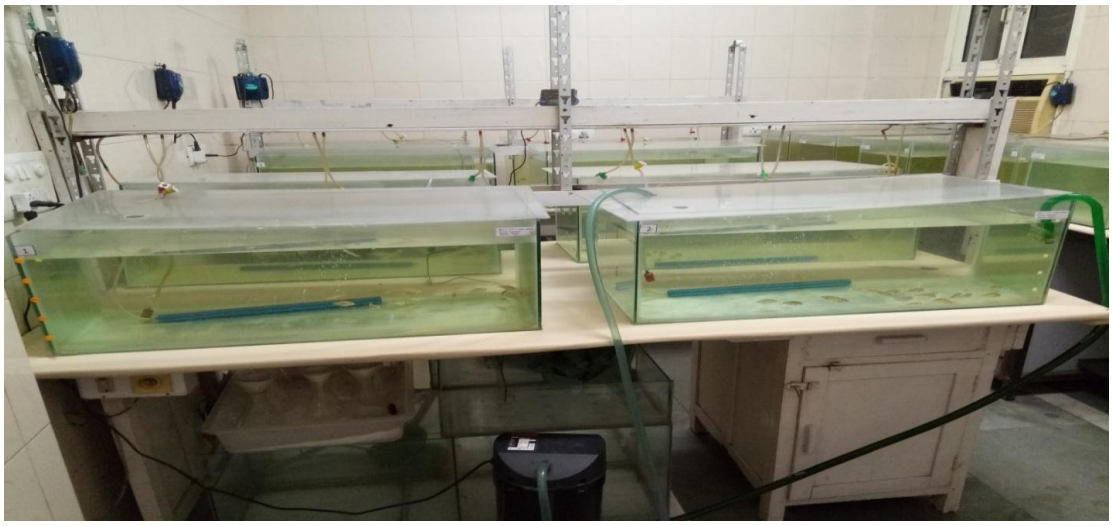

Fig.2. The culture of fish in the wet laboratory facility.

Fig.3. The culture of fish in hapas kept inside the pond.

3.2. Ingredients used for the formulation of fish diets

Fish diets were prepared using various ingredients. The dried fish *Harpadon nehereus* (Bombay duck) were procured from Ghazipur Fish Market, New

Delhi, India. Wheat four (Aashirvaad Atta, ITC Limited, Bangalore, India), cod liver oil (Universal Medicare Pvt. Ltd, Mimbai, India), vitamin and mineral premixes (Piramal Pharma Limited, Mumbai, India) were procured from the local market. The leaves and seeds of *Achyranthes aspera* were supplemented in fish feeds (as immunostimulants) with basic ingredients. *A. aspera* was cultivated in the outdoor nursery facility (Figs.4a and b).

Fig.4(a). Growth of *Achyranthes aspera* plants in the experimental facility.

Fig.4(b). *Achyranthes aspera* with seeds.

The green juicy leaves were collected; properly cleaned with deionized water. Then dried in oven at 35°C and ground. The mature seeds were collected and cleaned manually and ground. All ingredients were sieved (500 µm) to get fine powder and stored at 4°C prior to diet formulation.

3.3. Formulation of feed and feeding of fishes

The Pearson's Square method was used for the formulation of fish diets. The dry fish powder was used as protein source and wheat flour was used as a carbohydrate source. The leaves and seeds of *A. aspera* were added at

various doses with the basic ingredients (Table 1). Three experimental diets were as follows: diets incorporated with 0.5% seeds, 0.25 and 0.5% leaves of *Achyranthes aspera*. In control diet, leaves and seeds were absent. All the ingredients were mixed properly with slightly warm water in appropriate amount for homogeneous spreading of all the ingredients. The pelleted diets of different sizes were prepared (Fig.5) using Twin-Screw Extruder, Basic Technology Pvt. Ltd. Kolkata, India (Fig.6). The diets were kept in feed drier (Hicon, India) for 3-4 h at 40°C. Then the pelleted diets were stored in a refrigerator for further use.

Table 1. Fish diets formulation and their proximate compositions.

Ingredient (g/kg diet)	**Experimental diets**			
	Control	**0.25% Leaves**	**0.5% Leaves**	**0.5% Seeds**
Composition of feed				
Fishmeal	651.6	651.6	651.6	651.6
Wheat flour	334.4	331.9	329.4	329.4
Cod liver oil	10.0	10.0	10.0	10.0
Vitamin and mineral premixes	4.0	4.0	4.0	4.0
Leaves powder	–	2.5	5.00	–
Seeds powder	–	–	–	5.0
Proximate composition (g/100 g, dry matter basis)				
Crude protein	36.96	37.05	36.98	37.02
Crude fat	8.64	9.24	7.82	8.31
Total carbohydrates	40.06	34.47	37.05	37.21
Moisture	7.14	11.09	10.03	9.43
Ash	7.20	8.15	8.12	8.03
Crude fibre	4.1	6.8	6.5	4.4
Energy (kcal/100 g)	385.84	369.24	366.50	371.71

Fig.5. Twin-Screw Extruder has been used for the preparation of fish diets.

Fig.6. Experimental pelleted diet for fish.

Fish were acclimated in wet laboratory facility for 15 days and then transferred to the experimental systems (aquarium/hapas). Fish were fed with control diet during acclimation. Then feeding starts with different test and control diets. Three replicates were used for each feeding regime. Feeding rates ranged from 3-5% of body weight each day. Fish were fed at 9:00 am and 5:00 p.m. throughout the study period.

3.4. Performance of fish

3.4.1. Survival rate (%)

The survival rate of fish was analyzed by calculating the difference between the numbers of fish at the start of experiment and the number of fish during harvesting. The formula:

$$\text{Survival rate (\%)} = \frac{\text{No. of fish harvested}}{\text{No. of fish stocked}} \times 100$$

3.4.2. Cumulative mortality rate (%)

The cumulative mortality rate was calculated for fish challenged with *Aeromonas hydrophila.* The cumulative mortality rate was observed from day of challenge

and was continued for 10 days. It was presented as a percentage of the initial fish population. Any abnormal sign on fish body was observed for 10 days.

3.4.3. Final average weight (g)

The initial weight of individual fish and final weight of individual fish at the time of sampling were measured using electronic balance (AUX220, Shimadzu, Japan). The average of final weight of fish was expressed in gram using following formula:

$$\text{Average weight} = \frac{\text{Average weight of fish}}{\text{Number of fish}}$$

3.4.4. Specific growth rate (%)

The following formula was used to calculate specific growth rate (SGR):

$$\text{SGR (\%)} = \frac{\ln(\text{final weight}) - \ln(\text{initial weight})}{\text{Experimental period}} \times 100$$

3.4.5. Feed conversion ratio

The feed conversion ratio was calculated using the formula:

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed consumed}}{\text{Body weight gain}}$$

3.5. Biochemical assays

3.5.1. Proximate composition

The moisture content of various feed ingredients was analyzed following the standard method (AOAC, 2000). The sample was kept in an oven at 105°C for 24 h for the estimation of moisture content. The oven dried sample was kept in a muffle furnace (Hicon, India) at 550°C for the estimation of ash content. The crude protein content was analyzed using an automated micro-Kjeldahl system

(Pelican Instruments, Chennai, India) (Fig.7). The nitrogen content of the sample was estimated first and then multiplied with *the conversion factor (N x 6.25).* Crude lipid content was assayed gravimetrically using the standard method (Folch *et al.,* 1957). Carbohydrate content was estimated by the subtraction method (Merrill and Watt, 1973). Energy value was determined (Merrill and Watt, 1973) using the following formula.

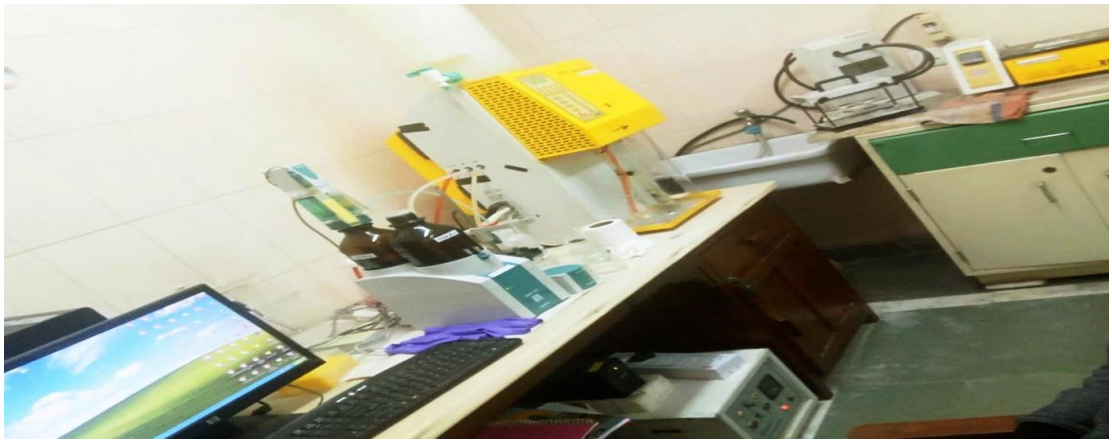

Fig.7. Kjeldahl system, Pelican Instruments, Chennai, India.

Carbohydrate = 1000-[(Moisture+Protein+Lipid+Ash) contents of 1 kg feed]

Energy (kcal/100 g) = [(Crude protein g/100g×4)+(Crude lipid g/100 g×9)+(Total carbohydrate g/100 g×4)]

3.5.2. Amino acids analysis

The amino acid profiles of various samples were analyzed with the help of Automatic Amino Acid Analyzer (L-8900, Hitachi, Japan) (Fig.8). The samples were hydrolyzed using 6N HCl at 110°C for 24 h for all amino acids except, cysteine, methionine and tryptophan (Chakrabarti *et al.,* 2018). The sample was oxidized with performic acid and then subjected to 6N HCl treatment, followed by the analysis of the sulfur-containing amino acids. The sample was hydrolyzed

with 4M methanesulfonic acid and 0.2% 3-(2-aminoethyl) for the analysis of tryptophan. Nitrogen evaporator (PCi Analytic Private Limited, Mumbai, India) was used to dry the digested sample. The HCl (0.02 N) was added to the sample to obtain a protein concentration of 0.5 mg/ml. The sample (1.5 ml) was kept in the auto sampler. A cation-exchange resin column (4.6 mm ID x 60 mm L) with 3 mm particle size was used for the separation of amino acids.

Fig.8. Automatic Amino Acid Analyzer, L-8900, Hitachi (Japan).

The reaction temperature was 135°C, the column temperature was between 30 and 70°C, and the ninhydrin flow rate was 0.35 ml per min. All amino acids were detected at 570 nm, with the exception of proline and hydroxyproline, which were measured at 440 nm. The concentration of 38 amino acids, except glutamine and tryptophan, amino acids mixture type B and type AN-2 were measured as g/kg and compared to a reference solution (Wako Pure Chemical Industries Ltd., USA). Quantification was carried out based on the chromatogram peaks areas (Fig.9). Fresh standard solutions were prepared for glutamine and tryptophan (Sigma-Aldrich, USA) before analysis.

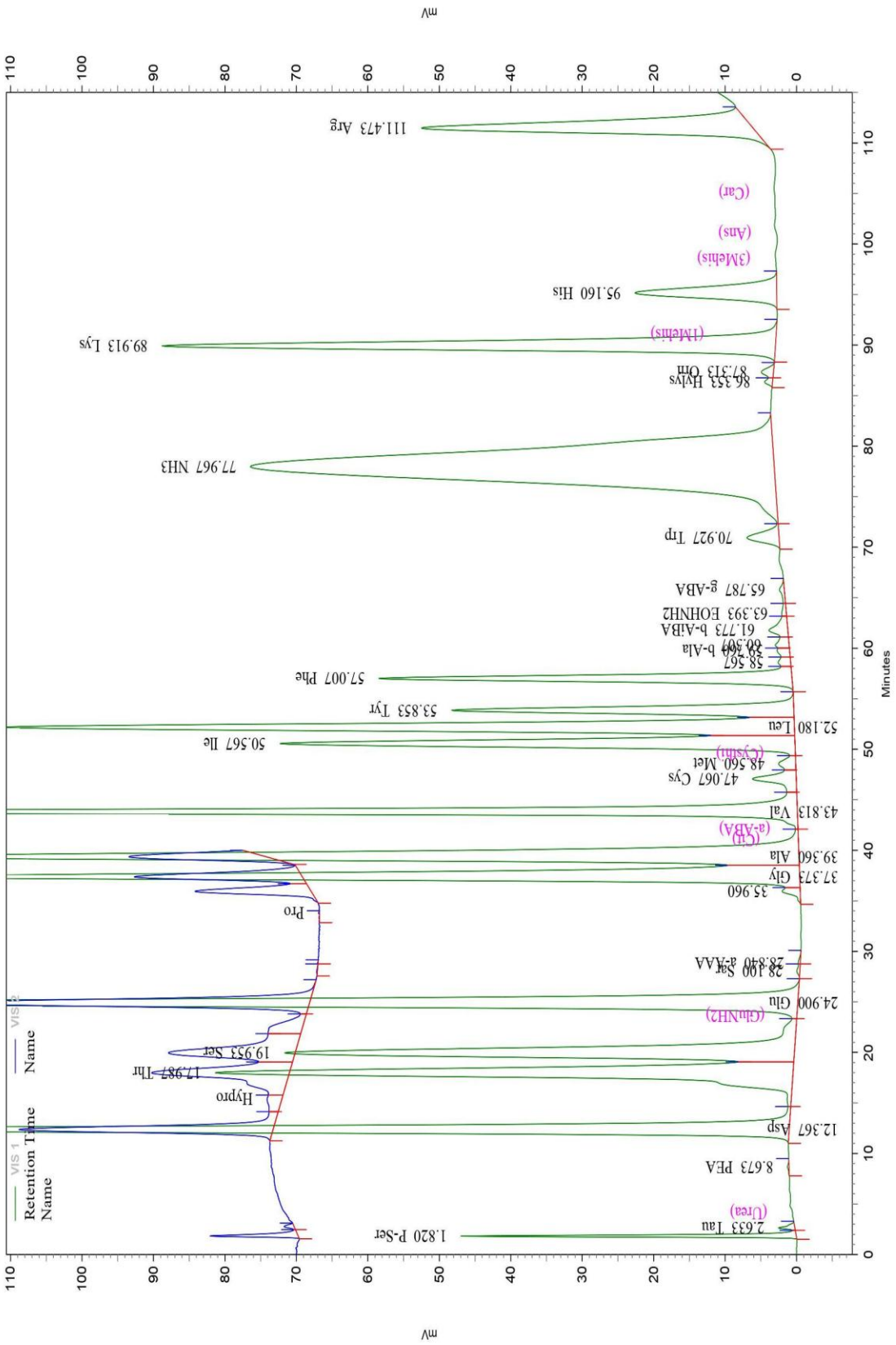

Fig.9. Chromatogram shows the amino acid profile of *Achyranthes aspera*.

3.5.3. Fatty acids analysis

The fatty acids compositions of various feed ingredients were analyzed with Gas Chromatograph (GC)-Flame Ionization Detector, Clarus 580, PerkinElmer, Waltham, USA (Fig.10). The column was Zebronics wax (Phenomenex, UK) and measured 60 m in length, 0.32 mm inside diameter x0.25 µm in thickness. The sample was dried at 40°C and ground. The total lipid fraction was extracted from 1 g of sample through homogenization in chloroform/ methanol (v/v, 2:1) using a tissue disrupter (Ultra-Turrax, Fisher Scientific, UK). The lipid content was determined gravimetrically (Folch *et al.,* 1957).

Fig.10. Clarus 580 (Gas Chromatography) PerkinElmer, Waltham, USA.

Crude lipid (1 mg) isolated from each sample was mixed with 2 ml of methylation reagent, 1 ml of toluene and 1 ml of heptadecanoic acid (C17:0) internal standard. After proper mixing the lipid sample was flushed with nitrogen. The fatty acid methyl ester (FAME, 1 mg/ml) was prepared from the extracted lipid by transesterification using sulphuric acid in methanol at 50°C for 16 h (Christie, 2003). The FAME was extracted and purified (Tocher and

Harvie, 1988). The FAME was separated and quantified by gas liquid chromatography. Different components of the sample were separated on the basis of their boiling point and interaction with the stationary phase of the column (Fig.11).

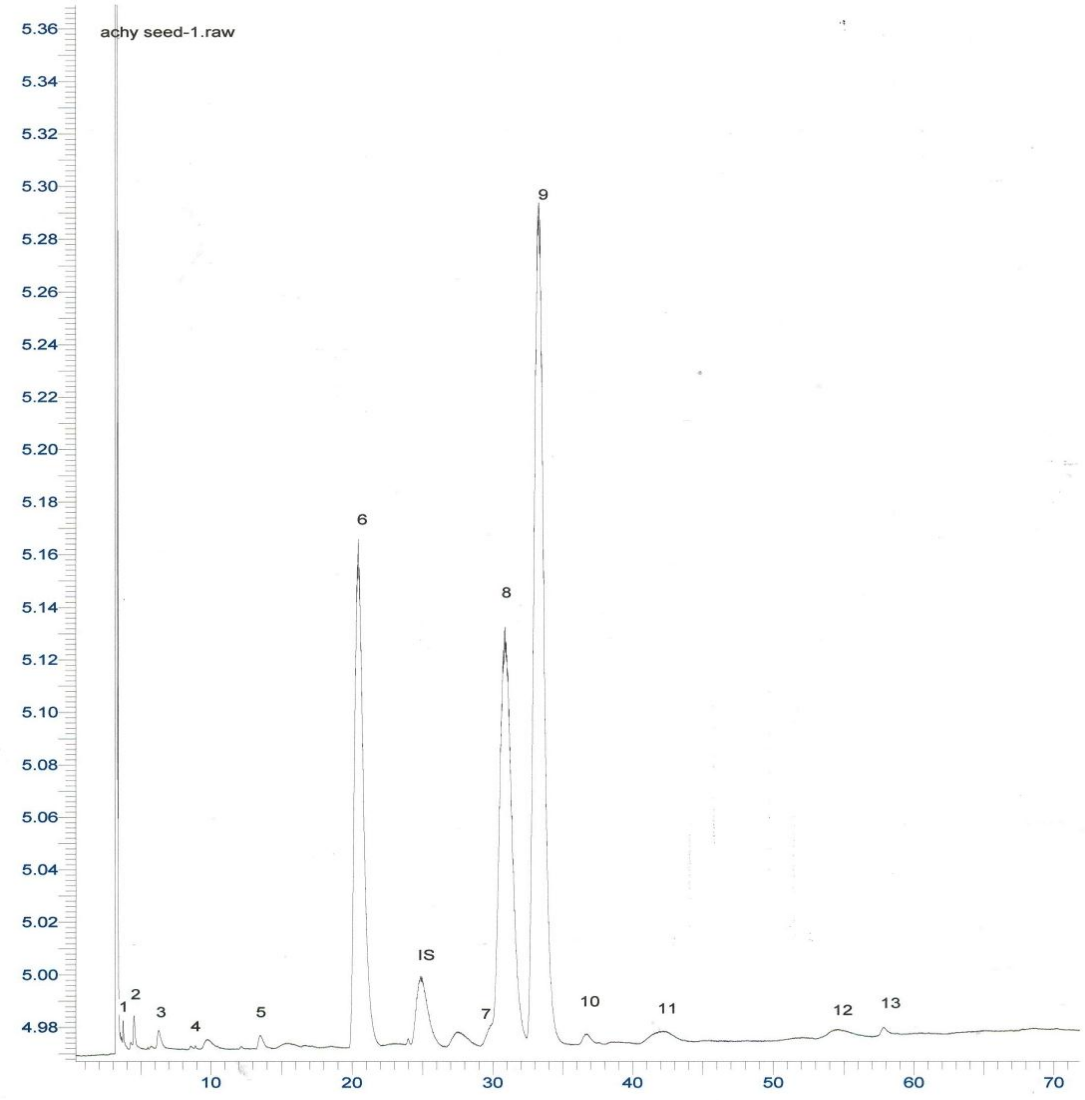

Fig.11. Chromatogram showed the fatty acid profile of *Achyranthes aspera*.

The data were collected from pre-installed programme software (TotalChrom Workstation Ver6.3, PerkinElmer, USA). Standard (Sigma-Aldrich, USA) was used for the identification of fatty acids.

3.5.4. Vitamin analysis

Vitamin contents of various feed ingredients were analyzed with Ultra High Performance Liquid Chromatography (UHPLC) (ThermoFisher Scientific, USA) with C18 column (3 µM, 150 x 4.6 mm). The fat soluble vitamins were analyzed following the standard method (Qian and Sheng, 1998). In an extraction tube (10 ml), 4 ml of hexane was added to 1 g sample and flushed with nitrogen gas. The sample was centrifuged for 5 min at 1500 x *g*. In a vial (10 ml), supernatant (1 ml) was transferred and it was evaporated using a nitrogen evaporator. The residue was dissolved in n-butanol (0.3 ml) and injected to the UHPLC. Mobile phase used for fat soluble vitamins was methanol and the flow rate was 1 ml/min. The vitamin levels were recorded at 254/290 nm.

The water soluble vitamins were analyzed (Sami *et al.,* 2014). The dry sample (2 g) was hydrolyzed with 0.1N H_2SO_4 at 121°C for 30 min for the estimations of thiamine (B_1), riboflavin (B_2), pyridoxine (B_6) and cobalamin (B_{12}). After incubation at room temperature, 2.5M sodium acetate was mixed with the hydrolyzed sample and pH of the sample was 4.5. The enzyme takadiastase (50 mg) was added to the sample and incubated at 35°C for 12 h. Then the sample was filtered with Whatman filter paper (no. 4) and distilled water was added to maintain the volume 50 ml. Sample (2 g) was treated with a solution of 0.3M meta-phosphoric acid and 1.4M acetic acid for the estimation of vitamin C. The sample was centrifuged for 15 min at 9000 *x g* and filtered with whatman filter paper. Mobile phase used for water soluble vitamins was a mixture of 0.1M potassium acetate (33 ml, pH 4.9) and 67 ml acetonitrile with water (1:1 ratio); the flow rate was 1 ml/min. The micro-pore (0.45 µm) syringe

filter was used to filter the samples before injection; then placed into the UHPLC vial. Then sample (20 µl) was injected automatically in UHPLC system. The absorbance was recorded at 254 nm at 25°C.

3.5.5. Minerals analysis

The mineral compositions of macrophytes were assayed using inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7900, USA) following standard protocol at the Instrumentation Facility of Indian Institute of Technology (IITD), New Delhi. The ground sample (150 mg) was taken in a closed digestion vessel and 8 ml of suprapure 69% nitric acid (HNO_3, Merck, USA) was added to the vessel. Sample was digested in microwave digestion system (Multiwave PRO; Anton Paar, Austria). Digested sample was cooled at room temperature and transferred into a measuring cylinder. The Milli-Q ultrapure water was added to maintain the volume 40 ml. The sample was filtered through 0.2 µM syringe filter (ThermoFisher Scientific, USA) in a glass vial. A 20 µl sample was injected through autosampler in the ICP-MS. The standard solution for each mineral was supplied with the equipment (Agilent Technologies, USA). It was diluted with Milli-Q ultrapure water containing 1% HNO_3 to make concentrations of 20, 40, 60, 80, 100, 250, 500, 1000 µg/l. The calibration (standard) curve was prepared. The blank was prepared with Milli-Q ultrapure water containing 1% HNO_3.

3.6. Immunization of fish

The fish were immunized with chicken red blood cells (c-RBC) on 0-day/immunization day (Fig.12). Chicken red blood cells (c-RBC) were collected

in Alsever's solution one day before immunization, washed thrice with phosphate buffered saline (PBS, pH 7.4) and made to 20% (v/v) suspension in the same solution. Chicken red blood cells (c-RBC) were used as antigen to the experimental fishes. Tricaine methanesulfonate (MS-222, Sigma, USA) was used to anaesthetize the fish prior to immunization. The c-RBC suspension in PBS was injected intraperitoneally. PBS buffer was injected into one group of fish (sham control). Then fish were released into the appropriate tanks or hapas. Various tissues and blood samples were collected on 7^{th}, 14^{th} and 21^{st} days after immunization.

Fig.12. Immunization of *Labeo rohita* with c-RBC at CIFE Rohtak Centre.

3.7. Challenge of fishes

3.7.1. Challenge of fish with *Aeromonas hydrophila*

In Luria Bertani broth (LB-broth), containing 100 g/ml ampicillin, the *Aeromonas hydrophila* were cultured till mid log phase (12 h). After 80 days of initial feeding, fish were anesthetized with tricaine methanesulfonate then virulent *A*.

hydrophila (5×10^6 CFU/ml) was injected intraperitoneally with sterilized syringes with 26 G×½ (0.45×13 mm) needles (Hindustan Syringes & Medical Devices Ltd.). Fish were introduced in the separate tanks (Fig.13) to avoid the contamination in pond. The 1x PBS buffer (100 µl, pH 7.4) were injected in sham control fish. The mortality of fish was recorded for 10 days at 12 h interval. Various tissues and blood samples were collected after bacterial challenge.

Fig.13. *Clarias batrachus* introduced in the separate tanks after bacterial challenge.

3.7.2. Challenge with UV-B radiation

Fish were challenged with UV-B radiation (280-320 nm). The Philips tube light (TL 20/12 RS, Holland) was used as a source of UV-B radiation. The pre-burned tubes (100 h) were fixed above the aquaria (1 tube/aquarium) and covered with dark black plastic sheet to avoid reflections between aquarium and outside light. The dose of UV-B radiation was 157 µW/cm^2. Three replicate were used for each feeding scheme. The duration of exposure was 15 min and was maintained for 7 days at a rate of 15 min per day. The Sun photometer,

Microtops II of Solar Light (USA) was used for the measurement of UV-B irradiation. Fish were kept under fluorescent lights (20 W Philips) with no UV components for the experimental and control groups. The photoperiods were 12 h for both dark and light. Various tissues and blood samples were collected after 7 days of UV-B exposure.

3.8. Samples collection

Tricaine methanesulfonate was used to anaesthetize fish before sampling. The blood sample was drawn from the caudal vein using sterilized syringe (1 ml) fitted with 26G×½ (0.45×13 mm) needle. The syringe and needle were rinsed with 2.7% ethylenediamine tetraacetic acid (EDTA) solution. The blood samples were allowed to clot in sterilized micro centrifuge tube and stored in a refrigerator at 4°C. After 12 h samples were centrifuged at 400 × *g* at 4°C for 10 min, serum was collected in sterile tube (PCR tube) and stored at -20°C for various immunological estimations. The tissues of individual fishes were collected and stored at -80°C for various enzymatic studies. Similarly, different tissues (100 mg) were kept in TRIzol® Reagent (ThermoFisher Scientific, USA) for gene expression analysis.

3.9. Biochemical assay

3.9.1. Total tissue protein

The total protein of different tissues was analyzed using standard method (Lowry *et al.,* 1951). The tissue (100 mg) was homogenized in 1.5 ml micro-centrifuge tube with 1 ml of PBS (1X, pH 7.4) and centrifuged at 10,000 × *g* at 4°C for 15 min. The supernatant (25 µl) was added in the 96 well absorbance

plate, then 125 µl reagent C (50 ml reagent A+1 ml reagent B) mixed in the plate and incubated for 10 min at 25°C. After incubation, 12.5 µl reagent E (Dilute folin reagent + distilled water, 1:1 ratio) was added in each well of the plate and incubated for 30 min. The absorbance (OD) was taken at 750 nm using 96-well (U-bottom) plate with microplate multimode reader (Synergy H1, Hybrid Reader, BioTek, USA).

3.9.2. Serum lysozyme

Serum lysozyme activity was analyzed using following standard method (Siwicki, 1989). *Micrococcus luteus* solution (20 mg/100ml of 0.02M sodium acetate buffer, pH 5.5) was mixed with 10 µl of serum. The initial absorbance was recorded at 450 nm and incubated for 1 h at 25°C. After incubation, final absorbance was taken at 450 nm. The activity was expressed in mg/ml. The Hen's egg lysozyme (Sigma, USA) was used as standard.

3.9.3. Myeloperoxidase activity

The myeloperoxidase activity of serum was measured using standard method (Quade and Roth, 1997). The hank's balanced salt solution (HBSS, without Ca^{2+} or Mg^{2+}) 90 µl was added in each well of 96-well microplate containing 10 µl of serum. After that, 35 µl of mixture of 20 mM TMB, Genei and 5 mM H_2O_2, 1:9 dilution was added to each well. The assay mixture was incubated for 2 min; then 35 µl 4M H_2SO_4 was added to stop the reaction. The color of the sample was changed to yellowish. The optical density (OD) was measured at 450 nm. The 1,3,5-trinitrobenzene (TNB, Sigma, USA) was used as standard.

3.9.4. Hemagglutination titer

The hemagglutination assay was estimated using standard method (Dash *et al.,* 2000) for the antigen-specific antibody response. The chicken blood (c-RBC) was collected in Alsever's solution (1:3) and was kept for 24 h at 4°C. The sample was cleaned three times with PBS (pH 7.4), centrifuged for 10 min at 3,000 rpm at 4°C, and then 2 ml of c-RBC was added to 98 ml of PBS to make a 2% (v/v) solution. In a 96-well U-bottom ELISA plate, 50 µl serum was serially diluted with PBS. In each well 50 µl of c-RBC (2%) was added and incubated at room temperature for 60 min and then overnight at 4°C. The antibody titer for hemagglutination was defined as the reciprocal of the highest dilution that represented agglutination.

3.9.5. Nitric oxide synthase

Nitric oxide synthase (NOS) level of various tissues were measured using standard method (Lee *et al*., 2003). The tissue (100 mg) was homogenized in 1.5 ml microcentrifuge tube with 1 ml of PBS buffer (pH 7.4) and centrifuged at 10,000 x *g* for 15 min at 4°C. The sample (supernatant, 100 µl) was taken into ELISA well plate and was mixed with griess reagent (equal volume of 1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid). The mixture was incubated 10 min at 25°C and the absorbance was recorded at 540 nm. The NOS activity was recorded as µmol mg/tissue. The reference substance (standard) was sodium nitrite.

3.9.6. Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) assay indicates the tissue lipid oxidation. The tissue sample (100 mg) was washed in chilled 0.9% NaCl

and homogenized in chilled potassium chloride (KCl) solution (1 g in 900 µl). In a micro-centrifuge tube (MCT), 25 µl homogenate was mixed with 25 µl sodium dodecyl sulphate, 187.5 µl acetic acid (20%), 187.5 µl thiobarbituric acid (0.8%) and 75 µl of distilled water. The mixture was heated in a water bath (Hicon, India) at 95°C for 1 h. Then the sample was cooled, the MCT tube was filled with 125 µl distilled water and 625 µl of n-butanol:pyridine mixture (15:1). The whole reaction mixture was shaken vigorously and centrifuged at 800 x *g* at 25°C for 10 min. Top organic layer was collected and the absorbance of the sample (200 µl) was measured at 532 nm. The external standard was 1,1,3,3-tetramethoxypropane (TMP). The KCL (1.15%) was used as blank. The TBARS was expressed as nmol MDA/mg tissue (Ohkawa *et al.,* 1979).

3.9.7. Carbonyl protein

The tissue carbonyl protein level was estimated using standard method (Lenz *et al.,* 1989). The tissue (100 mg) was homogenized in 1 ml of potassium phosphate (PBS) buffer (50 mM) containing with pH 7.0, 0.5 mM EDTA, and 10 µl 100µM PMSF. In a micro-centrifuge tube, 250 µl homogenate with 500 µl 10% trichloroacetic acid (TCA) was taken and centrifuged at 13,000 x *g* for 5 min at 25°C. Pellet was collected and mixed with 1 ml 10mM 2,4-dinitrophenylhydrazine (DNPH) and incubated for 1 h at 25°C. After incubation, mixture was centrifuged (13,000 x *g*) at 25°C for 5 min. The pellet was cleaned with 1 ml mixture of ethanol and butylacetate (1:1). The supernatant was then discarded. The procedure of washing of pellet was repeated thrice. Final pellet was centrifuged at 13,000 x *g* for 5 min at 25°C and dissolved in 1.5 ml of guanidine hydrochloride (6M). The supernatant (200 µl) was used for the

estimation of carbonyl protein at 370 nm. In the control, 1 ml 2M HCl was used instead of 1 ml of 10mM DNPH. The expression for the carbonyl protein was nmol/mg protein. The molar coefficient of extinction was $22x10^3$/M/cm.

3.9.8. Superoxide dismutase

The superoxide dismutase (SOD) was measured in tissues (Kakkar *et al.,* 1984). After blending the tissue (100 mg) with 1 ml of chilled sodium phosphate buffer (0.1M, pH 7.4), the sample was centrifuged at 10,500 x *g* at 4°C for 20 min. The reaction mixture was composed of 300 µl of 0.052M sodium pyrophosphate (Na_3PO_4) buffer (pH 8.3), 25 µl of 186 µM phenazine methosulphate, 75 µl of 300 µM nitroblue tetrazolium (NBT), 50 µl of sample (10% post mitochondrial supernatant), 250 µl distilled water and 50 µl of 780 mM NADH (nicotinamide adenine dinucleotide). It was added into the micro-centrifuge tube and incubated for 90 sec at 30°C. After the incubation, 250 µl of glacial acetic acid (CH_3COOH) was added to stop the reaction. The activity was measured at 560 nm and expressed as units/mg protein.

3.10. Water quality parameters

The major water quality parameters like, dissolved oxygen (DO), temperature, pH, ammonia (NH_3) and conductivity were monitored regularly. Water temperature, pH, dissolved oxygen and conductivity were measured using specific probes of Multi-parameter Meter (HACH, HQ40D, USA). The temperature, pH, dissolved oxygen, ammonia and conductivity ranged from 27°C-30°C, 7.4-8.3, 4.50-6.0 mg/l, 0.001-0.13 mg/l and 580-623 µS/cm, respectively during experimental period.

3.11. Relative mRNA expressions

The various immune and stress related genes *viz.*, *lysozyme C, lysozyme G,* tumor necrosis factor-α (*TNF-α*), interleukin 10 *(IL-10),* interleukin-1β *(IL-1β),* toll-like receptor 4 (*TLR-4*) and *β-actin* were studied in rohu *Labeo rohita* and tumor necrosis factor-α (*TNF-α*), inducible nitric oxide synthase (*iNOS*), nuclear factor-kB (*NF-kB*), bcl-2-associated X protein (*BAX*), cytochrome complex (*cytochrome c*), superoxide dismutase-c (*SOD*-c), *caspase 3, caspase 9* and b-cell lymphoma 2 (*BCL 2*) were studied in magur *Clarias batrachus*. The RNA was extracted from the tissue. The NanoDrop spectrophotometer (ThermoFisher Scientific, USA) was used to check the purity of RNA (260:280). The RNA quality was tested in gel electrophoresis with 1% agarose. The RNA (1 µg) was first treated with 1U of DNase to avoid contamination of DNA. Reverse transcription with random primers was carried out using the high capacity cDNA reverse transcription kit (Applied Biosystems, ThermoFisher Scientific, USA). The cDNA confirmation was checked with housekeeping gene (*β-actin*) through PCR-amplification.

Fig.14. RT-PCR, QuantStudio 6 Flex, Applied Biosystems, ThermoFisher, USA used for the study.

The relative quantification of various genes was performed through real-time PCR (Applied Biosystems, QuantStudio 6 Flex, USA) (Fig.14) with the help of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, ThermoFisher Scientific, USA). The primers for specific genes were designed using NCBI tool (Tables 2 and 3).

Table 2. Target genes and their sequence of primers of rohu used in qPCR analysis.

Target gene	Primer	Primer sequence (5'-3')	Accession/ Reference
Lysozyme C	Forward reverse	CGATGATGGCACTCCAGGT CATGCTTTCAGTCCTTCGGC	EF203085.1
Lysozyme G	Forward reverse	CAATGGCTTTGGCCTCATGC CACGTGGGAAACTTTGCTCTG	KC934746.1
TNF-α	Forward reverse	GGCGGCTTGAAAGTAGTGGA TATGCAGAACGTCGTGGTCC	FN543477.1
IL-10	Forward reverse	GCTCAGTGCAGAAGAGTCGAC CCCGCTTGAGATCCTGAAATATA	Banerjee *et al*. (2015)
IL-1β	Forward reverse	GTACCCCACAAAACATCGGC CAAGAGCAGTTTGGGCAAGG	AM932525.1
TLR-4	Forward reverse	CTAAGAAAGTGCTTGGGCTTCAT GGTTTGTGGCAATAATGGCTTTC	KX218428.1
β-actin	Forward reverse	GACTTCGAGCAGGAGATGG CAAGAAGGATGGCTGGAACA	Mohanty and Sahoo (2008)

A serial cDNA dilution and the construction of a standard curve (Fig.15) were used to test the effectiveness and specificity of the primers. The composition of reaction mixture (10 µl) was as follows: cDNA (1 µl, 1:3 ratio with DEPC), 2x SYBR™ Green PCR Master Mix (5 µl), forward and reverse primers (0.25 µl each, 2.5 mol) and nuclease-free water (3.5 µl). Real-time PCR (RT-PCR) programme setup were as follows: 10 min at 95°C, followed by 35/40 cycles for 15 sec at 95°C and then 1 min at 60°C. Each sample was run in duplicate, and

a NTC (non-template control) without cDNA was also performed. Melt curve (Fig.16) analysis was used to verify the qPCR's specificity. For the melt curve study, the following conditions were maintained: 15 sec at 95°C, 1 min at 60°C, and 95°C for 15 sec. After completing the programme, the threshold cycle (C_T) value of each well was recorded and analysis was performed using standard $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The amplified primers were considered with 100% efficiency. The housekeeping *β-actin* gene was used for the normalization of target genes. The band was observed under UV-visualizer system (UV2020-B, TOPBIO, Taiwan) (Fig.17). The data of treatment groups were compared to the control group.

Table 3. Target genes and the sequence of primers of magur used in qPCR analysis.

Target gene	Primer	Primer sequence (5'-3')	Accession number
iNOS	Forward	ATGGGCACTGAGATTGGAGC	KT180212
	Reverse	CTTCGTCCTTCCACAGCGAT	
SOD-C	Forward	CATGGTGGACCACGTGATGA	KF444052
	Reverse	TGATTGAGTGAGGCCCAAGC	
NF-κB	Forward	CTCGGTGAAGCGAGGAAGAG	MG571500
	Reverse	CATTCCGGCTCCGACTCTC	
Bcl-2	Forward	ATTACTGAAGACGCAGCCCC	KC907874
	Reverse	CGAGAGCAGGACGGTGTAAA	
BAX	Forward	GTAGTGTCGCGGAACGAACT	KT003584
	Reverse	TTTAGTCGGCTGGAGCGATG	
TNF-α	Forward	TCTCAGGTCAATACAACCCGC	KM593875
	Reverse	GAGGCCTTTGCGGAAAATCTTG	
Cytochrome-C	Forward	TATACCCACCCCTTGCAGGA	KT835312
	Reverse	GGAGATGGCTGGCGGTTTTA	
Caspase 9	Forward	GAGGAAGTCGGGCTTTCACA	KP261050
	Reverse	AGGCTGTGTAGGGACAGGAA	
Caspase 3	Forward	TTGGAGGAACACACTGACCG	KC921209
	Reverse	AGGCAGACTCAAAGTCCAGC	
β-actin	Forward	GAGCACCCTGTCCTGCTTAC	EU527190
	Reverse	GTACAGGGACAGCACAGCC	

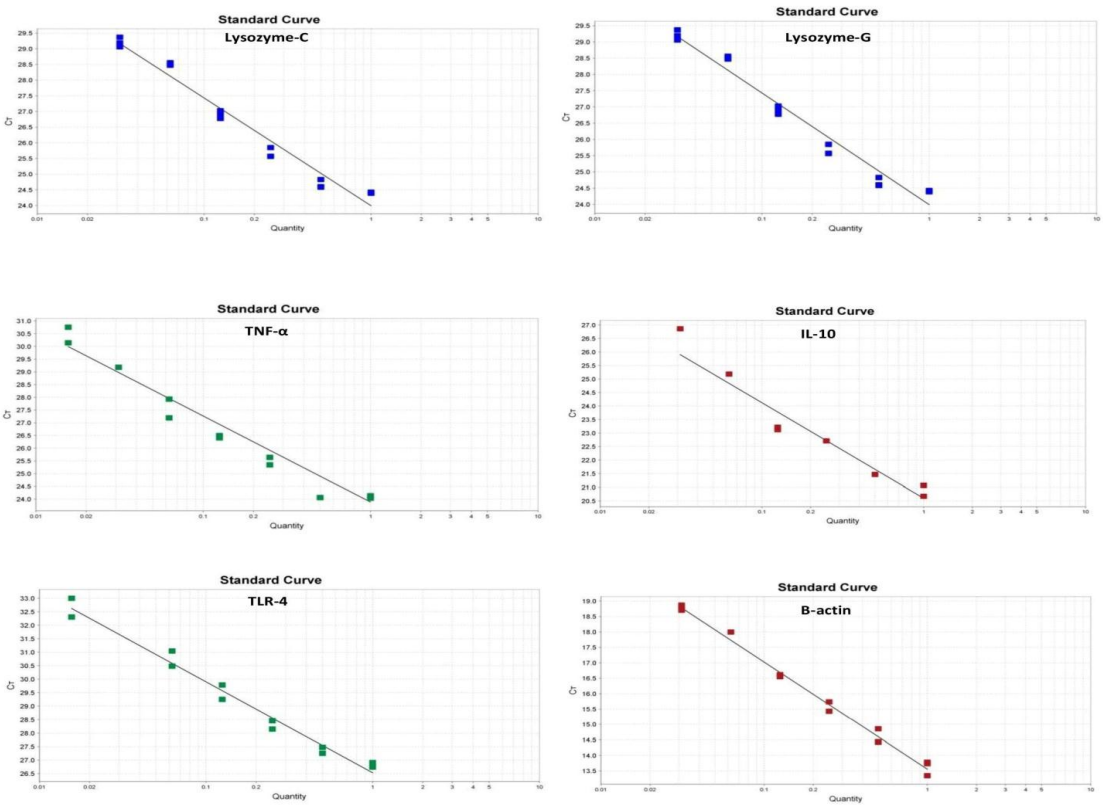

Fig.15. The standard curves of reference and target genes.

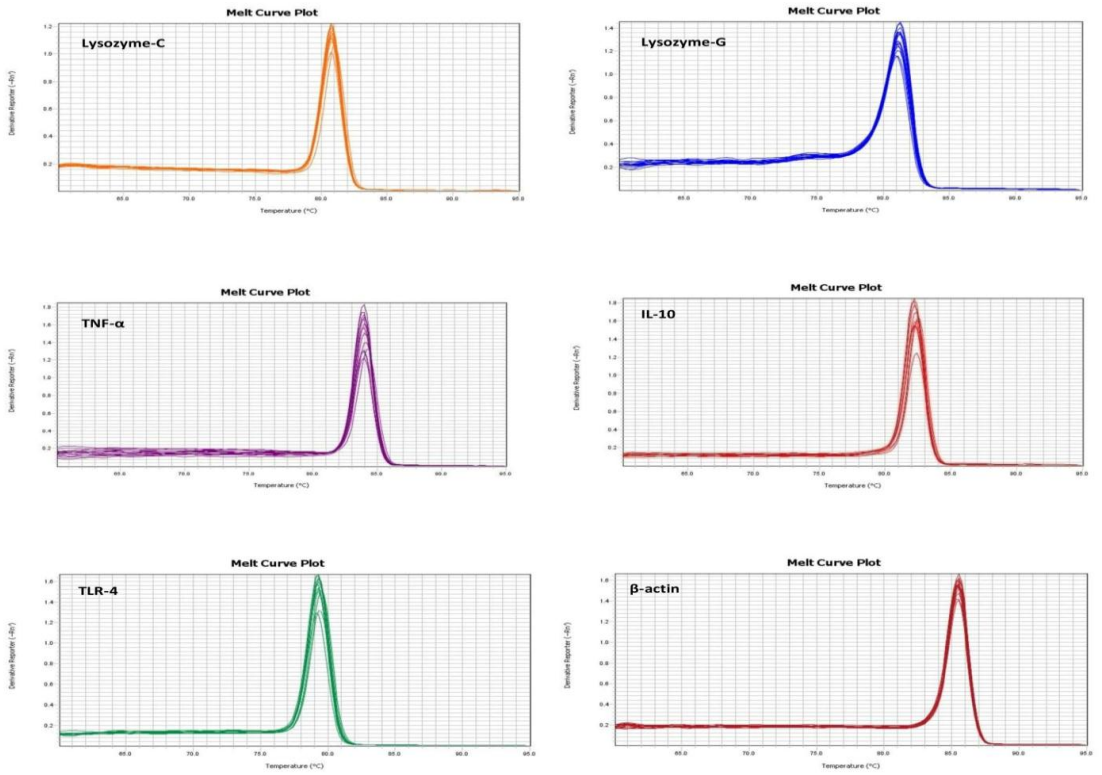

Fig.16. The melting curves of reference and target genes.

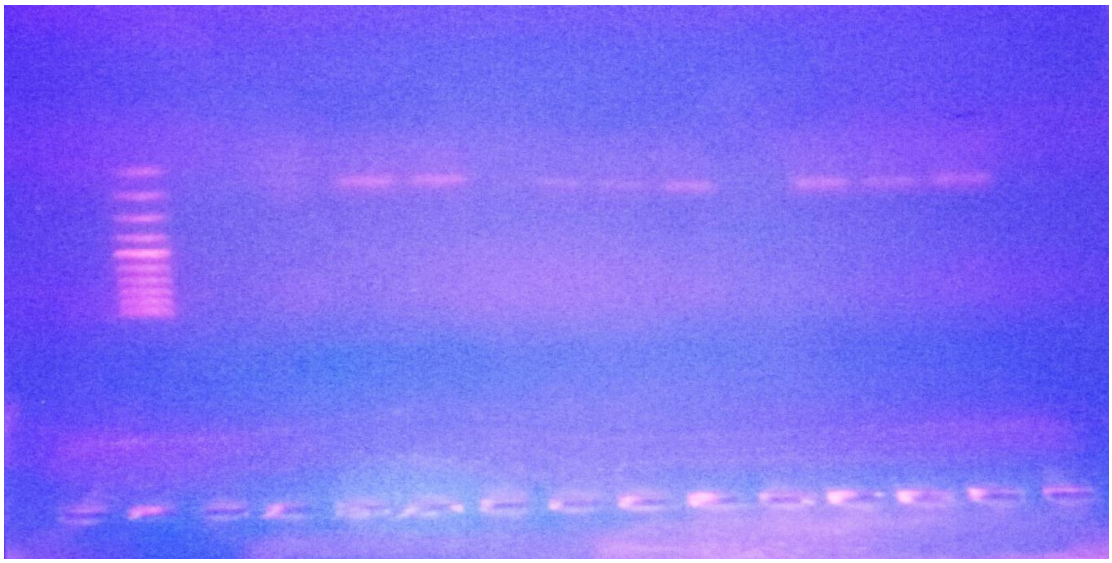

Fig.17. PCR bands observed under UV-visualize system.

3.12. Statistical analysis

All data related to growth parameters, enzymatic assays and mRNA expressions were given as means ± standard error. The Kolmogorov-Smirnova test was implemented to check the normality of the individual data (Massey Jr, 1951). The significance value $P>0.05$ indicates normality in all parameters except, hemagglutination titer. The data of significance value of hemagglutination titer was $P<0.05$ in all treatment groups. The non-parametric Kruskal-Wallis test was used to study the differences in hemagglutination titer among different days as well as the treatments. The one way ANOVA analysis was performed among all treatments and also for various days of sampling (7, 14 and 21) for all parameter in the treatment groups. The one-way multivariate analysis of variance ((one-way MANOVA)) was used to find out significant differences in the parameters among different treatments for the exposed and unexposed groups in UV-B experiment. The differences between two or more groups or between-subjects effects on multiple variables at once assessed with

Wilks' lambda test (Wilks, 1932). Partial eta squared (η^2) was performed to measure and compare the effect size in the different variables (Keppel, 1991). Further, Tukey HSD post-hoc analysis confirmed the pair-wise significance among different treatments/groups/days for each parameter (Bland and Altman, 1995). An independent sample t-test was used to compare the exposed and unexposed groups. Statistical analysis was performed using SPSS 26.0 version (South Asia (P) Ltd., Bangalore, India) software. The significance level was accepted as $P<0.05$.

Chapter 4

Results

4.1. The study of biochemical compositions of leaves and seeds of *Achyranthes aspera*

In the outdoor facility, *Achyranthes aspera* plants were grown. The seeds and leaves were collected, cleaned properly with water, dried at 35°C and ground. The matured seeds were collected, manually cleaned, ground and sieved (300 µm). The fine powder was used for various assays.

4.1.1. Biochemical analysis

The proximate composition study revealed that *A. aspera* seeds had significantly (P<0.05) higher crude protein and crude lipid than the leaves (Table 1). The amount of ash in leaves was significantly (P<0.05) higher than the seeds.

Table 1. The proximate compositions of *A. aspera* seeds and leaves (g/100 g).

Parameters	Seeds	Leaves
Crude protein	29.68 ± 0.27^{a}	23.05 ± 0.05^{b}
Crude lipid	4.36 ± 0.01^{a}	2.50 ± 0.08^{b}
Moisture	4.66 ± 0.04^{b}	7.88 ± 0.05^{a}
Ash	2.74 ± 0.05^{b}	15.47 ± 0.12^{a}
Carbohydrate	55.34 ± 1.11^{a}	54.32 ± 1.02^{a}
Energy (kcal/g)	3.79 ± 0.02^{a}	3.32 ± 0.01^{b}

4.1.2. Amino acid profile

The histidine, isoleucine, lysine, methionine and arginine levels were significantly (P<0.05) higher in seeds than the leaves (Table 2a). The leucine, tryptophan and valine levels were significantly (P<0.05) higher in leaves than

the seeds. All non-essential amino acids (except, alanine and tyrosine) contents were significantly (P<0.05) lower in leaves than the seeds. Alanine content was significantly (P<0.05) lower in seeds than the leaves. The phosphoserine and glutamine were present in seeds and absent in the leaves (Table 2b).

Table 2a. Essential amino acids compositions of *A. aspera* (g/100 g dry weight).

Amino acids	**Seeds**	**Leaves**
Arginine (Arg)	3.08 ± 0.57[a]	1.83 ± 0.06[b]
Histidine (His)	0.92 ± 0.01[a]	0.55 ± 0.03[b]
Isoleucine (Ile)	1.38 ± 0.01[a]	1.31 ± 0.07[b]
Leucine (Leu)	2.06 ± 0.01[b]	2.21 ± 0.13[a]
Lysine (Lys)	1.73 ± 0.04[a]	1.33 ± 0.07[b]
Methionine (Met)	0.61 ± 0.02[a]	0.50 ± 0.04[b]
Phenylalnine (Phe)	1.54 ± 0.09[a]	1.52 ± 0.08[a]
Threonine (Thr)	1.35 ± 0.01[a]	1.34 ± 0.05[a]
Tryptophan (Trp)	0.17 ± 0.17[b]	0.71 ± 0.00[a]
Valine (Val)	1.53 ± 0.01[b]	1.80 ± 0.11[a]
∑ Essential amino acids	14.37 ± 1.92[a]	13.11 ± 0.64[b]

The α-amino adipic acid and hydroxylysine were present in leaves. These amino acids were absent in seeds. Taurine was found in both leaves and seeds and was significantly (P<0.05) higher in leaves compared to the seeds.

Table 2b. Non-essential amino acids compositions of *A. aspera* (g/100 g dry weight).

Amino acids	**Seeds**	**Leaves**
Alanine (Ala)	1.17 ± 0.02^{b}	1.54 ± 0.08^{a}
Aspartic acid (Asp)	3.03 ± 0.02^{a}	2.67 ± 0.14^{b}
Cystine (Cys)	0.48 ± 0.08^{a}	-
Glutamate (Glu)	7.22 ± 0.01^{a}	3.44 ± 0.19^{b}
Glycine (Gly)	2.83 ± 0.02^{a}	1.61 ± 0.09^{b}
Proline (Pro)	1.35 ± 0.05^{a}	1.18 ± 0.06^{b}
Serine (Ser)	1.59 ± 0.01^{a}	1.16 ± 0.03^{b}
Tyrosine (Tyr)	1.33 ± 0.05^{a}	1.33 ± 0.06^{a}
Cystathionine (Cysthi)	0.30 ± 0.01^{b}	0.32 ± 0.05^{a}
Glutamine ($GluNH_2$)	0.01 ± 0.00^{a}	-
Hydroxylysine (Hylys)	-	0.04 ± 0.01^{a}
Hydroxyproline (Hypro)	1.48 ± 0.04^{a}	0.10 ± 0.01^{b}
Phosphoserine (p-ser)	0.13 ± 0.02^{a}	-
Phospho ethanol amine (PEA)	0.04 ± 0.01^{a}	0.04 ± 0.02^{a}
Taurine (Tau)	0.08 ± 0.01^{b}	0.13 ± 0.01^{a}
α- Amino adipic acid (α-AAA)	-	0.01 ± 0.00^{a}
β-Alanine (β-Ala)	0.26 ± 0.01^{a}	0.18 ± 0.01^{b}
β- Amino isobutyric acid (β-AiBA)	0.17 ± 0.04^{b}	0.19 ± 0.12^{a}
γ- Animo-n-butyric acid (γ-ABA)	0.13 ± 0.07^{b}	0.19 ± 0.01^{a}
1-Methylhistidine (1 Mehis)	0.09 ± 0.01^{a}	0.11 ± 0.02^{a}
∑ Non-essential amino acids	21.66 ± 0.42	14.24 ± 0.26

4.1.3. Fatty acid profile

Total saturated fatty acid content (SFAs) was 2-fold higher in seeds compared to leaves (Table 3). The study showed that monounsaturated fatty acid (MUFAs) content was 9-fold higher in seeds compared to the leaves. The

saturated fatty acids, carproic, caprylic, capric and lauric acids were present in seeds, whereas absent in leaves. Arachidic acid was present in leaves, whereas absent in the seeds. Palmitic acid was the dominant SFA in both seeds and leaves. The maximum level was found in seeds. Palmitoleic and gonodoic acids were absent in seeds and leaves, respectively.

Table 3. Fatty acid profile of *A. aspera* seeds and leaves (mg/100 g).

Fatty acids	**Formula**	**Seeds**	**Leaves**
Saturated fatty acids			
Caproic acid	6:0	7.88 ± 0.02^{a}	-
Caprylic acid	8:0	18.05 ± 0.16^{a}	-
Capric acid	10:0	25.56 ± 0.29^{a}	-
Lauric acid	12:0	5.50 ± 0.37^{a}	-
Myristic acid	14:0	22.82 ± 0.16^{a}	6.81 ± 0.32^{b}
Palmitic acid	16:0	1780.41 ± 8.14^{a}	910.71 ± 2.32^{b}
Stearic acid	18:0	1.97 ± 0.20^{b}	4.59 ± 0.83^{a}
Arachidic acid	20:0	-	6.24 ± 0.18^{a}
Lignoceric acid	24:0	22.07 ± 1.37^{a}	10.92 ± 0.81^{b}
∑SFA		1884.28 ± 7.84^{a}	939.27 ± 3.66^{b}
Monounsaturated fatty acids			
Palmitoleic acid	16:1 n-7	-	45.83 ± 0.83^{a}
Oleic acid	18:1 n-9	1863.34 ± 9.25^{a}	164.81 ± 0.62^{b}
Gondoic acid	20:1 n-9	104.25 ± 0.88^{a}	-
Erucic acid	22:1 n-9	61.42 ± 0.11^{a}	13.94 ± 0.80^{b}
∑ *MUFA*		*2029.01 ± 8.33^{a}*	224.58 ± 1.69^{b}
n-6 poly unsaturated fatty acids			
Linoleic acid	18:2 n-6	3342.05 ± 8.05^{a}	604.87 ± 2.58^{b}
∑ *n-6 PUFA*		*3342.05 ± 8.05^{a}*	*604.87 ± 2.58^{b}*
n-3 poly unsaturated fatty acids			
α-Linolenic acid	18:3 n-3	29.66 ± 0.50^{b}	1058.07 ± 3.71^{a}
∑ *n-3 PUFA*		*29.66 ± 0.50^{b}*	*1058.07 ± 3.71^{a}*

In seeds, oleic acid content was 11-fold higher compared to the leaves. Significantly (P<0.05) higher level of linoleic acid, the n-6 poly unsaturated fatty acid (n-6 PUFA) was found in seeds (3342.05±8.05 mg/100 g) compared to the leaves. The α-linolenic acid, the n-3 poly unsaturated fatty acids (n-3 PUFA) content was significantly (P<0.05) higher in leaves (1058.07±3.71 mg/100 g) compared to the seeds.

4.1.4. Vitamins

The vitamin A (retinol) was present in both seeds and leaves (Table 4). Vitamin D2 (ergocalciferol) and vitamin E (tocopherol) levels were significantly (P<0.05) higher in leaves than seeds. Among water soluble vitamins, B_1 (thiamine) and B_6 (pyridoxine) were present in the seeds, whereas absent in leaves. Vitamin B_2 (riboflavin) and B_{12} (cobalamin) contents were significantly (P<0.05) higher in seeds than leaves. Vitamin C (ascorbic acid) was present in seeds and absent in the leaves.

Table 4. Vitamin contents of seeds and leaves of *A. aspera* (mg/100 g, dry weight).

Vitamins	Seeds	Leaves
Fat soluble		
Retinol (A)	0.26 ± 0.08[a]	0.26 ± 0.09[a]
Ergocalciferol (D_2)	1.49 ± 0.01[b]	69.53 ± 0.53[a]
Tocopherol (E)	0.62 ± 0.07[b]	34.92 ± 0.40[a]
Water soluble		
Thiamine (B_1)	2.16 ± 0.15[a]	-
Riboflavin (B_2)	137.43 ± 6.17[a]	25.81 ± 2.75[b]
Pyridoxine (B_6)	2.93 ± 0.19[a]	-
Cobalamin (B_{12})	79.83 ± 8.40[a]	1.34 ± 0.06[b]
Ascorbic acid (C)	166.40 ± 3.41[a]	-

4.1.5. Mineral composition

All macro, trace and ultra-trace minerals (except, zinc) contents were significantly (P<0.05) higher in leaves than the seeds (Table 5). Zinc content was significantly (P<0.05) higher in the seeds than the leaves. In leaves, sodium, potassium, calcium and magnesium levels were 0.15±0.01, 27.50±0.32, 2.10±0.12 and 5.70±0.17 mg/100 g, respectively. Iron content was 76.82±4.15 and 296.73±11.40 µg/100 g of seeds and leaves, respectively. Significantly (P<0.05) higher level of cobalt (0.23±0.04 µg/g) was found in leaves compared to the seeds.

Table 5. Minerals composition of *A. aspera* seeds and leaves (dry weight).

Minerals	Seeds	Leaves
Macro minerals (mg/g)		
Calcium (Ca)	0.17 ± 0.01[b]	2.10 ± 0.12[a]
Magnesium (Mg)	2.18 ± 0.01[b]	5.70 ± 0.17[a]
Potassium (K)	6.35 ± 0.04[b]	27.50 ± 0.32[a]
Sodium (Na)	0.06 ± 0.01[b]	0.15 ± 0.01[a]
Trace minerals (µg/g)		
Aluminium (Al)	41.07 ± 4.16[b]	333.36 ± 16.62[a]
Cadmium (Cd)	0.10 ± 0.04[b]	0.62 ± 0.34[a]
Copper (Cu)	7.67 ± 0.19[b]	9.49 ± 0.57[a]
Iron (Fe)	76.82 ± 4.15[b]	296.73 ± 11.39[a]
Lead (Pb)	0.21 ± 0.02[b]	1.32 ± 0.06[a]
Manganese (Mn)	30.20 ± 0.63[b]	43.09 ± 1.58[a]
Molybdenum (Mo)	0.28 ± 0.02[b]	0.66 ± 0.10[a]
Strontium (Sr)	3.39 ± 0.26[b]	58.10 ± 1.54[a]
Zinc (Zn)	41.77 ± 0.18[a]	36.05 ± 2.18[b]
Ultra-trace minerals (µg/g)		
Chromium (Cr)	2.18 ± 0.38[b]	8.43 ± 1.39[a]
Cobalt (Co)	0.09 ± 0.01[b]	0.23 ± 0.04[a]
Nickel (Ni)	1.35 ± 0.44[b]	3.03 ± 0.23[a]
Tin (Sn)	0.18 ± 0.04[b]	0.38 ± 0.03[a]

4.2. Evaluation of immunostimulatory properties of leaves and seeds of *Achyranthes aspera* in rohu *Labeo rohita*

Indian major carp rohu *Labeo rohita* fry (1.9±0.08 g) were cultured in hapas (2.0 mx1.5 mx1.5 m) set inside the pond. Rohu fry were fed with two experimental diets enriched with 0.5% seeds (D1) and 0.5% leaves (D2) of *A. aspera.* One group of rohu was fed with the control diet (D3) without the plant ingredient. Feed was given at the rate of 5% of body weight. The duration of study was 60 days. Then fish were anaesthetized with tricaine methanesulfonate and were injected intraperitoneally with 60 µl of 20% (v/v) chicken-RBC. PBS buffer alone was injected to one group of fish (sham control). Fish were released into the respective hapa after immunization. The blood and tissue samples were collected on 7, 14, and 21 days after immunization.

4.2.1. Average weight, specific growth rate and feed conversion ratio

Dietary supplements of plants had a favorable effect on the growth of rohu. In all four days of sampling, the average final weight was significantly (P<0.05) higher in the fish fed with the diet supplemented with 0.5% seeds (D1) than other two diets fed fish (Fig.1). At the time of immunization (0-day), the average weight of D2 and D3 diets fed fish was not significantly (P>0.05) different. Minimum average weight was found in D3 diet fed fish on 7th, 14th and 21st days after immunization. The average weight of fish increased gradually with the duration of the culture period regardless of feeding regimes. A similar trend was also observed with specific growth rate (SGR). The highest SGR was found in D1 diet fed rohu in all sampling days. SGR showed a decreasing trend with the age of fish in all these treatments (Fig.2). Significantly (P<0.05) lower

feed conversion ratio (FCR) was observed in D1 diet fed rohu than other diets fed fish throughout the study period. FCR ranged from 0.44-0.50, 0.49-0.56 and 0.52-0.61 in rohu fed with D1, D2 and D3 diets, respectively (Fig.3).

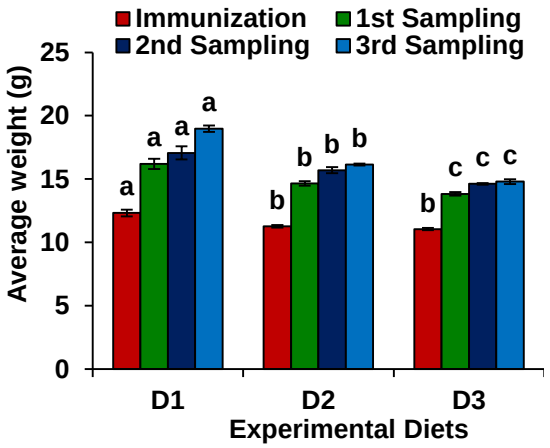

Fig. 1. Average weight of *L. rohita* cultured under different feeding regimes.

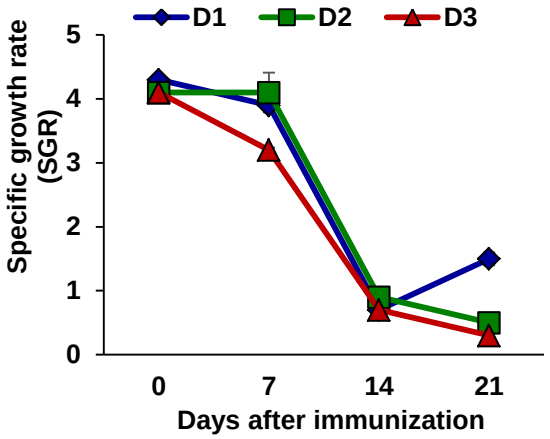

Fig. 2. Specific growth rate of *L. rohita* cultured under different feeding regimes.

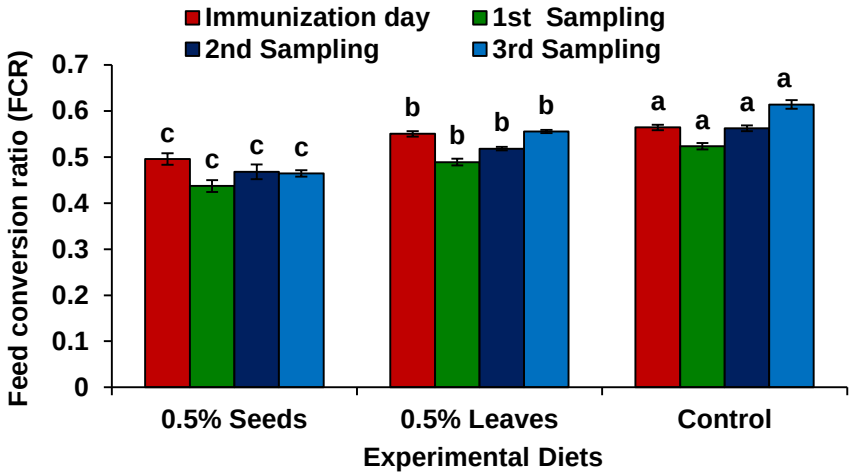

Fig. 3. Feed conversion ratio of *L. rohita* cultured under different feeding regimes.

4.2.2. Biochemical assays

The blood and tissue samples were collected on 7th, 14th, and 21st days after immunization. In D1 diet fed rohu, the serum lysozyme activity was significantly

(*P*<0.05) higher comparison to the other diets fed fish. The activity was minimum in D3 diet fed fish throughout the study period. Highest activity was observed on day-7 after immunization than the other days of sampling regardless of feeding regimes (Fig.4). Myeloperoxidase activity showed the similar trend. Significantly (*P*<0.05) higher myeloperoxidase activity was observed in D1 diet fed rohu than the other diets fed fish. Highest activity was recorded on day-7 after immunization. Then the activity gradually reduced regardless of treatments (Fig.5).

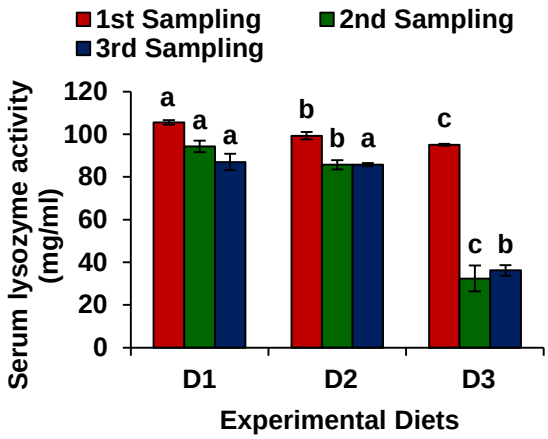

Fig.4. Serum lysozyme activities found in *L. rohita* cultured under different feeding regimes.

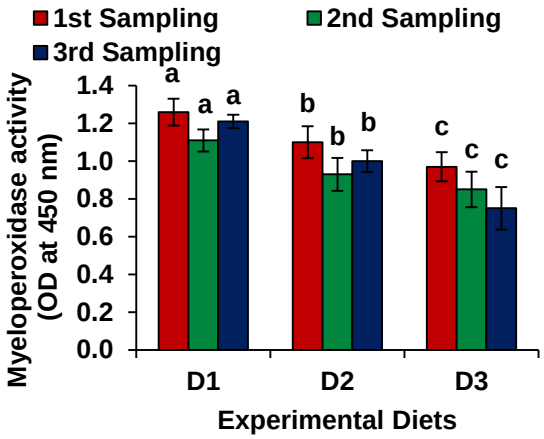

Fig.5. Myeloperoxidase activities found in *L. rohita* cultured under different feeding regimes.

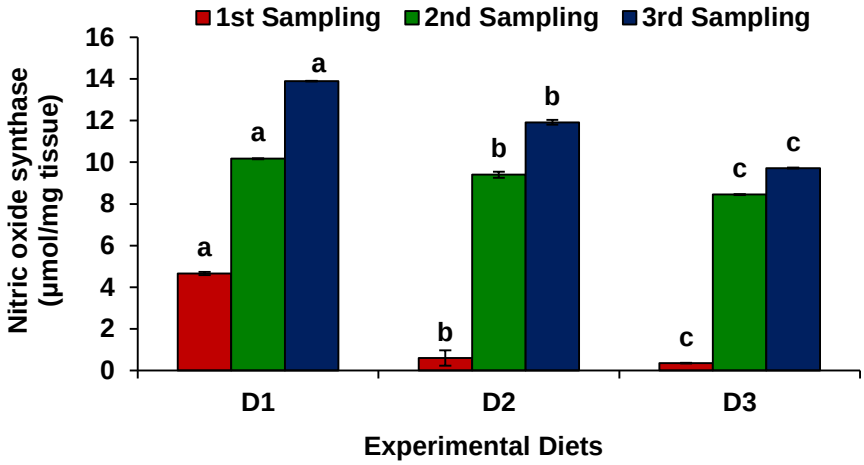

Fig.6. Nitric oxide synthase activity found in *L. rohita* cultured under different feeding regimes.

The nitric oxide synthase (NOS) level in hepatopancreas was also significantly (P<0.05) higher in D1 diet fed rohu than the other diets fed fish. Highest activity was observed on day-14 after immunization than other two days of sampling in all treatments (Fig.6). The seed supplemented diet (D1) fed rohu showed considerably (P<0.05) reduced TBARS levels compared to other treatments (Fig.7). Significantly (P<0.05) lower carbonyl protein level was seen in D1 diet fed rohu than the other diets fed fish (Fig.8). TBARS and carbonyl protein levels were minimum on day-7 after immunization and then the level gradually increased with the duration of culture period regardless of feeding regimes. TBARS and carbonyl protein levels were maximum in D3 diet fed rohu.

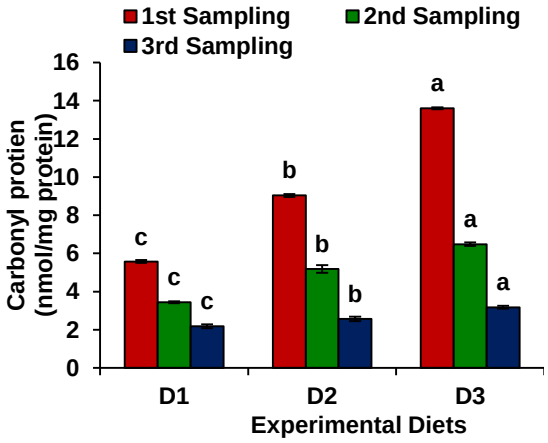

Fig.7. Carbonyl protein level found in *L. rohita* cultured under different feeding regimes.

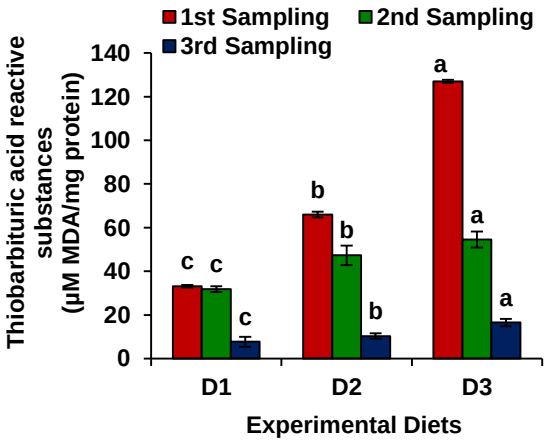

Fig.8. TBARS level found in *L. rohita* cultured under different feeding regimes.

4.2.3. Relative mRNA expression

Expression of various immune-related genes supported the physiological study. There were significant (P<0.05) up-regulation of *lysozyme C* and *lysozyme* G in hepatopancreas of rohu fed D1 diet compared to other diets fed fish (Figs.9 and 10). The *lysozyme C* expression was maximum on day-

21 after immunization in leaves and seeds incorporated diets fed rohu than the fish cultured in same feeding regimes on days-7 and 14. The expression of *lysozyme C* was higher than the *lysozyme G* in the hepatopancreas of rohu in all feeding regimes.

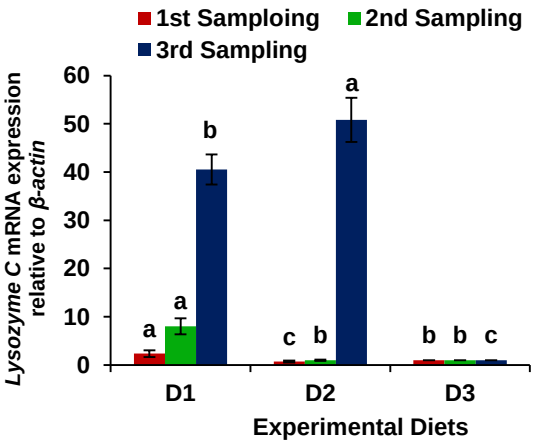

Fig.9. Expression of *lysozyme C* in *L. rohita* cultured under different feeding regimes.

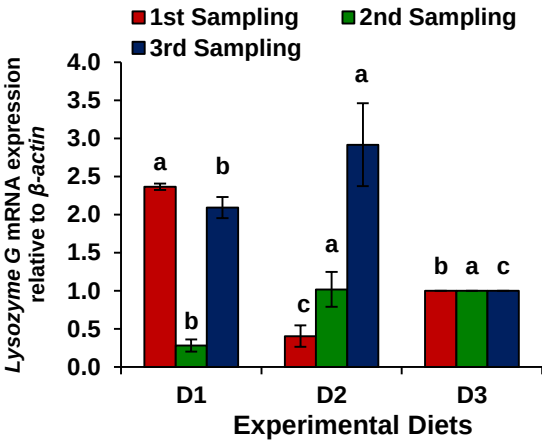

Fig.10. Expression of *lysozyme G* in *L. rohita* cultured under different feeding regimes.

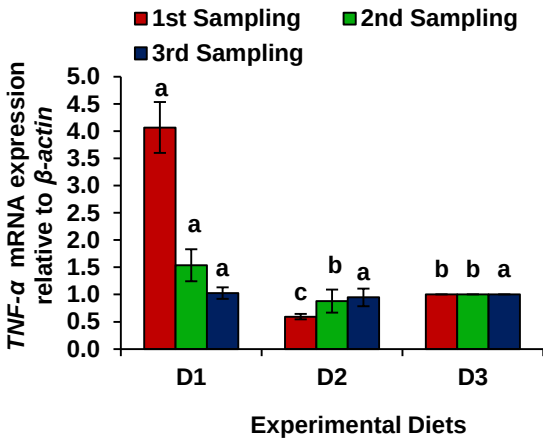

Fig.11. Expression of *TNF-α* in *L. rohita* cultured under different feeding regimes.

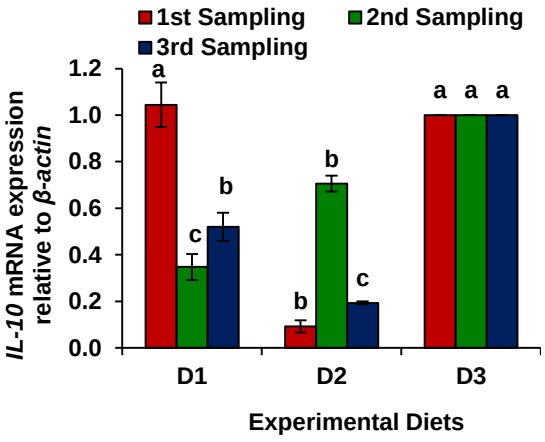

Fig.12. Expression of *IL-10* in *L. rohita* cultured under different feeding regimes.

Significantly (P<0.05) higher expression of *TNF-α* was seen in D1 diet fed rohu than other treatments (Fig.11). The activity was highest on day-7 after

immunization. Then the activity gradually decreased in seeds and leaves enriched diets fed rohu. In enriched diets fed rohu, *IL-10* expression was significantly (P<0.05) lower than the control diet fed fish (Fig.12). Significantly (P<0.05) higher expression of *TLR-4* was found in D1 diet fed rohu than the other treatments on day-7 after immunization. Then the expression was down-regulated in D1 treatment. In D2 diet fed rohu, the expression was up-regulated on day-21 after immunization (Fig.13). The expression of *IL-1β* was significantly (P<0.05) higher in D3 diet fed rohu than the other treatments throughout the study period (Fig.14).

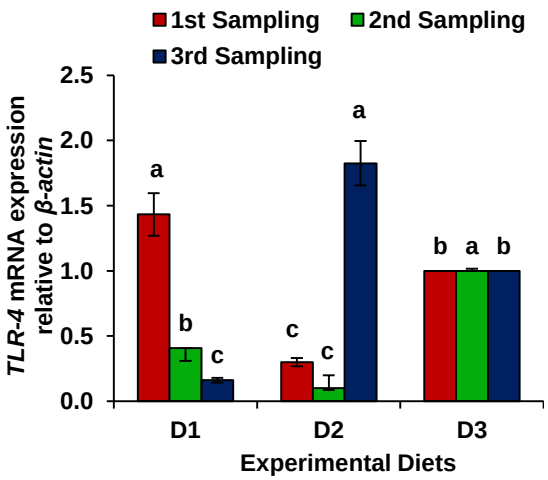

Fig.13. Expression of *TLR-4* in *L. rohita* cultured under different feeding regimes.

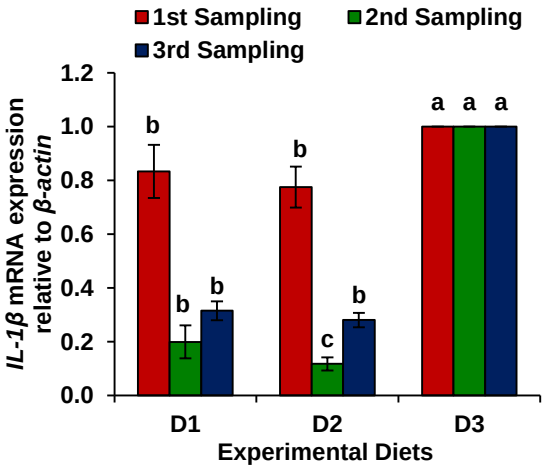

Fig.14. Expression of *IL-1β* in *L. rohita* cultured cultured under different feeding regimes.

4.3. Performance of *Achyranthes aspera* leaves and seeds supplemented diets fed rohu *Labeo rohita* and challenged with *Aeromonas hydrophila* in pond conditions

Indian major carp *rohu Labeo rohita* fry (25 fish/hapa) were cultured in hapas (2.0 mx1.5 mx1.5 m) set inside the pond. The two experimental test diets incorporated with 0.5% seeds (D1) and leaves (D2) of *A. aspera*, and control diet (D3), were fed to rohu fry (1.9±0.08). Three replicates were used for each feeding regime. Food was given once daily 9.00 a.m. @ 5% of body weight. After 80 days of initial feeding, fish were anesthetized with tricaine methanesulfonate and injected intraperitoneally (100 µl) with live *Aeromonas hydrophila* (5×10^6 CFU/ml). One group of fish was injected with PBS buffer and this served as sham control. The mortality of fish was recorded for 10 days at 12 h interval. Various tissue and blood samples were collected for the study.

4.3.1. Cumulative mortality of fish

The first mortality was recorded in D2 diet fed rohu within 12 h of challenge with bacterial pathogen. After 24 h of challenge, the mortality rates were 2, 4 and 10% in D1, D2 and D3 diets fed groups, respectively. In control, 50% fish died within five days of challenge. Significantly (P<0.05) higher cumulative mortality rate was found in control diet fed rohu than other treatments (Fig.15). The cumulative mortality rates were 28, 48 and 76% in D1, D2 and D3 diets fed rohu, respectively.

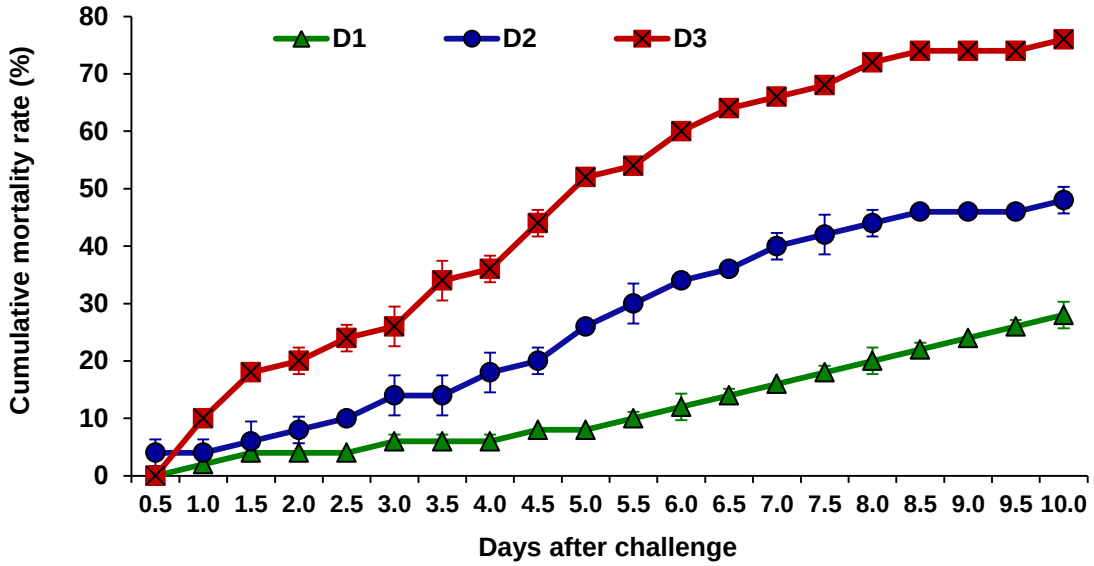

Fig.15. Cumulative mortality rate of *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila*.

4.3.2. Average weight

Feeding of fish with enriched diets showed a positive impact on the growth of rohu. The average final weight was significantly (P<0.05) higher in rohu fed with 0.5% seeds enriched diet (D1) than other diets fed fish. The average final weight of D1 diet fed rohu was 6.5 and 12.5% higher than D2 and D3 diets fed fish, respectively (Fig.16).

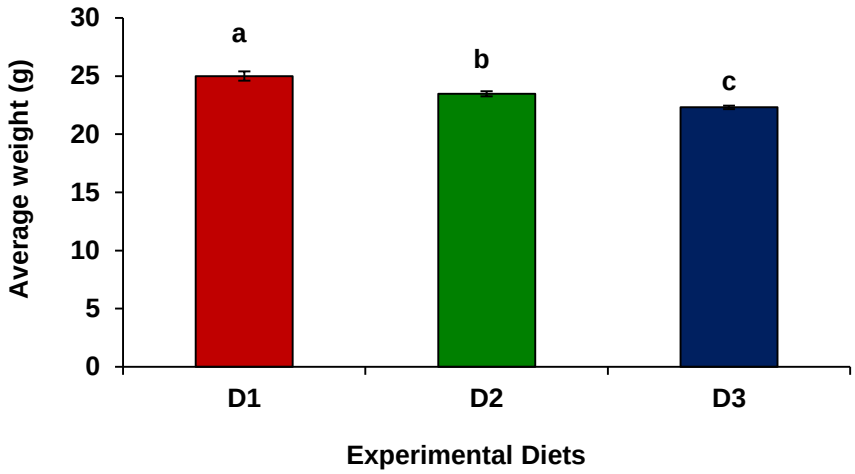

Fig.16. Average weight of *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila.*

4.3.3. Biochemical assays

4.3.3.1. Myeloperoxidase and nitric oxide synthase levels

The myeloperoxidase activity was higher (P<0.05) in the D1 and D2 diets fed rohu compared to the D3 diet fed fish (Fig.17). Nitric oxide synthase levels in hepatopancreas and kidney were significantly (P<0.05) higher in D1 diet fed fish than the others. The level was 5-7 folds higher in hepatopancreas compared to the kidney of rohu fed with same diet (Fig.18).

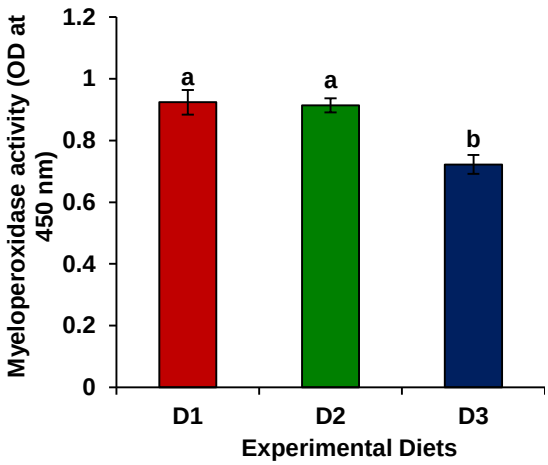

Fig.17. Myeloperoxidase activity of *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila.*

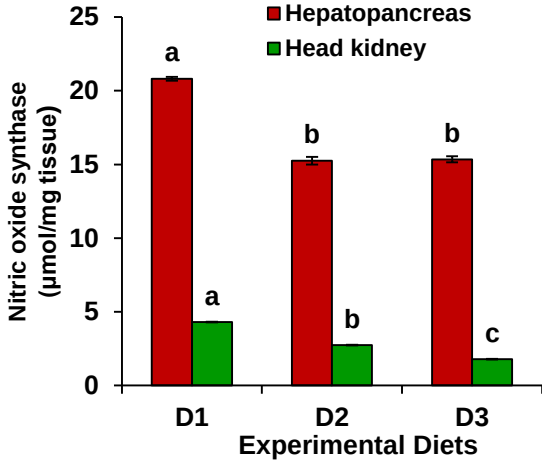

Fig.18. Nitric oxide synthase activity found in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila.*

4.3.3.2. Oxidation of tissue lipids and proteins

The TBARS level was significantly (P<0.05) lower in the hepatopancreas and kidney of D1 diet fed rohu comparison to the others. Similarly, the level of carbonyl protein was significantly (P<0.05) lower in D1 diet fed fish than others. The maximum levels of carbonyl protein and TBARS were observed in D3 diet fed fish. The levels were higher in hepatopancreas compared to the kidney of rohu cultured in the same feeding regime (Figs.19 and 20).

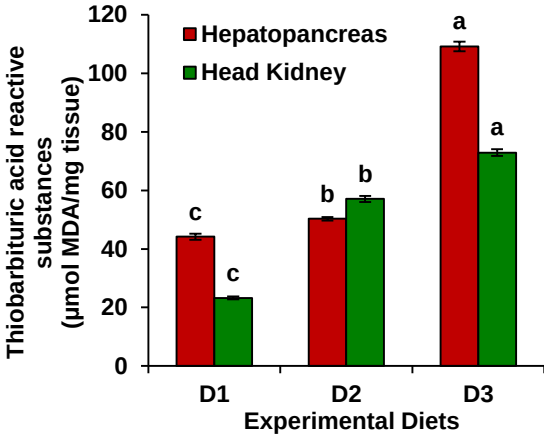

Fig.19. Thiobarbituric acid reactive substances (TBARS) found in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila.*

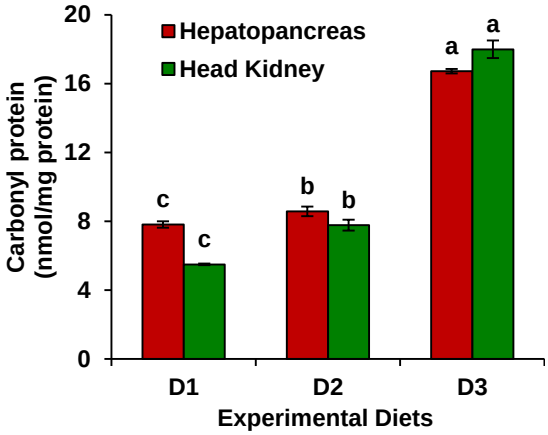

Fig.20. Carbonyl protein found in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila.*

4.3.4. Relative mRNA expression

Expressions of various immune-related genes supported the physiological study. The expressions of *lysozyme C* and *lysozyme G* were significantly (P<0.05) higher in hepatopancreas of seeds (D1) supplemented diet fed fish, than other treatments. This treatment was followed by D2 diet fed rohu and minimum level was found in control diet (D3) fed fish (Fig.21).

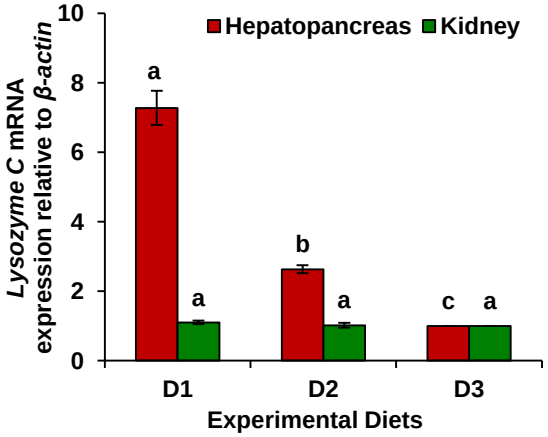

Fig.21. Expression of *lysozyme C* in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila.*

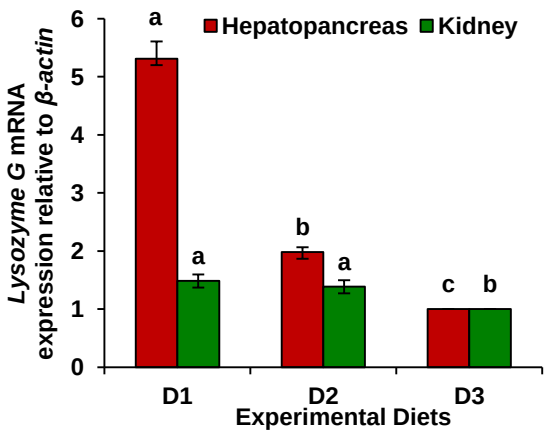

Fig.22. Expression of *lysozyme G* in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila.*

The expression of *lysozyme C* was higher than the *lysozyme G* in the hepatopancreas of rohu cultured in the same feeding regime. *Lysozyme G* level was significantly (*P*<0.05) higher in kidney of rohu fed with D1 and D2 diets compared to the D3 diet fed fish (Fig.22). In hepatopancreas of D1 diet fed rohu, the expression of *TNF-α* was significantly (*P*<0.05) higher than other treatments. This treatment was followed by D2 and D3 diets fed rohu (Fig.23). In kidney, the expression of *TNF-α* was significantly (*P*<0.05) higher in D1 and D2 diets fed rohu than D3 diet fed fish. The expression of *TNF-α* was 2-6 folds higher in hepatopancreas compared to kidney of rohu cultured in same feeding regime. There was up-regulation (*P*<0.05) of *IL-10* in hepatopancreas of D1 diet fed fish than others (Fig.24). This treatment was followed by D2 diet fed fish. In kidney, *IL-10* was up-regulated (*P*<0.05) in D1 diet fed rohu and it was down-regulated in D2 diet fed fish than the control diet fed fish. In hepatopancreas of D1 diet fed rohu, *IL-1β* was up-regulated (*P*<0.05) compared to others (Fig.25).

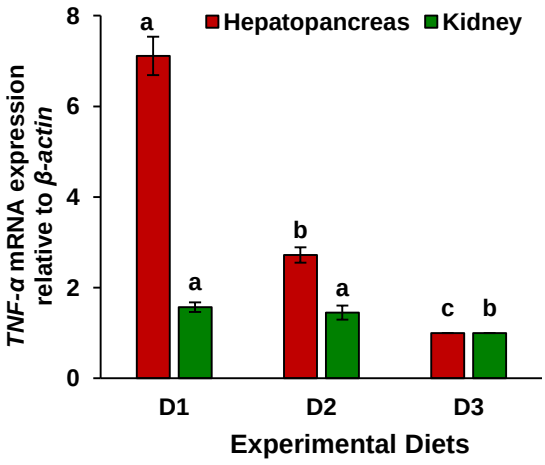

Fig.23. Expression of *TNF*-α, in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila*.

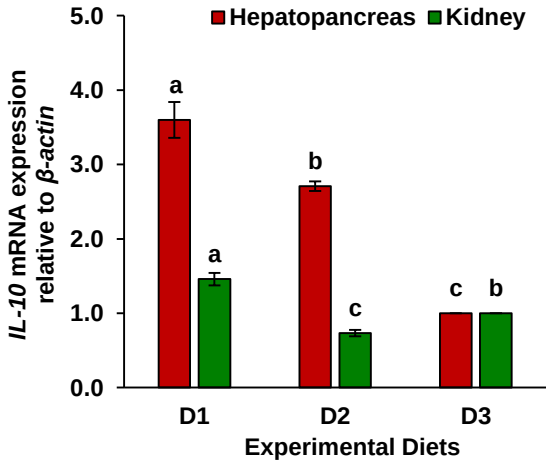

Fig.24. Expression of *IL-10* in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila*.

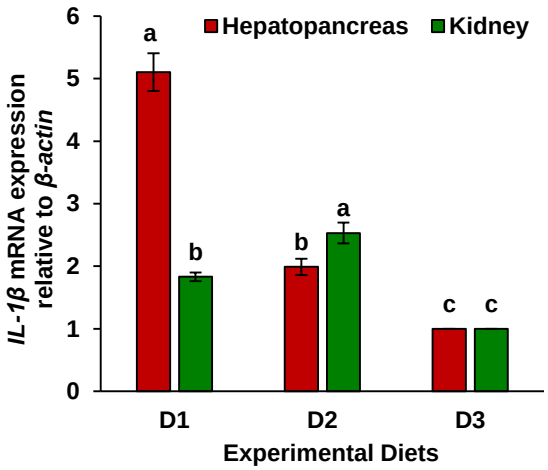

Fig.25. Expression of *IL-1β* in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila*.

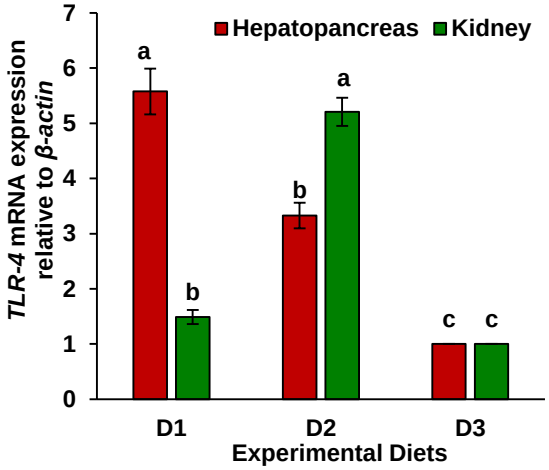

Fig.26. Expression of *TLR-4* in *L. rohita* cultured under different feeding regimes and challenged with *A. hydrophila*.

Whereas in kidney, significantly (P<0.05) higher expression of *IL-1β* was found in D2 diet fed fish than other treatments. Similar trend was also found with *TLR-4*. *TLR-4* was up-regulated in the hepatopancreas and kidney of D1 and D2 diets fed rohu, respectively (Fig.26).

4.4. Evaluation of immunostimulatory properties of leaves and seeds of *Achyranthes aspera* in magur *Clarias batrachus* in laboratory conditions

Two experiments were conducted to evaluate the immunostimulatory effects of *Achyranthes aspera* in Asian catfish magur *Clarias batrachus* were cultured in wet laboratory conditions.

4.4.1. First experiment

Clarias batrachus were cultured in plastic tanks (110 l) and stocking was density was 20 fish/tank. Magur were fed with experimental diet containing 0.5% seeds of *A. aspera* (D1) and control diet (D2) without any plant ingredient. Fish were fed @ 5% of body weight for 60 day. Three replicates were used for both feeding regime. After 60 days, the fish were anaesthetized with tricaine methanesulfonate and were injected intraperitoneally with 20% (v/v) chicken-RBC. PBS buffer was injected to one group of fish (sham control). Then the fishes were released into the appropriate tank. Blood and tissue samples were collected on day-7 after immunization.

4.4.1.1. Growth and survival of fish

The average final weight was significantly (P<0.05) higher in 0.5% seeds supplemented diet fed magur than the control diet fed fish (Fig.27). There was no mortality of fish during the study period.

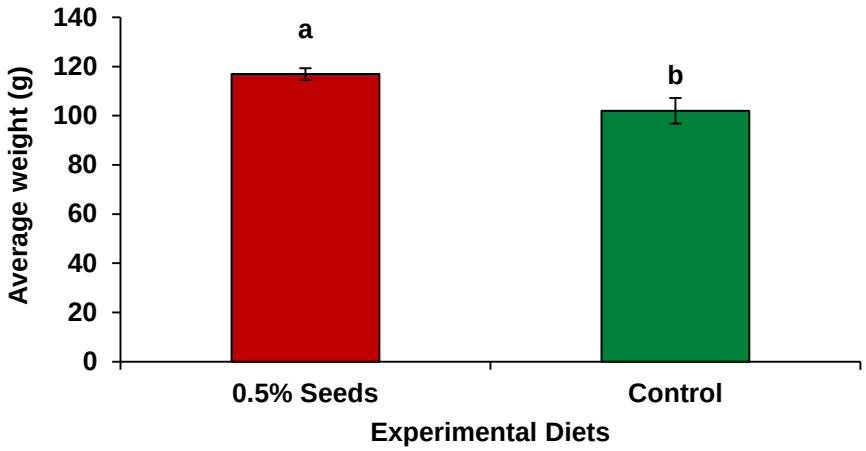

Fig.27. Average weight of *C. batrachus* cultured under two different feeding regimes.

4.4.1.2. Biochemical assays

4.4.1.2.1. Immunological parameters

Serum lysozyme level was significantly (P<0.05) higher in 0.5% seeds incorporated diet fed magur than the control diet fish (Fig.28) on day-7 after immunization. Like serum lysozyme, myeloperoxidase level was significantly (P<0.05) higher in 0.5% seeds enriched diet fed magur than the control diet fed one (Fig.29).

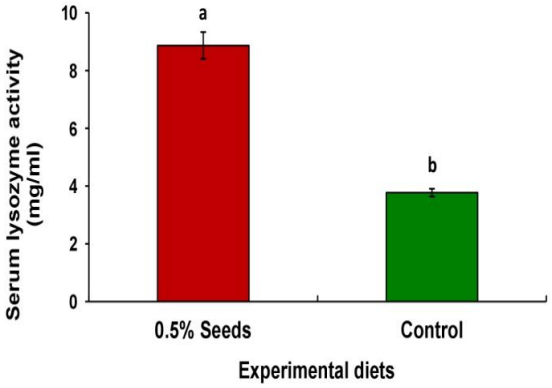

Fig.28. Serum lysozyme activity found in *C. batrachus* cultured under two feeding regimes.

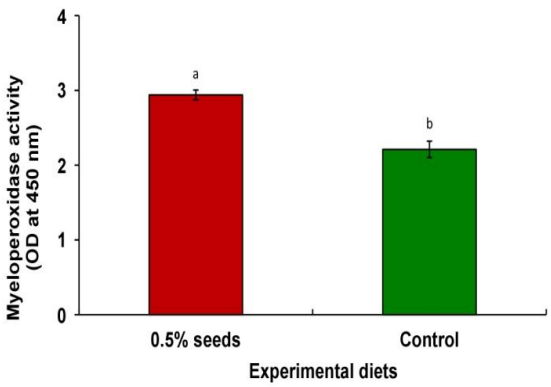

Fig.29. Myeloperoxidase activity found in *C. batrachus* cultured under two feeding regimes.

Similarly, nitric oxide synthase (NOS) level was significantly (P<0.05) higher in hepatopancreas of 0.5% seeds enriched diet fed magur than the control diet fed magur on day-7 after immunization (Fig.30).

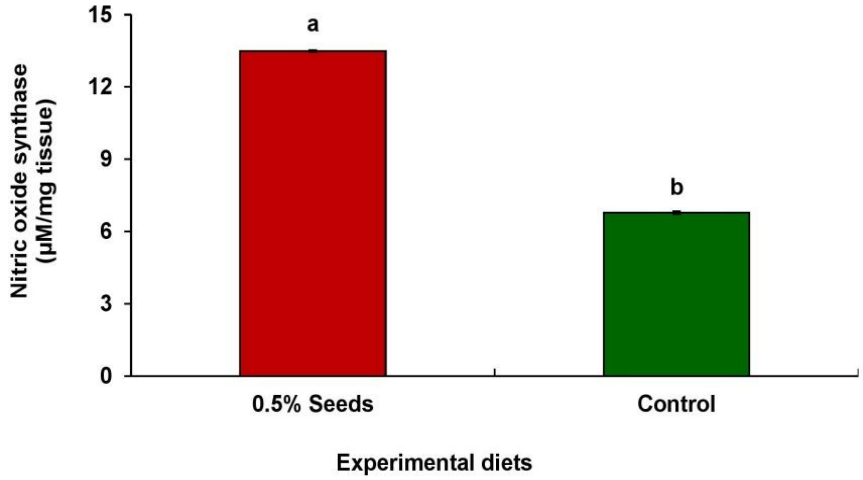

Fig.30. Nitric oxide synthase level found in *C. batrachus* fed with two different diets.

4.4.1.2.2. Thiobarbituric acid reactive substances and carbonyl protein

The thiobarbituric acid reactive substances (TBARS) level was significantly (P<0.05) higher in control diet fed magur than fish fed 0.5% seeds supplemented diet on day-7 after immunization (Fig.31).

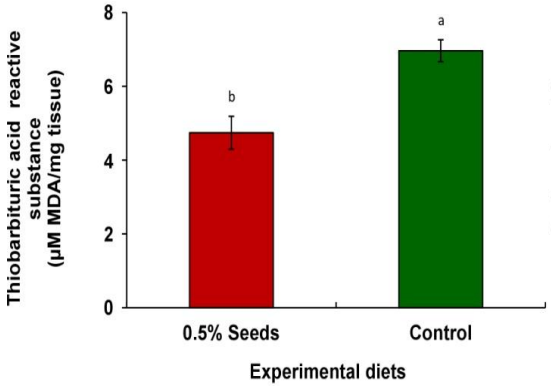

Fig.31. Thiobarbituric acid reactive substances (TBARS) level found in hepatopancreas of *C. batrachus.*

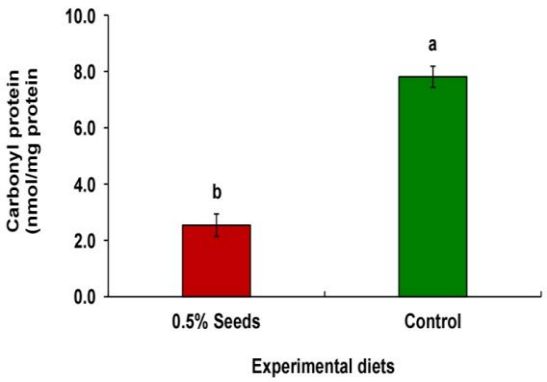

Fig.32. Carbonyl protein levels found in hepatopancreas of *C. batrachus.*

Similarly, carbonyl protein level was significantly (*P*<0.05) higher in hepatopancreas of control diet fed fish compared to 0.5% seeds supplemented diet fed group on day-7 after immunization (Fig.32).

4.4.1.3. Relative mRNA expression

The expressions of *IL-1β, iNOS* and *NF-kB* were studied in four tissues *viz*., liver, kidney, gills and arborescent organ (ARO). The expressions of *IL-1β, iNOS* and *NF-kB* were significantly (*P*<0.05) higher in 0.5% seeds supplemented diet fed magur compared to control diet fed fish in both liver and kidney (Figs.33 and 34). There was no significant (*P*>0.05) difference in expression level of *NF-kB* in gills and ARO of magur. The expressions of *IL-1β* and *iNOS* were significantly (*P*<0.05) higher in 0.5% seeds incorporated diet fed magur compared to control diet fed fish (Figs.35 and 36).

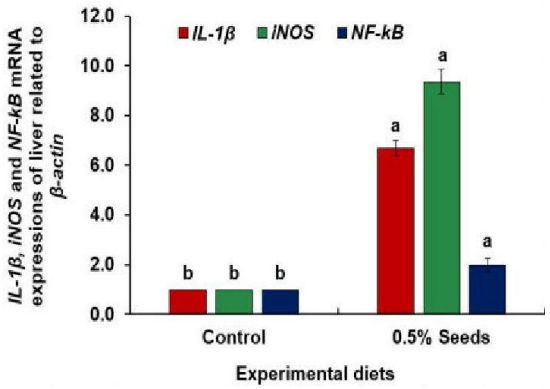

Fig.33. Expressions of *IL-1β, iNOS* and *NF-kB* in liver of *C. batrachus* immunized with c-RBC.

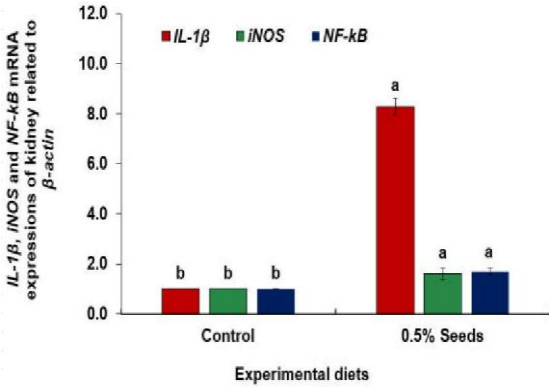

Fig.34. Expressions of *IL-1β, iNOS* and *NF-kB* in kidney of *C. batrachus* immunized with c-RBC.

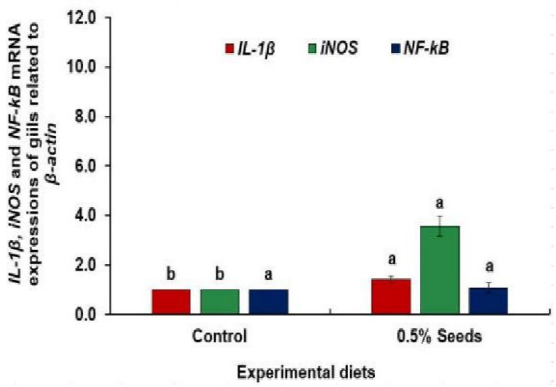

Fig.35. Expressions of *IL-1β, iNOS* and *NF-kB* in liver, kidney, gills and arborescent organ (ARO) of *C. batrachus* immunized with c-RBC.

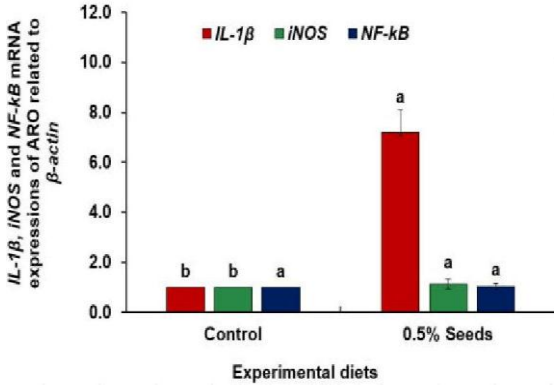

Fig.36. Expressions of *IL-1β, iNOS* and *NF-kB* in liver, kidney, gills and arborescent organ (ARO) of *C. batrachus* immunized with c-RBC.

4.4.2. Second experiment

In the second experiment, magur *Clarias batrachus* fry (352.4±8.0 mg) were cultured in aquarium (60 l) maintained in the wet laboratory (12 fish/aquarium). Fish were fed with three test diets (D1, D2, and D3) enriched with 0.5% seeds, 0.5% leaves, 0.25% leaves of herb *A. aspera* and a control diet (D4) devoid of any plant materials. After 60 days of feeding, fish were anaesthetized with tricaine methanesulfonate and were injected intraperitoneally with 20% (v/v) chicken-RBC. PBS buffer only was injected to one group of fish (sham control). Then the fishes were introduced into the appropriate tank. Blood and tissue samples were collected on days 7th, 14th and 21st after immunization.

4.4.2.1. Average weight, specific growth rate and feed conversion ratio

The final average weight was significantly (P<0.05) higher in D1 diet fed fish than other three treatments throughout the experimental period (Fig.37). Highest growth of fish was found on day-21 after immunization regardless of feeding schemes.

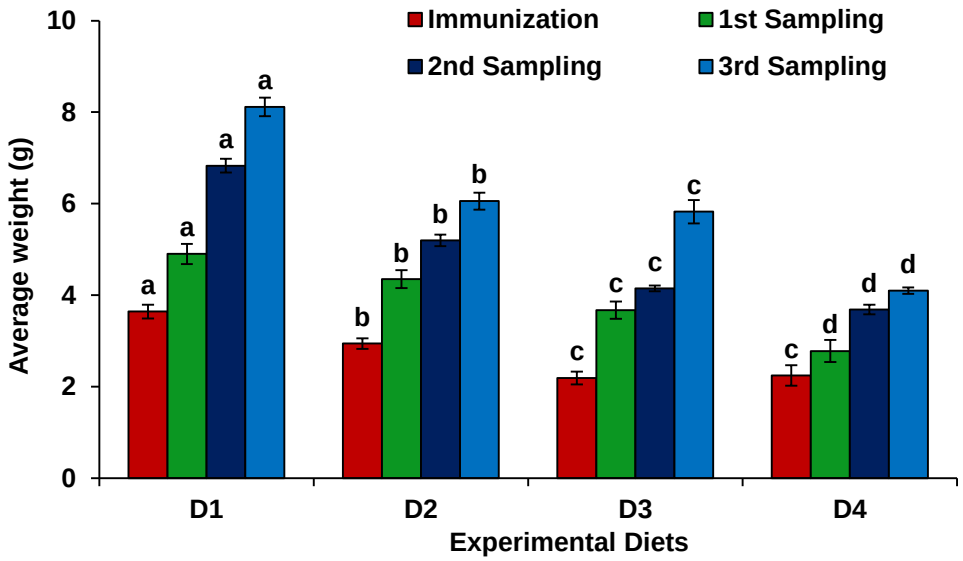

Fig.37. Average weight of *C. batrachus* cultured under different feeding regimes.

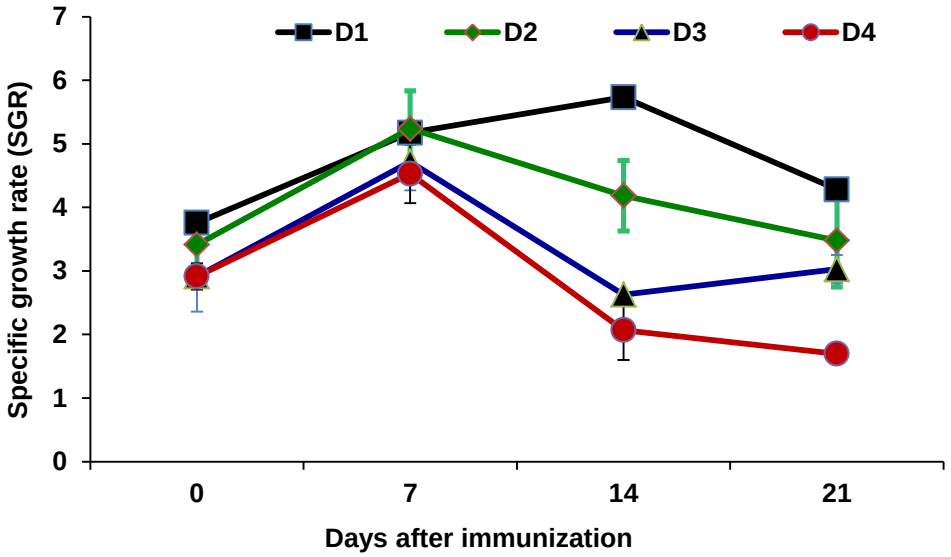

Fig.38. Specific growth rate *C. batrachus* cultured under different feeding regimes.

The specific growth rate (SGR) was significantly (P<0.05) higher in D1 diet fed magur than other treatments on the day of immunization and on day-14 after immunization (Fig.38). The FCR was significantly (P<0.05) lower in D1 than other treatments on the day of immunization and on day-14 after immunization (Fig.39).

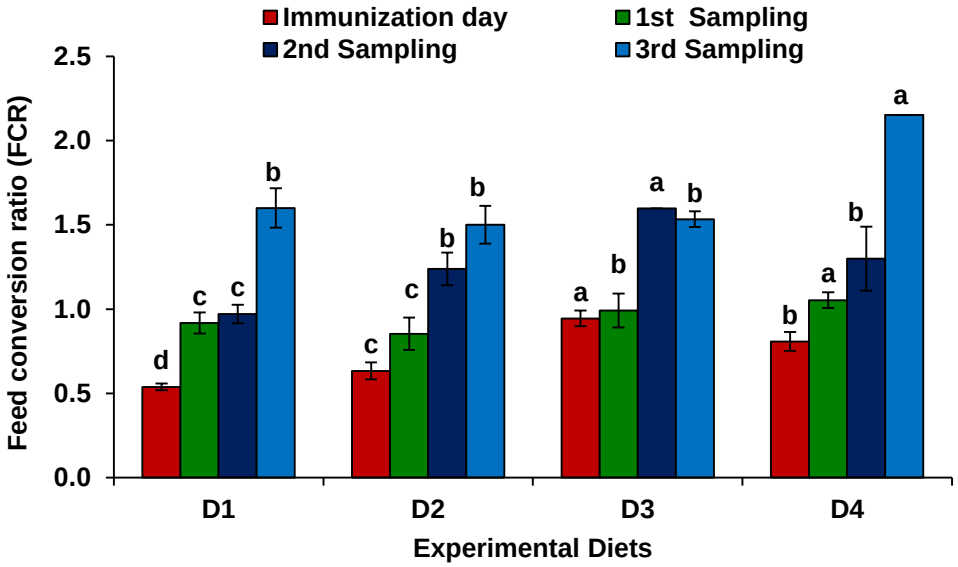

Fig.39. Feed conversion ratio of *C. batrachus* cultured under different feeding regimes.

4.4.2.2. Biochemical assays

4.4.2.2.1. Serum lysozyme, myeloperoxidase, hemagglutination titer and nitric oxide synthase

Serum lysozyme, myeloperoxidase, hemagglutination titer and nitric oxide synthase levels of magur cultured in four treatments were estimated on three different days after immunization. The serum lysozyme levels were 1.10-1.52, 1.07-4.35 and 1.01-2.88 folds higher on days-7, 14 and 21 after immunization, respectively in D1 compared to other treatments (Fig.40). The levels were significantly (P<0.05) higher in D1 and D3 than other two feeding regimes in all three days of sampling. Similarly, 1.08-1.30, 1.15-1.42 and 1.16-2.01 folds higher myeloperoxidase levels were observed in D1 (P<0.05) diet fed magur than other treatments on day 7th, 14th and 21st after immunization, respectively. The hemagglutination titer level was maximum in D1 diet fed fish throughout the study period (Fig.41).

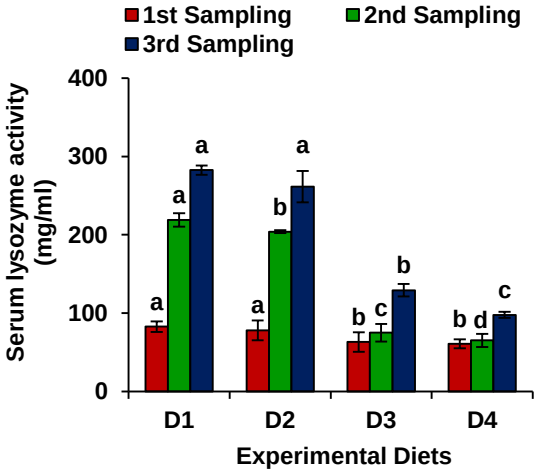

Fig.40. Serum lysozyme activity found in *C. batrachus* cultured under different feeding regimes.

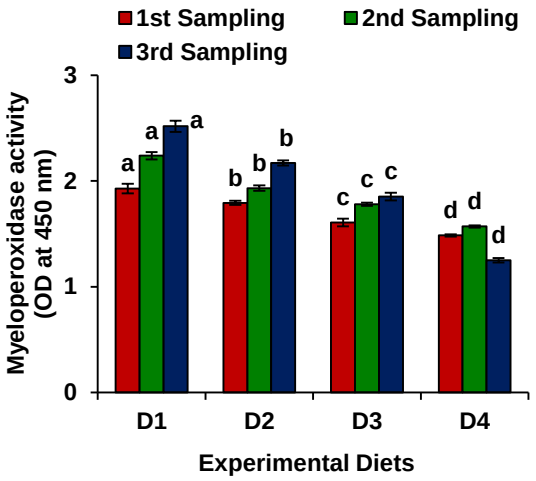

Fig.41. Myeloperoxidase activity found in *C. batrachus* cultured under different feeding regimes.

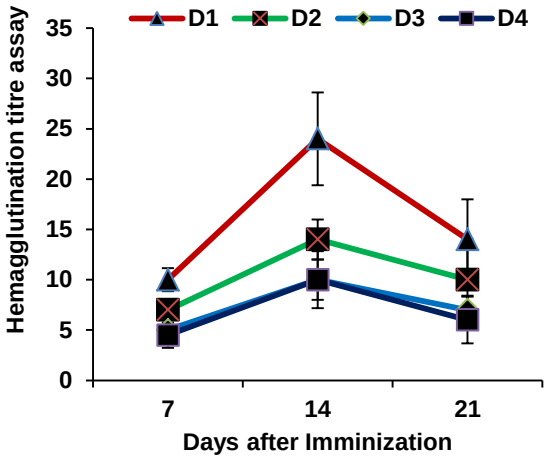

Fig.42. Hemagglutination titer level found in *C. batrachus* cultured under different feeding regimes.

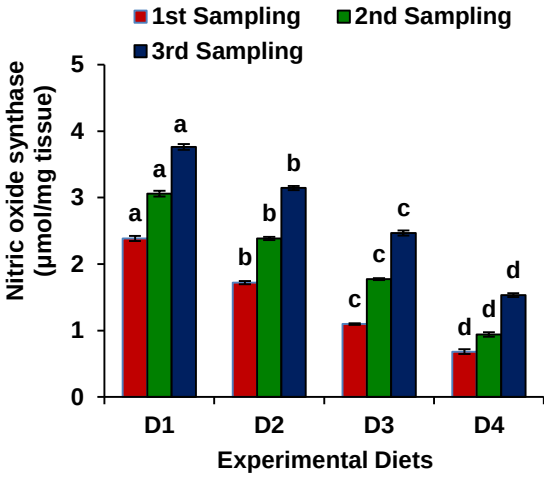

Fig.43. Nitric oxide synthase activity found in *C. batrachus* cultured under different feeding regimes.

The days of immunization also influenced the study parameters. Serum lysozyme and myeloperoxidase levels were significantly (P<0.05) higher on day-21 regardless of treatments, except the control diet fed (D4) magur. In the D4 diet fed fish, lysozyme and myeloperoxidase levels increased on day-14. In all these treatments, highest hemagglutination titer level was found on day-14 after immunization (Fig.42).

The nitric oxide synthase levels in liver of D1 diet fed magur was significantly (P<0.05) higher than the other diet fed fish (Fig.43). Similar trend was observed in kidney of magur. Highest nitric oxide synthase level (P<0.05) was found in D1 diet fed fish. The NOS levels in liver and kidney increased gradually with the days of immunization. Highest (P<0.05) nitric oxide synthase level was found on day-21 after immunization in all these treatments.

4.4.2.2.2. Superoxide dismutase

Superoxide dismutase (SOD) level in liver was significantly (P<0.05) lower in D1 diet fed magur than other treatments throughout the study period. In D1 and D2 diets fed magur, SOD level gradually decreased (except in D2, on day-14 after immunization) with the days of immunization. In D3 and D4 diets fed magur, SOD level gradually increased and it was significantly (P<0.05) higher on day-21 after immunization (Fig.44).

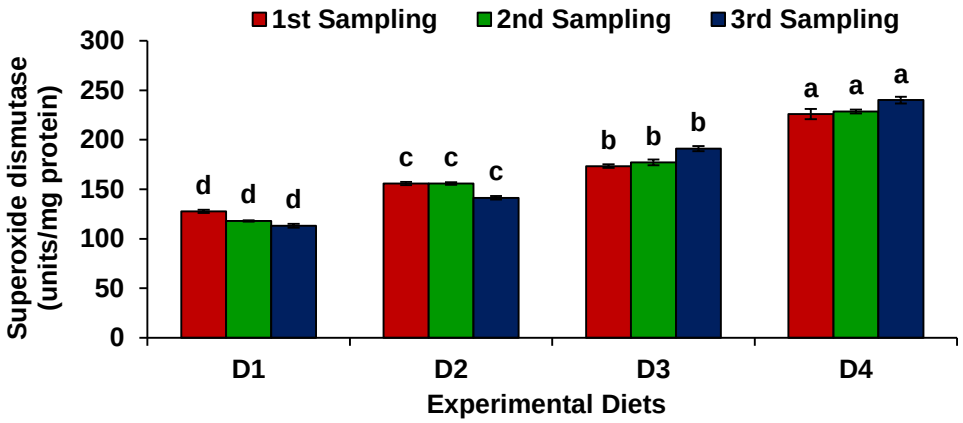

Fig.44. Superoxide dismutase level found in *C. batrachus* cultured under four different feeding regimes.

4.4.2.2.3. Thiobarbituric acid reactive substances and carbonyl protein

The thiobarbituric acid reactive substances (TBARS) level was 1.95-7.15, 1.70-6.05 and 1.90-6.97 folds lower on days-7, 14 and 21, respectively in the liver of

D1 diet fed magur than other treatments. In the kidney of D1 diet fed fish, TBARS level was 2.24-6.06, 2.18-7.54 and 2.30-9.15 folds lower compared to other diets fed fish on days-7, 14 and 21, respectively. TBARS levels in both liver and kidney of control diet fed fish were significantly (P<0.05) higher comparison to the experimental diets fed fish throughout the study period. Similarly, in both liver and kidney, carbonyl protein levels were significantly (P<0.05) lower in D1 compared to other treatments throughout the study period (Figs.45 and 46).

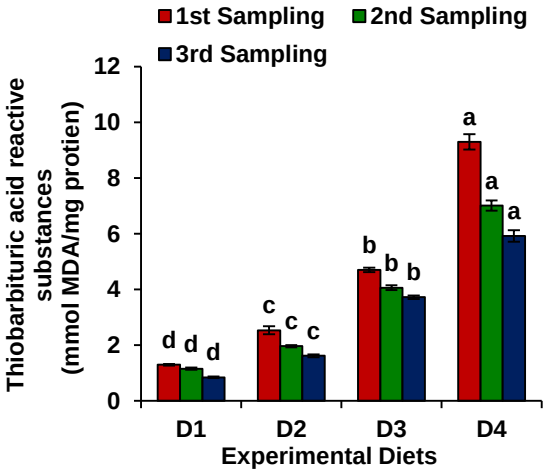

Fig.45. Thiobarbituric acid reactive substances (TBARS) found in *C. batrachus* cultured under different feeding regimes.

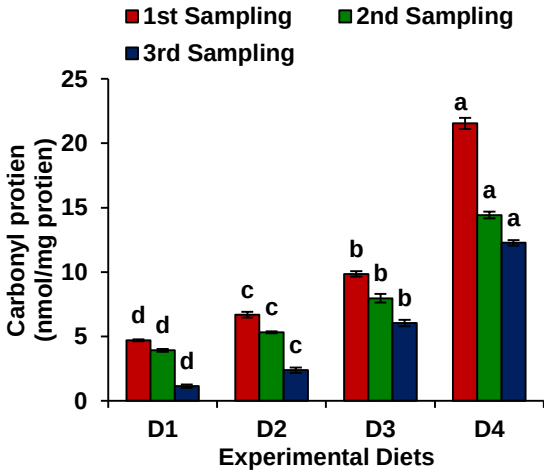

Fig.46. Carbonyl protein found in *C. batrachus* cultured under different feeding regimes.

The effect of immunization days was also found in this study. In liver and kidney, TBARS levels gradually decreased (P<0.05) with the days of immunization regardless of treatments. The carbonyl protein showed the similar trend. In all these feeding schemes, carbonyl level was maximum on day-7 and it was minimum on day-21 after immunization.

4.4.2.3. Relative mRNA expression

In kidney and liver of magur, *TNF-α, iNOS* and *NF-kB* expressions were significantly (*P*<0.05) higher in D1 diet fed fish than the diet fed fish. Treatment D3 was followed by the D1. In D2 treatment, *NF-kB* was down-regulated in kidney on days-7 and 14 after immunization compared to the control diet fed fish. On day-21 after immunization, there were up-regulations of all these genes in D2 treatment (Fig.47 a-c).

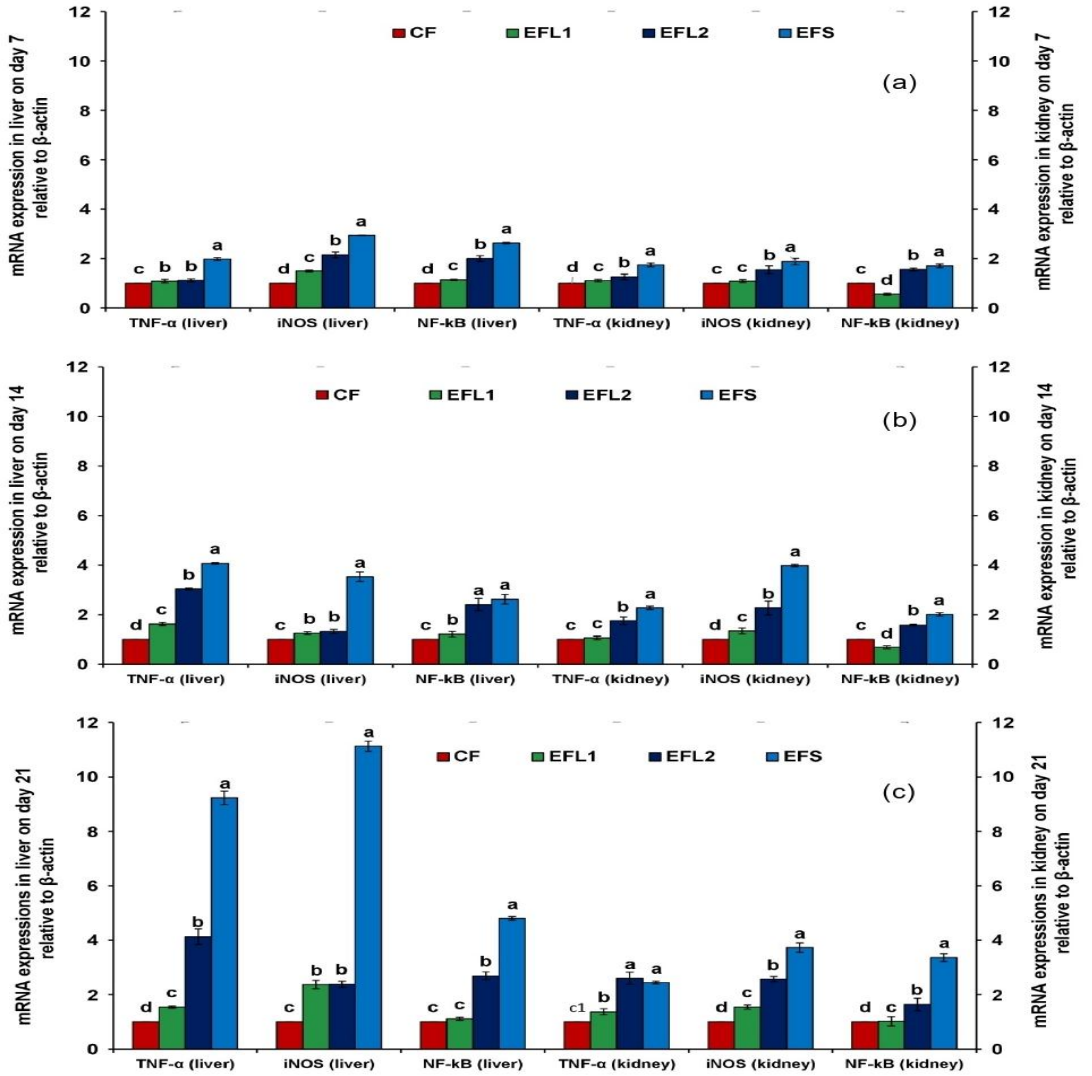

Fig.47. Expressions of *TNF-α*, *iNOS*, *NF-kB* in liver and kidney of four different diets fed *C. batrachus* on (a) day-7, (b) day-14 and (c) day-21 after immunization. The relative expression of the target gene was expressed as fold changes in comparison to the group receiving a control diet after being normalized to the expression of *β-actin* (an internal control). Vertical bars with different superscripts are significantly (*P*<0.05) different (n=3).

4.5. Evaluation of performance of Asian catfish *Clarias batrachus* fed with leaves and seeds of *Achyranthes aspera* supplemented diets and exposed to UV-B radiation in laboratory conditions

Asian walking catfish magur *Clarias batrachus* fry (352.4±8.0 mg) were cultured in aquarium (10 fish/aquarium, 20 l) maintained inside the wet laboratory. Fish were fed with control diet (D1) without any plant ingredients and experimental diets enriched with 0.25% leaves (D2), 0.5% leaves (D3) and 0.5% seeds of *Achyranthes aspera* with other diet ingredients. Fish were fed @ 5% of body weight at 9.00 a.m. and 5.00 p.m. Six replicates were used for each feeding regime. After 83 days of feeding, magur (three replicates/ treatment) were exposed to UV-B irradiation. The other three replicates of the same feeding scheme remained unexposed to UV-B (served as positive control). The dose of UV-B was 157 µW/cm^2 during the exposure.

The fish were exposed for 7 days (15 min/day).

4.5.1. Survival rate, final weight and specific growth rate

The number of fish was recorded in all treatments after UV-B exposure. The survival rate of fish was not affected with UV-B exposure, all fish survived. Among UV-B exposed fish, significantly (P<0.05) higher final weight was found in 0.5% seed incorporated diet (D4) fed magur compared to the other treatments (Fig.48). The final weight was minimum in control diet (D1) fed fish. Specific growth rate (SGR) of magur was maximum and minimum in D4 and D1 treatments, respectively. Similar trend was observed in unexposed fish (Fig.49).

The final weight was higher in unexposed fish than UV-B exposed ones in the respective feeding regime.

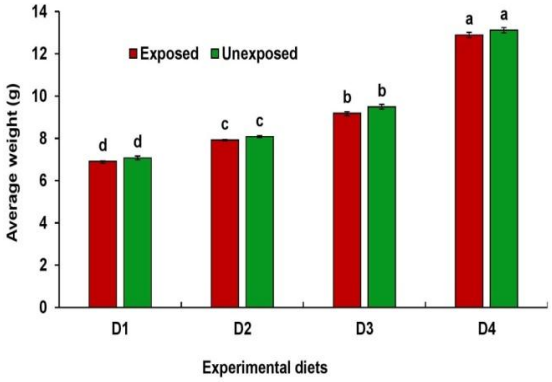

Fig.48. Average weight of *C. batrachus* cultured under different feeding regimes.

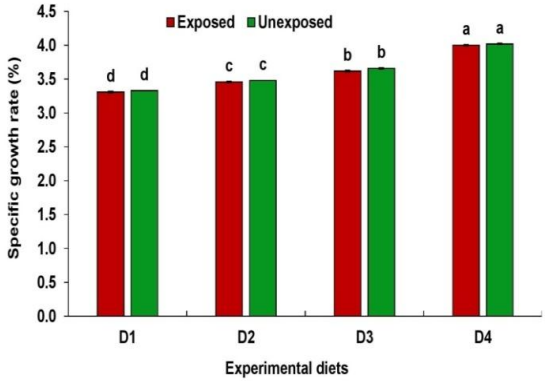

Fig.49. Specific growth rate of *C. batrachus* cultured under different feeding regimes.

4.5.2. Biochemical assays

4.5.2.1. Lysozyme, myeloperoxidase and hemagglutination titer

Among the exposed fish, serum lysozyme level was significantly (P<0.05) higher in 0.5% seed enriched diet (D4) fed magur than others (Fig.50). The 0.5% leaves incorporated diet (D3) fed fish followed D4 diet fed one. Similar trend was also observed with unexposed fish. Significantly (P<0.05) higher myeloperoxidase levels were found in D4 and D3 diets fed exposed fish than other diets fed exposed fish. Among unexposed magur, highest myeloperoxidase level was observed in D4 diet fed fish followed by D3, D2 and D1 treatments (Fig.51). Highest hemagglutination titer level was found in D4 diet fed exposed fish. Similar trend was found in the unexposed one (Fig.52). Myeloperoxidase level was significantly (P<0.05) higher in exposed fish than the unexposed fish in the respective treatment.

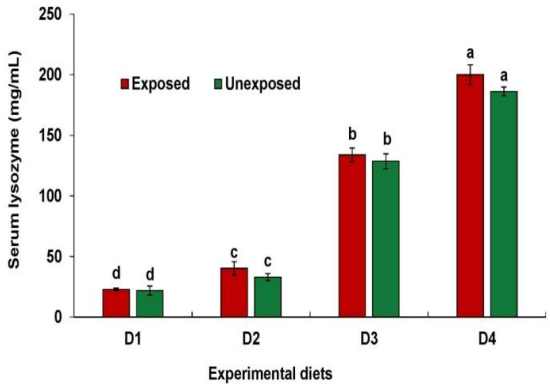

Fig. 50. Serum lysozyme activity found in *C. batrachus* cultured under different feeding regimes.

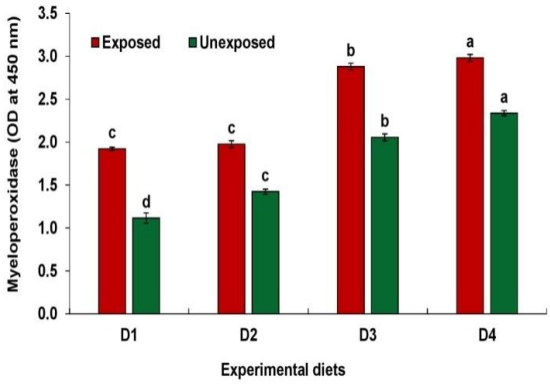

Fig. 51. Myeloperoxidase activity found in *C. batrachus cultured* under different feeding regimes.

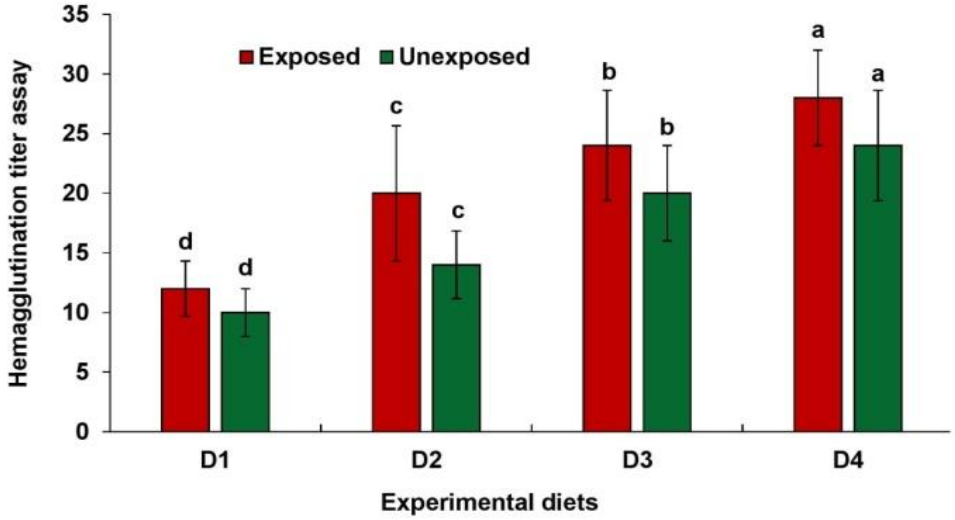

Fig.52. Hemagglutination activity found in *C. batrachus* cultured under different feeding regimes.

4.5.2.2. Nitric oxide synthase, superoxide dismutase, thiobarbituric acid reactive substances and carbonyl protein

In the liver of exposed fish, significantly (P<0.05) higher NOS level was seen in D4 diet fed magur than other diets fed fish (Fig.53). This treatment was followed by D3, D2 and D1. Similar trend was observed with unexposed fish. Significantly (P<0.05) higher superoxide dismutase level was found in liver of

0.5% seeds incorporated diet fed magur than other treatments of both exposed and unexposed groups (Fig.54).

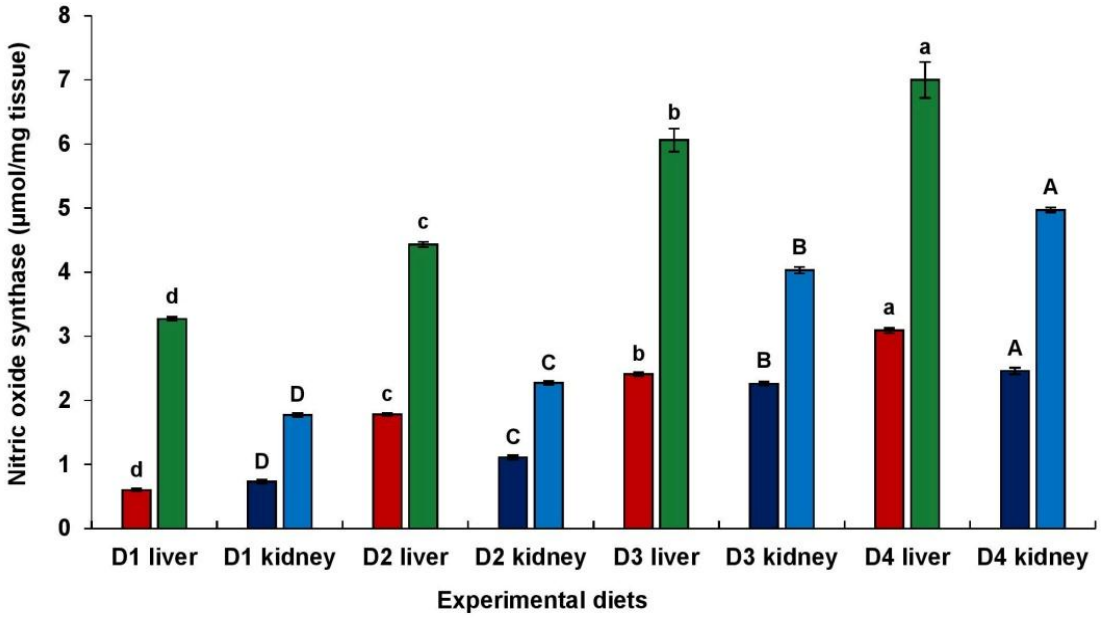

Fig.53. Nitric oxide synthase level found in different diets fed *C. batrachus.*

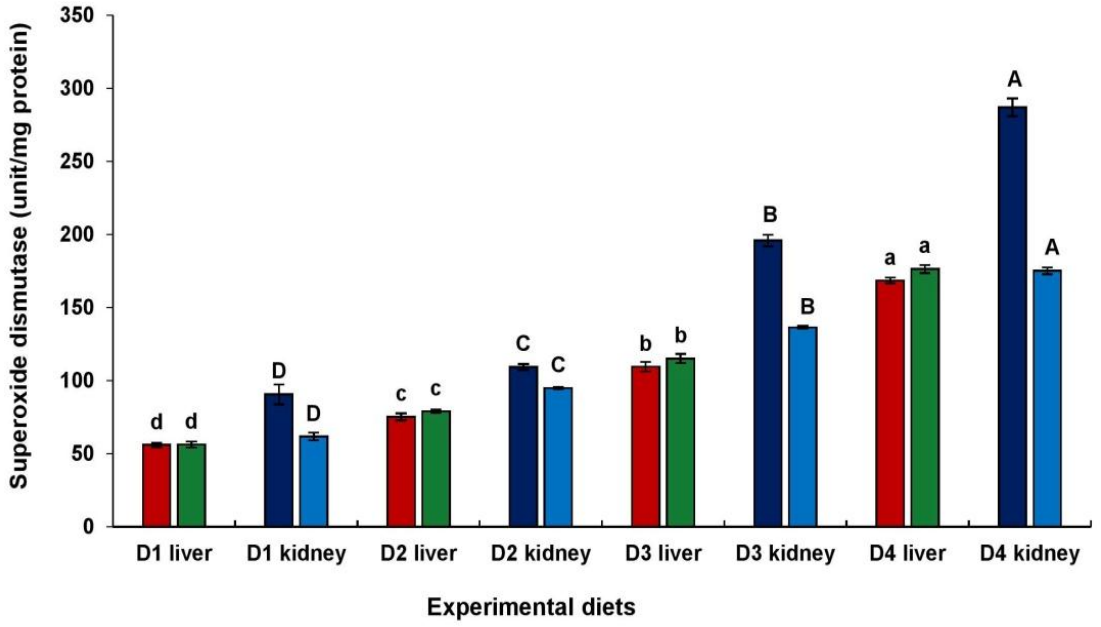

Fig.54. Superoxide dismutase activity found in different diets fed *C. batrachus.*

Significantly (P<0.05) higher TBARS level was found in D1 diet fed magur than experimental diets fed fish. It is applicable for both UV-B exposed and unexposed fish.

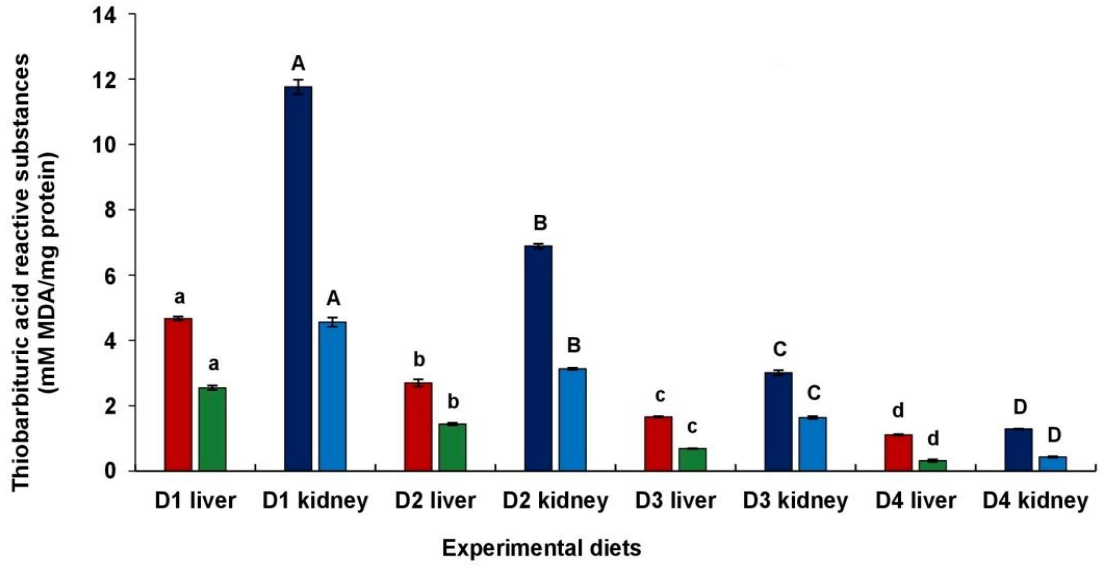

Fig.55. Thiobarbituric acid reactive substances activity found in different diets fed *C. batrachus.*

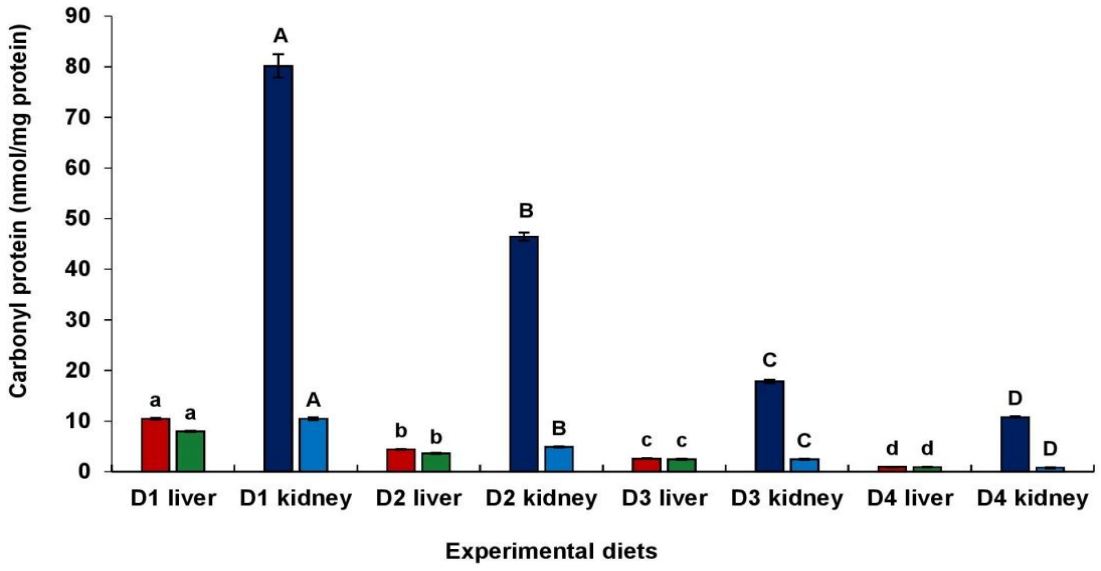

Fig.56. Carbonyl protein found in different diets fed *C. batrachus.*

In head kidney of both exposed and unexposed fish, significantly (P<0.05) higher superoxide dismutase and nitric oxide synthase levels were observed in D4 treatment than others. An inverse relationship was observed between the increasing dose of leaves in diets and TBARS levels in the liver of fish (Fig.55). The carbonyl protein level in liver showed inverse relationship with the inclusion level of leaves in the diets of fish. It was significantly (P<0.05) higher in the D1 diet fed fish than other treatments (Fig.56). Significantly (P<0.05) higher TBARS and carbonyl protein (CP) levels were observed in control diet (D1) fed of UV-B exposed and unexposed fish than the other experimental diets fed ones. An inverse relationship was recorded between the increasing dose of leaves in diets and TBARS and carbonyl protein levels in head kidney of magur.

The independent sample t-test was performed to compare the exposed and unexposed fish. It is interesting to see that NOS level was significantly (P<0.05) higher and TBARS level was significantly (P<0.05) lower in liver of unexposed group than exposed fish in the respective treatment. Carbonyl protein levels in D1 and D2 were significantly (P<0.05) lower in liver of unexposed magur than exposed fish in the respective treatment. There was no significant difference in carbonyl protein level in liver of D3 and D4 diets fed exposed and unexposed fish. In head kidney of unexposed fish, NOS level was significantly (P<0.05) higher and superoxide dismutase, TBARS and carbonyl protein levels were significantly (P<0.05) lower than UV-B exposed fish in the respective treatment.

4.5.3. Relative mRNA expression

In head kidney and liver of both UV-B exposed and unexposed magur, there were up-regulations of *TNF-α*, *iNOS*, *NF-kB, BAX, cytochrome c, SOD-c, caspase 3, caspase 9, BCL-2* in *A. aspera* leaves and seeds incorporated diets fed fish than control diet fed fish. In liver, significantly (P<0.05) higher expressions of *TNF-α*, *NF-kB*, *BAX*, *SOD-c, caspase 3* and *caspase 9* were recorded in 0.5% seeds supplemented diet (D4) fed exposed magur than other diets fed exposed fish (Fig.57). The expressions of *iNOS* and *BCL-2* were significantly (P<0.05) higher in liver of 0.5% leaves (D3) and 0.5% seeds (D4) enriched diets fed exposed fish than other diets fed exposed ones.

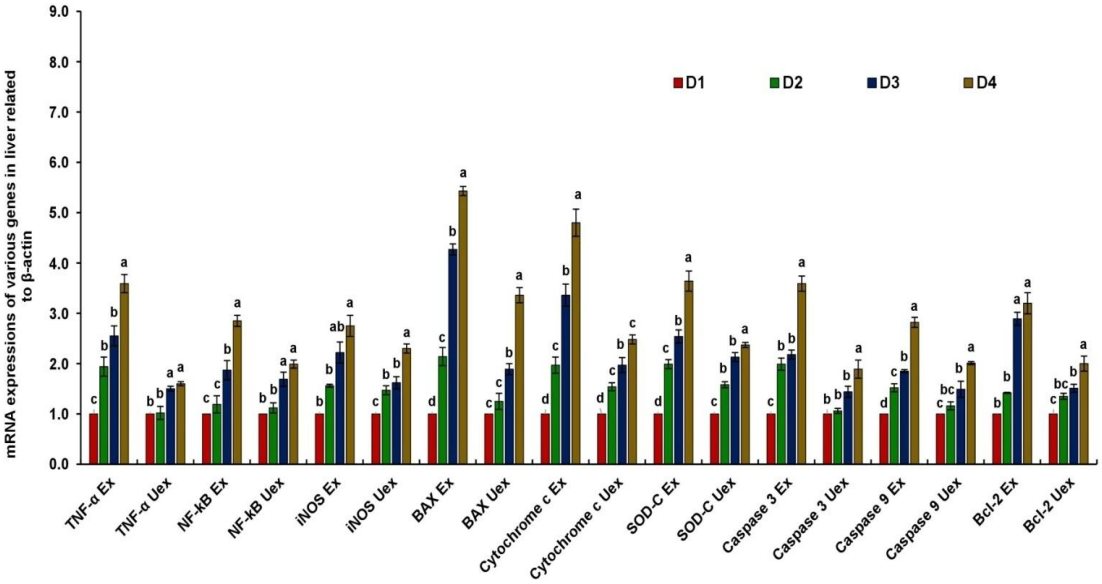

Fig.57. Fold changes in mRNA expression of various genes in liver of *C. batrachus* cultured under different feeding regimes.

In head kidney of UV-B exposed 0.5% seeds supplemented diet fed fish (D4), significantly (P<0.05) higher expressions of *TNF-α*, *NF-kB*, *iNOS, BAX*, *SOD-c, caspase 3*, *caspase 9* and *BCL-2* were recorded compared to other diets fed exposed fish (Fig.58). Among unexposed fish, significantly (P<0.05)

higher expressions of *TNF-α*, *BAX*, *SOD-c, caspase 9* and *BCL-2* were found in D4 compared to other treatments. The independent sample t-test showed that the expressions of all genes in liver (except *NF-kB* in D2 and D3, *iNOS* in D2 and D4, *caspase 9* in D3, *BCL-2* in D2) were significantly (P<0.05) higher in UV-B exposed fish than unexposed ones.

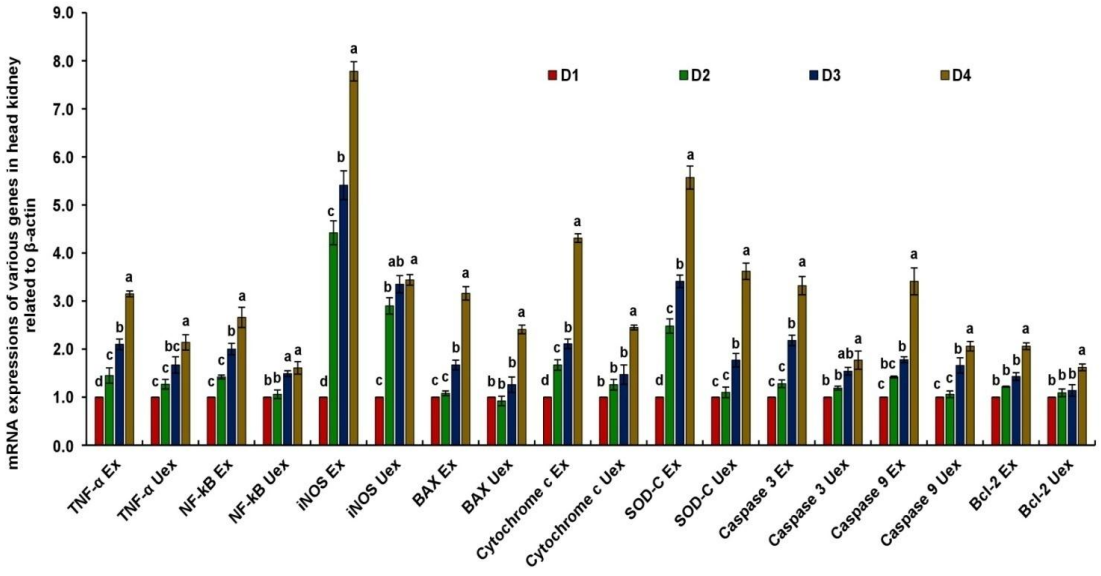

Fig.58. Fold changes in mRNA expression of various genes in head kidney of *C. batrachus* cultured under different feeding regimes.

Similarly in head kidney, significantly (P<0.05) higher expressions were observed in exposed fish (except, *BAX* in D2 and D3, *caspase 3* in D2, *caspase 9* in D3 and *BCL-2* in D2 and D3) compared to the unexposed ones.

Chapter 5

Discussion

5.1. Composition of leaves and seeds of *Achyranthes aspera*

The crude protein, crude lipid and carbohydrate contents were significantly higher in seeds of *Achyranthes aspera* compared to the leaves, whereas maximum ash content was found in leaves. Several studies showed variations in the biochemical composition of leaves of *A. aspera.* In Bangladesh, the proximate composition of leaves of *A. aspera* (wild variety) was assayed. The protein, fat, ash and carbohydrate contents of leaves were 18.13±1.67, 1.88±0.20, 21.43±0.33 and 39.91±1.85 mg/100 g, respectively (Rana *et al.,* 2019). In the present investigation, protein and lipid contents were higher and ash content was lower in nursery grown leaves compared to the wild collection in Bangladesh. The protein, lipids and ash levels were 25.76±3.71, 4.89±0.41 and 3.73±0.04 g/100 g, respectively in raw leaves (Joy *et al*., 2017). The biochemical composition varied depending on the environmental conditions, where the plant grew. Among essential amino acids such as histidine, isoleucine, lysine, methionine and arginine contents were significantly higher in seeds compared to leaves in the present study. Leucine, tryptophan and valine levels were significantly higher in leaves compared to the seeds. All non-essential amino acids (except, alanine and tyrosine) contents were significantly higher in seeds compared to leaves. The phosphoserine and glutamine were present in seeds and absent in the leaves. Whereas, α-amino adipic acid and hydroxylysine were found in leaves and absent in seeds. The highest amount of taurine was found in leaves. The amino acids, leucine, isoleucine, phenylalanine and valine contents of *A. aspera* seeds were comparable to

Bengal gram and methionine and cysteine levels of seeds were greater than those of most pulses (Goyal *et al.,* 2007).

The comparative study of fatty acid profiles of seeds and leaves showed that total saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) were around 2 and 9-fold higher in seeds compared to the leaves. Palmitic acid was the dominant SFA in both seeds and leaves. In seeds, oleic acid content was 11-fold higher compared to the leaves. Higher levels of linoleic acid, the n-6 polyunsaturated fatty acid (n-6 PUFA) and α-linolenic acid, the n-3 polyunsaturated fatty acids (n-3 PUFA) were found in seeds and leaves, respectively. Higher saturated, monounsaturated and n-6 PUFA contents were observed in seeds, whereas, n-3 PUFA level was higher in the leaves. Daulatabad and Ankalgi (1985) recorded the fatty acid composition of seeds. They reported that oleic (22.6%) and linoleic acids (49.4%) were dominant unsaturated fatty acids and palmitic acid (18.6%) was the major constituent of the saturated fatty acids; lauric (0.4%), myristic (1.2%), stearic (4.4%), arachidic (1.6%) and behenic (1.8%) acids were present in small amount in the seeds. The fatty acid profile of seeds of the present investigation is comparable with the previous data. In the present assay, oleic, linoleic and palmitic acids contents were 25.57, 45.87 and 24.44%, respectively. The arachidic and behenic acids were absent in seeds. In the present study, the arachidic acid was found in the leaves.

There were some differences in the vitamin composition between seeds and leaves, like, vitamin D2 and vitamin E were higher in leaves compared to

seeds; vitamins B1 and B12 were higher in seeds. The absence of vitamins B1, B6 and C in leaves was conspicuous. Fatima *et al*. (2014) measured various vitamins in the whole plant collected from the local market. In the whole plant, vitamins B1, B2, B3 and B6 contents were 0.27, 0.28, 0.58 and 0.27 mg/100 g, respectively and vitamin B9 content was 39 µg/100 g. These values were lower compared to the present study. In the present study, 17 minerals (four macro, nine trace and four ultra-trace minerals) were observed in both leaves and seeds. In the whole plant, different minerals, viz. calcium, sodium, potassium, magnesium, iron, copper, zinc etc. were observed (Fatima *et al*., 2014). Total 14 minerals were reported in the leaves of the plant; Ca and K were absent (Joy *et al*., 2017). All macro, trace and ultra-trace minerals contents (except, zinc) were significantly higher in leaves compared to seeds. Zinc content was significantly higher in the latter compared to former one.

5.2. The performance of fish

The role of medicinal plants on the health status of rohu and magur was evaluated in the present study. This is an important area for research in the field of aquaculture. Various feeding trials were carried out in wet laboratory and in pond conditions. Supplementation of *A. aspera* seeds and leaves at various concentrations resulted in increased growth and specific growth rate (SGR) in both fishes. It indicated the beneficial impact of the plant ingredients on fishes. The presence of secondary metabolites in the leaves and seeds (Raju *et al.,* 2022) and essential fatty acids (*viz*. linolenic acid and oleic acid) in the seeds enhanced the growth rate of rohu and magur (Chakrabarti *et al.,* 2012; Sharma *et al.,* 2021). Enhanced growth was found in *Basella alba* leaf

extract supplemented diet fed Nile tilapia *Oreochromis niloticus* (Chakraborty *et al.,* 2015) and in *Coriandrum sativum* extract incorporated diet fed rainbow trout *Oncorhynchus mykiss* (Farsani *et al.,* 2019). The incorporation of Cornelian cherry *Cornus mas* L. fruit extract in the diet of common carp (Ahmadifar *et al.,* 2022) and bitter leaf *Vernonia amygdalina* extract in the diet of Nile tilapia (Dandi *et al.,* 2022) boosted the average weight and SGR.

The feed conversion ratio (FCR) was consistently reduced in enriched diets fed rohu and magur in the present study. It indicated that enriched diets were utilized efficiently by both the fishes. Similarly lower FCR values were also observed in oregano *Origanum heracleoticum* incorporated diet fed channel catfish *Ictalurus punctatus* (Zheng *et al.,* 2009), nutmeg *Myristica fragrans* extract incorporated diet fed common carp and mooseer *Allium hirtifolium* extract supplemented diet fed rainbow trout (Rashidian *et al.,* 2022a a and b).

5.3. Impact of enriched diets on the survival of the fishes challenged with bacteria

The supplementation of plant ingredients (leaves and seeds) enhanced the survival rate of rohu challenged with *Aeromonas hydrophila*. After bacterial infection, mortality rate of rohu was observed for 10 days. The lower mortality rate was recorded in *A. aspera* seeds and leaves enridhed diets fed fish comparison to the control diet fed fish. Seeds supplemented diets fed rohu showed the lowest mortality rate, followed by 0.5% leaves supplemented diets fed fish. Similar results were found with *A. aspera* seeds enriched diets fed rohu fingerlings (challenged with *A. hydrophila*) (Rao *et al.,* 2006) and with coriander

Coriandrum sativum seed extract supplemented diet fed rainbow trout (challenged with *Yersinia ruckeri*) in the laboratory conditions (Farsani *et al.,* 2019).This suggested that herbal compounds boosted the resistance of fish to bacterial infection.

5.4. Impact on immune system of fish

Fish have an intrinsic immune system that serves as a primary protection mechanism (Magnadóttir, 2006). Wide variations in the innate immune parameters like, myeloperoxidase, nitric oxide synthase, serum lysozyme and hemagglutination titer were observed in both rohu and magur fed with the *A. aspera* seeds and leaves supplemented diets and control diet. In the present study, the increased levels of serum lysozyme, myeloperoxidase and nitric oxide synthase in seeds and leaves enriched diets fed fishes demonstrated the immunostimulatory effect of the plant ingredients. Lysozyme is a cationic enzyme and it can lyse both Gram-positive and Gram-negative bacteria. Lysozyme protects the fish from bacterial pathogens (Alexander and Ingram, 1992; Saurabh and Sahoo, 2008). In the present study, serum lysozyme level was always higher in plant ingredients incorporated diets fed rohu and magur. The highest level of lysozyme was observed in 0.5% seeds supplemented diet fed fishes. The use of herbal components and extracts increased lysozyme levels in a variety of fish, *viz*. *A. aspera* seeds incorporated diet in rohu (Rao *et al.,* 2006), hayata leaf *Citrus depressa* meal enriched diet in barramundi *Lates calcarifer* (Shiu *et al.,* 2016), henna *Lawsonia inermis* extract incorporated diet in common carp (Soltanian and Fereidouni, 2016), Assam tea *Camellia sinensis* extract incorporated diet in Nile tilapia (Van Doan *et al.,* 2019) and

water hyacinth *Eichhornia crassipes* leaf extract supplemented diet in rainbow trout (Rufchaei *et al.,* 2020). The bactericidal activity of lysozyme helps to control the infection (Chakrabarti *et al*., 2014; Giri *et al*., 2015b).

Myeloperoxidase (MPO) is an essential enzyme which plays a significant role in destroying microorganisms (Johnston Jr, 1978). The higher level of myeloperoxidase aids in the destruction and expulsion of invading pathogens from the host body (Yano, 1992; Dalmo *et al.,* 1997). Myeloperoxidase activity was increased in *A. a*spera seeds and leaves supplemented diets fed rohu and magur. MPO activity was highest in 0.5% seeds supplemented diet fed fish. MPO activity in leaves supplemented diets fed fish increased in a dose-dependent manner. Myeloperoxidase showed phagocytic, chemotactic and bactericidal actions in fish neutrophils (Roth, 1993). Nile tilapia fed with gotu kola *Centella asiatica* supplemented diets showed higher myeloperoxidase levels compared to the control diet fed fish (Srichaiyo *et al.,* 2020).

This nitric oxide synthase plays significant role in fish defense (Rombout *et al.,* 2005). The inducible nitric oxide synthase (iNOS) catalyses the production of nitric oxide (NO). Lipopolysaccharides were found in the cell walls of Gram-negative bacteria and they trigger an innate and adaptive immunological response that was employed to elicit increased nitric oxide generation (Sarkar *et al.,* 2008). The NOS level was always higher in *A. aspera* seeds and leaves enriched diets fed rohu and magur than control diets fed fishes. Seeds supplemented diet fed rohu and magur showed higher levels of these enzymes and leaves enriched diet fed fishes followed the seed supplemented diet fed

one. Similar result was also found in catla fed with *A. aspera* seeds supplemented diets (Chakrabarti *et al.,* 2014). Enhanced level of nitric oxide was found in herbal medicine *Sophora flavescen*s incorporated diet fed Nile tilapia (Wu *et al.,* 2013) and anthracenedione incorporated diet fed striped dwarf catfish *Mystus vittatus* (Harikrishnan *et al*., 2019). In the present study, nitric oxide synthase level was found in the following order: 0.5% seeds>0.5% leaves>0.25% leaves>control in the UV-B exposed magur. It showed the influence of seeds and leaves supplemented diets in magur. The earlier study showed that supplementation of *A. aspera* seeds at 0.5% level raised the nitric oxide level in UV-B exposed catla and rohu larvae (Singh *et al.,* 2013a; Sharma *et al.,* 2015).

Lysins, agglutinins, precipitins and C-reactive protein (CRP) all aid in the prevention of disease in fish before particular immune responses develops. In the present study, the hemagglutination titer level (antigen-specific antibody) was higher in rohu and magur fed with *A. aspera* seeds and leaves incorporated diets. The highest hemagglutination titer was always reported in 0.5% seeds supplemented diet fed rohu and magur. Similarly, enhanced hemagglutination titer level was found in *A. aspera* seed extract supplemented diet fed common carp (Chakrabarti *et al.*, 2012) and levamisole incorporated diet fed magur (Kumari and Sahoo, 2006b).

5.4.1. Oxidative stress in fish

Carbonyl protein and thiobarbituric acid reactive substances (TBARS) are oxidative products of tissue lipid and protein and their increased amounts

indicate stress in fish (Schieber and Chandel, 2014). Carbonyl groups in higher concentrations suggest tissue injury (Stadtman and Oliver, 1991). Pollutants in the environment caused oxidative stress in fish (Lushchak, 2016). Reactive oxygen species are responsible for lipid peroxidation, which is a well-known process of oxidative damage (Devasena *et al.,* 2001). Lipid peroxidation can be caused by the end products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (Bohr *et al.,* 2004; Liu *et al.,* 2020). In the present study, the thiobarbituric acid reactive substances level was always lower in rohu and magur fed with *A. aspera* seeds and leaves supplemented diets. It indicated that enriched diets played a significance role in reducing oxidative stress in rohu and magur. Similar effect was also observed in aloe vera *Aloe indica* supplemented diet fed GIFT-tilapia *Oreochromis niloticus* (Gabriel *et al.,* 2015) and in banana *Musa acuminata* peel flour incorporated diet fed rohu (Giri *et al.,* 2016).

Carbonyl proteins induction is used as a biomarker of oxidative stress (Parvez and Raisuddin, 2005). The lower carbonyl protein level was always seen in rohu and magur fed with *A. aspera* seeds and leaves incorporated diets than control diet fed fishes. The lowest level of carbonyl protein was observed in 0.5% seeds enriched diet fed fish and 0.5% leaves incorporated diet fed fish followed this treatment. Similarly, reduced level of protein oxidation was found in garlic *Allium sativum* supplemented diet fed common carp (Naeiji *et al.,* 2013) and tea tree *Melaleuca alternifolia* oil incorporated diet fed silver catfish *Rhamdia quelen* (de Freitas Souza *et al.,* 2019). Protein oxidation also affected protein metabolism (Dabrowski and Guderley, 2002). It is clear that

incorporation of *A. aspera* seeds and leaves helped in reducing oxidative stress in rohu and magur.

5.4.2. Anti-oxidant enzymes in fish

The superoxide dismutase (SOD) activity indicates the antioxidant capacity of the animal. In the present study, *A. aspera* seeds and leaves supplemented diets enhanced the SOD level in magur. The antioxidant enzyme activity was maximum in 0.5% seeds supplemented diet fed fish and 0.5% leaves incorporated diet fed fish followed this treatment. It is observed that *A. aspera* seeds and leaves played important role in reducing the stress in fish by enhancing the level of anti-oxidant enzyme. Similarly higher level of SOD was also reported in banana peels flour incorporated diet fed rohu (Giri *et al.,* 2016), gelatinized carbohydrate incorporated diet fed magur (Talukdar *et al.,* 2019) and hydroethanolic extract of *Oliveria decumbens* supplemented diet fed Nile tilapia (Vazirzadeh *et al.,* 2019).

5.4.3. Relative mRNA expression of specific genes

The relative mRNA expression study of different immunological and stress related genes showed the impact of *A. aspera* leaves and seeds supplemented diets in fish. In the present study, the up-regulation and down-regulation of different immunological genes were observed in rohu and magur. The physiological investigation was confirmed by the expression of several immune-related genes. *Lysozyme C, lysozyme G* and the pro-inflammatory gene *TNF-α* expressions in the hepatopancreas were up-regulated in seeds supplemented diet fed rohu, followed by leaves supplemented diet fed fish. Similar result was

also observed in *A. aspera* seeds incorporated diet fed catla (Chakrabarti *et al.,* 2014). The increased expressions of *lysozyme C* and *lysozyme G* were observed in magur and rohu fed with *A. aspera* enriched diets (Singh *et al.,* 2020; Sharma *et al.,* 2021). In the present study, the anti-inflammatory gene *IL-10* was down-regulated in rohu. In rohu fed with guava leaves supplemented diets, *IL-1β* and *TNF-α* were up-regulated in the head-kidney, intestine and hepatopancreas, whereas *IL-10* was down-regulated (Giri *et al.,* 2015b). The feeding of rohu with ginger enriched diet resulted in an increase of *IL-10* (Sukumaran *et al.,* 2016). There was an inverse relationship between the pro-inflammatory and anti-inflammatory cytokine expressions in the anterior kidney and hepatopancreas of rohu fed with *A. aspera* seeds and leaves enriched diets. It indicated that enriched diets were regulating the cytokines responses in fish. Similar results were also reported in banana peels supplemented diet fed rohu (Giri *et al.,* 2016).

In case of magur, all genes were upregulated in both tissues, except *iNOS* and *BCL-2* in liver of the magur. The liver of 0.5% seeds and 0.5% leaves incorporated diets fed fish showed significantly higher expressions of these genes. *TNF-α* is a pro-inflammatory cytokine and the increased expression of *TNF-α* suggests the presence of an inflammatory condition (Secombes *et al.,* 2001). In guava *Psidium guajava* leaves and *A. aspera* seeds enriched diets fed rohu and magur, *TNF-α* expressions were significantly higher (Giri *et al.,* 2015b; Sharma *et al.,* 2021). In zebrafsh *Danio rerio* embryo/larvae exposed to UV-B, *TNF-α* was up-regulated (Banerjee and Leptin, 2014; Aksakal and Ciltas, 2018). In fish, nitric oxide synthase and inducible nitric oxide synthase (iNOS)

are essential immune-regulatory molecules. Feeding of common carp with Chinese foxglove *Rehmannia glutinosa* increased the expression of *iNOS* in tissues *viz*., liver and spleen (Wang *et al.,* 2015). Bisphenol produced oxidative stress in rohu larvae and suppressed the *NF-κB* signalling pathway, resulting in immunosuppression (Faheem *et al.,* 2020). In the present study, *NF-κB* was up-regulated in 0.5% leaves and seeds incorporated diets fed magur compared to the control and 0.25% leaves incorporated diets fed fish.

In normal physiological conditions, apoptosis is an important part of maintaining homeostasis and growth. However, particular habitats and dietary stressors can cause aberrant apoptosis in aquatic species (AnvariFar *et al.,* 2017). In the present study, UV-B irradiation increased the expression of genes like *NF-kB, BCL-2, caspase 3, caspase 9, BAX, and cytochrome c* implicated in apoptosis in magur. In the UV-B unexposed magur, the expressions were lower. In comparison to the control diet fed fish, enhanced expressions were observed in seeds and leaves enriched diets fed fish (particularly at the 0.5% level).

The expression of *caspase 3* was reported in *Enteromyxum scophthalmi* infected turbot *Scophthalmus maximus* (Losada *et al.,* 2014). The apoptotic genes were up-regulated in propiconazole-exposed zebra fish embryos like *BAX, BCL-2* (apoptosis regulator), *casp-3* and *casp-9* were detected (Zhao *et al.,* 2020). In rohu challenged with *A. hydrophila*, an increased level of *iNOS* and *caspase 3* +ve cells were found (Srivastava *et al.,* 2020). In mitochondria-mediated apoptosis signaling, the key initiator *caspase-9* played an important

role (Gao *et al.,* 2013). The expression of *caspase 3, caspase 8* and *caspase 9* was stimulated in common carp after exposure to paraquat (Ma *et al.,* 2018). Teprenone supplementation in the feed of Chinese seabass *Lateolabrax maculatus* protected the fish from hypoxia and oxidative stress-induced apoptosis (Dong *et al.,* 2021). In fish, the expression of pro-apoptotic signaling molecules such as *cytochrome c* (c*yt-c*), *caspase-9* and *caspase-3* were reduced, whereas the expression of *BCL-2* increased. After exposure to the pathogen *A. hydrophila*, the up-regulation of *iNOS*, *SOD-C, TNF-α*, *cytochrome c* and *caspase 9* were observed in seeds and leaves enriched diets fed magur (Sharma *et al.,* 2021).

Chapter 6

Summary and Conclusions

The increasing demand of aquacrops leads to the intensive aquaculture. Intensive aquaculture, however, makes fish more vulnerable to diseases that result into high mortality rate (Dawood *et al.,* 2016). The microbial diseases are responsible for the financial loss to aquaculture industry. The use of antibiotics to treat diseases may have unintended adverse effects. Fish respond to different infectious pathogens through both non-specific and specific immune systems. Indian major carp rohu *Labeo rohita* and Asian catfish magur *Clarias batrachus* are two commercially important cultivable species in India. Disease outbreaks result in financial losses to Indian aquaculture. The current investigation intends to investigate the impact of seeds and leaves of *Achyranthes aspera* enriched diets on the health of rohu *Labeo rohita* and magur *Clarias batrachus*. The health state of fishes was assessed using immunological enzymes, oxidative stress parameters, antioxidant enzymes and relative mRNA expressions of several genes. The experimental information and main findings of the present investigation are as follows.

The rohu *Labeo rohita* and magur *Clarias batrachus* were cultured in wet laboratory and in pond conditions. Fishes were fed with *Achyranthes aspera* leaves (0.25 and 0.5%) and seeds (0.5%) supplemented diets and control diet, without plant ingredients. Fish were either immunized with chicken RBC (c-RBC) or challenged with pathogenic *Aeromonas hydrophila*/ UV-B radiation.

The cumulative mortality rate of fish was monitored for 10 days in fish challenged with bacterial pathogen. The blood and tissue samples were collected for various assays. In immunization study, three sampling were conducted on 7th, 14th and 21st days after immunization of fishes.

Significantly (P<0.05) higher average weight and SGR were observed in enriched diets fed rohu and magur in the present study. The cumulative mortality rate was significantly (P<0.05) lower in the enriched diet fed fish than the control diet fed fish. It was evident that supplementation of leaves and seeds of *A. aspera* to diets enhanced the growth of fish and also gave them resistance to the bacterial infection. The feed conversion ratio (FCR) was always lower in plant incorporated diets fed fishes in comparison to the control group. Fish fed with *A. aspera* leaves and seeds incorporated diets had significantly (P<0.05) higher levels myeloperoxidase, nitric oxide synthase and lysozyme than control diet fed fish. It was evident that plant enriched diets stimulated the immune systems of fishes. Significantly (P<0.05) higher hemagglutination titers was seen in enriched diet fed fish than control diet fed fish. The bioactive substances *viz*., alkaloids, flavonoids, saponins, steroids, terpenoids, fatty acids, amino acids etc. present in leaves and seeds of *A. aspera* stimulated the immune system of fishes. Significantly (P<0.05) lower levels of thiobarbituric acid reactive substances (TBARS) and carbonyl protein were found in enriched diets fed fishes than the control diets fed fishes. Fish fed enriched diets had significantly (P<0.05) higher levels of superoxide dismutase than control diet fed fish. Thus, enriched diets enhanced the antioxidant system of fish.

The physiological investigation was supported by the relative mRNA expressions of several genes, including *lysozyme C*, *lysozyme G*, *TLR-4*, *NF-kB*, *TNF-α*, *IL-1β*, *IL-10*, *iNOS*, *SOD-C*, *caspase 9* and *BAX*, c*ytochrome-c, caspase 3, BCL-2*. The expressions of genes were tissue-specific. The

increased level of serum lysozyme was supported by the up-regulation of *lysozyme C* and *lysozyme G*. Serum lysozyme is an important enzyme of non-specific immune system. The up-regulation of *iNOS* and *SOD-C* in liver and head kidney of magur clearly indicated that the positive effects of *A. aspera* leaves and seeds supplemented diets in fish. *A. aspera* leaves and seeds played a significant role in apoptosis, as evidenced by the up-regulation of *caspase 9*, *BCL-2*, *BAX*, *cytochrome-c*, *TNF-α*, and *caspase 3* genes in the liver and head kidney of enriched diets fed magur. These genes are linked to the extrinsic and intrinsic pathways of apoptosis.

The current study demonstrates that dietary supplementation of medicinal herb *Achyranthes aspera* is a useful tool to avoid stress and control diseases in both carp and catfish. It may helps to reduce/avoid the application of antibiotics in aquaculture. The plant is widely available and therefore, its application is cost-effective.

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List of Publications

Research Publications

1. **Kumar, N.,** Sharma, J. G., Singh, S. P., Singh, A., HariKrishna, V., Chakrabarti, R. (2019). Validation of growth enhancing, immunostimulatory and disease resistance properties of *Achyranthes aspera* in *Labeo rohita* fry in pond conditions. ***Heliyon*** (*Elsevier*) 5 (2): e01246. https://doi.org/10.1016/j.heliyon.2019.e01246 (Impact factor: 3.776)

2. **Kumar, N.,** Sharma, J. G., Mittal, P., Chakrabarti, R. (2022). Effect of leaves and seeds of *Achyranthes aspera* as feed supplements on the immunological and stress parameters and related-gene expressions of Asian catfish (*Clarias batrachus*). ***Veterinary Research Communications*** (*Springer Nature*). https://doi.org/10.1007/s11259-022-09932-5 (Impact factor: 2.816)

3. **Kumar, N.,** Sharma, J. G., Kumar, G., Shrivastav, A. K., Tiwari, N., Begam, A., Chakrabarti, R. (2021). Evaluation of nutritional value of prickly chaff flower (*Achyranthes aspera)* as fish feed ingredient. ***Indian Journal of Animal Sciences*** (*ICAR*) 91 (3): 239-244. http://epubs.icar.org.in/ejournal/index.php/IJAnS/article/view/114152 (Impact factor: 0.500)

4. Sharma, J. G., **Kumar, N.,** Singh, S. P., Singh, A., HariKrishna, V., Chakrabarti, R. (2019). Evaluation of immunostimulatory properties of prickly chaff flower *Achyranthes aspera* in rohu *Labeo rohita* fry in pond conditions. ***Aquaculture*** (*Elsevier*) 505: 183-189. https://doi.org/10.1016/j.aquaculture.2019.02.065 (Impact factor: 5.135)

5. Sharma, J. G., **Kumar, N.,** Mittal, P., Chakrabarti, R. (2022). Evaluation of UV-B protective properties of leaves and seeds of *Achyranthes aspera* in Asian catfish *Clarias batrachus* (Linn.). ***Photochemical & Photobiological Sciences*** (*Springer Nature*). https://doi.org/10.1007/s43630-022-00222-2 (Impact factor: 4.328)

6. Kumar, G., Sharma, J. G., Goswami, R. K., Shrivastav, A. K., Tocher, D. R., **Kumar, N.,** Chakrabarti, R. (2022). Freshwater Macrophytes: Potential Source of Minerals and Fatty Acids for Fish, Poultry and Livestock. ***Frontiers in Nutrition*** (*Frontiers Media S.A.*). 9: 869425. https://doi.org/10.3389/fnut.2022.869425 (Impact factor: 6.590)

7. Kumar, G., Sharma, J. G., Goswami, R. K., Shrivastav, A. K., **Kumar, N.,** Chandra, S., Chakrabarti, R. (2021). The study of effect of vitamin C and *Achyranthes aspera* seeds enriched diets on the growth, biochemical composition, digestive enzyme activities and expressions of genes involved in the biosynthesis of fatty acids in Snow trout *Schizothorax richardsonii* (Gray, 1832). ***Journal of Applied Aquaculture*** (*Taylor & Francis Online*). https://doi.org/10.1080/10454438.2021.1985679

Conferences Presentations

1. **Kumar, N.,** Sharma, J. G., Chakrabarti, R. (2021) Assessment of Immunostimulatory Properties of *Achyranthes Aspera* Seeds and Leaves Supplemented Diets in *Clarias batrachus* Fry. ***International Colloquium on Regulatory Mechanisms underlying Behavior, Physiology and Development*** at Department of Zoology, University of Delhi, Delhi, India.

2. **Kumar, N.,** Singh, A., Sharma, J. G., Chakrabarti, R. (2018) **'**To evaluate the immunostimulatory properties of medicinal plant (*Achyranthes aspera)* supplemented diets fed with rohu, *Labeo rohita* in pond culture conditions' in ***"8th International Science Congress"*** M. M. University, Ambala, Haryana, India.

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Validation of growth enhancing, immunostimulatory and disease resistance properties of *Achyranthes aspera* in *Labeo rohita* fry in pond conditions

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Abstract

The immunostimulatory and disease resistance properties of herb *Achyranthes aspera* L. (Amaranthaceae) were evaluated in rohu *Labeo rohita* in pond. Rohu fry (1.9 ± 0.08 g) were cultured in hapas (25 hapa^{-1}) set inside a pond and were fed with two experimental diets containing 0.5% seeds (D1) and leaves (D2) of *A. aspera* and control diet (D3). Fish were challenged with *Aeromonas hydrophila* after 80 days. The cumulative mortality rate of fish was significantly ($P < 0.05$) higher in D3 (28−48%) compared to others. Average weight was significantly ($P < 0.05$) higher in D1 (6.5−12.5%) compared to other treatments. Myeloperoxidase and nitric oxide synthase levels were significantly ($P < 0.05$) higher in D1 and D2 compared to D3. Thiobarbituric acid reactive substances and carbonyl protein levels were significantly ($P < 0.05$) lower in hepatopancreas and kidney of D1 compared to others. In hepatopancreas, the expressions of lysozyme C, loysozyme G, TNF-α, IL-10 and IL-1β were

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significantly ($P < 0.05$) higher in D1 compared to others. This treatment was followed by D2. In kidney, lysozyme G and TNF-α levels were significantly ($P < 0.05$) higher in D1 and D2 compared to D3. Whereas, IL-10 and IL-1β were significantly ($P < 0.05$) down-regulated and up-regulated, respectively in kidney of D2. There was up-regulation ($P < 0.05$) of TLR-4 in hepatopancreas and kidney of D1 and D2 diets fed rohu, respectively compared to others.

Keywords: Immunology, Zoology

1. Introduction

The application of herbal immunostimulants has been increasing rapidly to control the disease in aquaculture [1]. Several plant ingredients show promising results viz. miers, *Tinospora cordifolia* leaf extract [2], mango, *Magnifera indica* kernel [3], *Solanum trilobatum* leaf [4], green tea [5], guava *Psidium guajava* leaves [6, 7], ginger *Zingiber officinale* [8]. All these studies are performed in the control laboratory conditions. Validation of these studies is required in the ponds, the most important source for aquaculture production in Asian countries. *Achyranthes aspera* L., a member of the Family Amaranthaceae is commonly found as weed in various parts of India. The bushy herb can be grown in the field regularly. Roots and seeds of *A. aspera* showed immunostimulatory and disease resistance properties in fishes in the laboratory conditions. The supplementation of *A. aspera* in diets stimulates specific and non-specific immune systems of *Catla catla* (catla), *Cyprinus carpio* (common carp) and *Labeo rohita* (rohu) [9, 10, 11, 12, 13]. The supplementation of seeds of *A. aspera* in diet also enhances the growth of fish. Even it protects the early larvae of carps from harmful UV-B irradiation [14, 15, 16, 17, 18].

The study of biochemical composition of seeds unveils the reason for beneficial effect of *A. aspera* on carps. Two glycosides of oleanolic acid, saponin A and B are present in the alcohol extract of seeds [19]. Chakrabarti et al. [20] reported the occurrence of ecdysterone and two essential fatty acids linolenic acid and oleic acid in the seeds. Ecdysterone is associated with the increased protein synthesis in skeletal muscle [21]. The amino acid profile study shows that leucine, isoleucine, phenylalanine and valine contents of seeds are equivalent to Bengal gram, whereas, sulphur amino acids methionine and cystine contents are higher compared to most pulses [22]. The long-chain polyunsaturated fatty acid (PUFA) plays important role in many physiological functions. So far, all experiments with *A. aspera* are conducted in laboratory conditions. The validation of laboratory study is most essential in the field conditions. Among various parts of the plant, only immunostimulatory properties of roots and seeds have been evaluated; the efficiency of leaves yet to be evaluated.

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The innate immune system is the baseline defence system of fish [23]. Myeloperoxidase and nitric oxide synthase reflect the status of the immune system of the species. The elevated levels of these parameters are indicators of healthy immune system of fish. The pattern recognition molecule, the toll-like receptor (TLR) is associated with certain activities of the innate immune system viz. production of cytokines, differentiation of cells, production of reactive nitrogen and oxidative radicals [24, 25]. Cytokines play an important role in the immune system by binding to specific receptors and setting off a cascade of events leading to induction, enhancement or inhibition of a number of cytokines-regulated genes [26]. Interleukin-1β is one of the pivotal early response pro-inflammatory cytokines that enables organisms to respond to any infection, inducing an inflammatory cascade, along with other defensive responses [23, 27]. TNF-α is associated with recruitment and activation of phagocytes [28]. The expression patterns of immune related genes were studied in rohu infected with *Edwardsiella tarda* [29] and *Aeromonas hydrophila* [10, 30, 31]. The information on the expression of immune related genes in fish fed with enriched diets and challenged with bacteria in pond conditions is lacking. The aim of the present study is to evaluate the immunostimulatory and disease resistance properties of seeds and leaves of *Achyranthes aspera* in *Labeo rohita* challenged with *Aeromonas hydrophila* in the pond conditions.

2. Materials and methods

2.1. Culture of fish and challenge with pathogen

Indian major carp *L. rohita* fry were procured from a fish farm, acclimated for 7 days and then fry (1.9 ± 0.08 g) were introduced in hapas (25 hapa^{-1}) set inside the pond (54.5 m × 30.5 m × 2.25 m) of Rohtak Centre, Central Institute of Fisheries Education (Indian Council of Agricultural Research), Haryana. Each hapa (2.0 m × 1.5 m × 1.5 m) was made of nylon net; the top of the hapa was covered with a net to avoid the escape of fish. Hapas were set inside the pond with bamboo stick. Rohu fry were fed with two experimental diets containing plant ingredients and control diet (Table 1). The experimental diets were prepared with 0.5% seeds (diet 1, D1) and leaves (diet 2, D2) of *A. aspera* with other ingredients; diet without plant ingredient served as control (diet 3, D3). *A. aspera* was grown in the outdoor facility; leaves and ripe seeds were collected regularly, cleaned, dried, ground and kept in the refrigerator for further use. Three replicates were used for each feeding regime. Food was given once daily (9.00 a. m.) at the rate of 5% of body weight. Major water quality parameters like temperature, pH, dissolved oxygen and conductivity were recorded regularly using HQ40d Multiparameter (Hach, USA) from four sides of the pond closer to the hapas. Water temperature and pH ranged from 28.5 to 31 °C and

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Table 1. Composition of experimental and control diets.

Ingredients	Diets		
	0.5% Seeds (D1)	0.5% Leaves (D2)	Control (D3)
Composition (g kg^{-1})			
Fish powder	482.76	482.76	482.76
Wheat flour	488.24	488.24	493.24
Cod liver oil	20.00	20.00	20.00
Vitamin & mineral pre-mix	4.00	4.00	4.00
Leaves	-	5.00	-
Seeds	5.00	-	-
Proximate composition (g 100 g^{-1})			
Crude protein	36.71	35.67	33.58
Crude fat	8.31	7.82	8.64
Carbohydrates	37.52	38.36	43.45
Ash	8.03	8.12	7.20
Moisture	9.43	10.03	7.14
Crude fibre	0.44	0.65	0.41
Energy (kcal 100 g^{-1})	371.7	366.5	385.8

8.4 to 9.16, respectively throughout the culture period. Dissolved oxygen level ranged from 7.11 to 9.62 mg L^{-1} in the pond. After 80 days of initial feeding, fish were anesthetized with MS 222 (Sigma, USA) and injected intraperitoneally (100 μL) with live *Aeromonas hydrophila* (5×10^6 CFU mL^{-1}). A group of fish injected with buffer served as sham control. All fish were introduced in the respective hapa and mortality of fish was recorded for 10 days at 12 h interval. The whole study was conducted following the guidelines of Institutional Animal Ethics Committee, IAEC (approved by Committee for the Purpose of Control and Supervision of Experiments on Animals, CPCSEA) (Reference: DU/ZOOL/IAEC-R/2015/08). The study was conducted following all regulations.

2.2. Collection of samples

After 10 days of challenge test, fish were anesthetized with MS 222 (Sigma), blood sample was drawn from the caudal vein of each fish using Dispo van 2 mL single use syringe (0.55 × 25 mm/24 × 1) and transferred in serological tube. Blood samples were allowed to clot at 4 °C overnight. The serum was then spun down at 400 × g for 10 min. Then the serum was stored in sterile tube at −20 °C until used for assays. Three replicates were used for each feeding regime. Hepatopancreas and head kidney were collected aseptically and were preserved at −80 °C for biochemical assays and gene expression study.

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2.3. Myeloperoxidase and nitric oxide synthase assays

Myeloperoxidase activity was measured following the standard method [32]. First 10 μL serum was taken in each well of microplate, then 90 μL of Hank's balanced salt solution (without Ca^{2+} or Mg^{2+}), 35 μL of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (Genei, India) and 5 mM H_2O_2 were added to each well of microplate. The mixture was incubated for 2 min and 35 μL of 4 M sulphuric acid was added to stop the reaction. The activity was recorded at 450 nm.

Nitric oxide synthase levels of tissues (hepatopancreas and head kidney) were recorded [33]. In 1 mL phosphate buffer (pH 7.4), 100 mg tissue was homogenized; centrifuged at 10000× g for 20 min at 4 °C. The supernatant, 100 μL was mixed with 100 μL Griess reagent and incubated 10 min at room temperature. The activity was recorded at 540 nm. The nitrite concentration was measured with the nitrite standard curve and expressed as mmol mg tissue^{-1}.

2.4. Oxidation of tissue lipids and proteins

Thiobarbituric acid reactive substances (TBARS) indicate the oxidation of tissue lipids (hepatopancreas/head kidney). In 9 mL of KCl (1.15%), 1 g tissue was homogenized. Then the sample was incubated at 100 °C for 1 h in acid medium containing 0.45% sodium dodecyl sulphate (SDS) and 0.6% thiobarbituric acid [34]. The sample was centrifuged at 800 x g at 4 °C for 15 min. The standard was prepared with 1, 1, 3, 3-tetramethoxy propane. The activity was measured at 532 nm and expressed as mmol MDA mg protein^{-1}.

Carbonyl protein is an indicator of tissue protein oxidation. A 100 mg tissue (hepatopancreas/head kidney) was homogenized in 1 mL of potassium phosphate buffer (50 mM, pH 7.0) containing 0.5 mM ethylenediaminetetraacetic acid and 100 μM of phenylmethlysulfonyl fluoride [35]. The homogenate (250 μL) was mixed with 0.5 ml of 10% TCA, centrifuged at 13000× g for 5 min and the pellet was used for the assay. The pellet was mixed with 10 mM dinitrophenyl hydrazine (1 mL, DNPH) dissolved in 2 M HCl. It was incubated 1 h at room temperature, centrifuged at 13000 x g for 5 min; the pellet was collected and washed thrice with 1 mL of ethanol-butylacetate (1:1, v/v). Then it was dissolved in 1.5 mL of 6 M guanidine hydrochloride, centrifuged at 13000× g for 5 min and the supernatant was collected. The optical density of the supernatant was measured at 370 nm. The result was expressed as nmol mg protein^{-1}. The molar extinction coefficient was 22 × 10^3 M^{-1} cm^{-1}. Total tissue protein was measured at 750 nm [36].

2.5. Cumulative mortality rate of fish

The mortality of rohu in each hapa was recorded at 12 h interval after the challenge with *A. hydrophila*. The number of fish was recorded and expressed in per cent with

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respect to the initial one. Finally, it was expressed as cumulative mortality rate of rohu.

2.6. Gene expression study

2.6.1. Total RNA isolation and cDNA synthesis

The hepatopancreas and head kidney (100 mg) of rohu were processed separately in TRIzol reagent (Ambion, Life Technologies, USA) for the extraction of total RNA following the protocol of manufacturer. In Synergy H1 Hybrid microplate reader (Biotek, USA), the concentration and purity of RNA was checked at 260 and 280 nm, using Take 3 plate. Then integrity of extracted RNA was checked in 1% agarose gel. Total RNA (1 μg), was treated with 1U of DNase I (Sigma-Aldrich, USA) to avoid any contamination of DNA. High capacity cDNA reverse transcription kit (Applied Biosystems, USA) with RNase inhibitor was used for the reverse transcription of DNase-treated RNA following the manufacturer's protocol. The product was stored at −20 °C for further gene expression study.

2.6.2. Quantitative real-time PCR analysis

The quantitative real-time PCR (qRT-PCR) was performed to amplify immune relevant target genes like, lysozyme G, lysozyme C, TNF-α, IL-10, IL-1β, TLR-4 and housekeeping gene, β-actin using QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). MicroAmp optical 96-well reaction plate (0.1 mL) was used for amplifications. Primers for different genes were either self-designed or collected from earlier published data (Table 2). The cDNA of all the treatments were diluted (1:1) in nuclease-free water. Amplifications were carried out with 10 μL reaction volume and that composed of 1.0 μL of diluted cDNA, 0.25 μL of Forward and Reverse primers (2.5 μM each), 5 μL of Universal 2X PowerUp SYBR Green Master Mix (Applied Biosystems), 3.5 μL of nuclease-free water. Amplifications were performed in duplicate wells under following conditions: initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec, annealing at 55 °C (for lysozyme G, lysozyme C, TNF-α) and 60 °C (for IL-10, IL-1β, TLR-4, β-actin), and extension at 72 °C for 1 min. The reaction carried out without cDNA template was a negative control. The qPCR specificity was verified with melt curve analysed at a 95 °C for 15 sec (1.6 °C sec^{-1}), 60 °C for 1 min (1.6 °C sec^{-1}) and 95 °C for 15 sec (0.15 °C sec^{-1}). The PCR efficiencies were determined by analysis of serial dilutions of cDNA. The efficiencies close to 100% allowed the application of $2^{-\Delta\Delta CT}$ method for calculation of relative gene expression of the target gene with that of reference gene β-actin [37]. The specificity of the amplification product was verified in 1% agarose gel.

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Table 2. Target genes and sequences of primers used for qPCR analysis.

Target gene	Primer	Primer sequence (5′-3′)	Accession number/reference
Lysozyme C	Lyso C Fw Lyso C Rv	CGATGATGGCACTCCAGGT CATGCTTTCAGTCCTTCGGC	EF203085.1
Lysozyme G	Lyso G Fw Lyso G Rv	CAATGGCTTTGGCCTCATGC CACGTGGGAAACTTTGCTCTG	KC934746.1
TNF-α	TNF-α Fw TNF-α Rv	GGCGGCTTGAAAGTAGTGGA TATGCAGAACGTCGTGGTCC	FN543477.1
IL-10	IL-10 Fw IL-10 Rv	GCTCAGTGCAGAAGAGTCGAC CCCGCTTGAGATCCTGAAATATA	[49]
IL-1β	IL-1β Fw IL-1β Rv	GTACCCCACAAAACATCGGC CAAGAGCAGTTTGGGCAAGG	AM932525.1
TLR-4	TLR-4 Fw TLR-4 Rv	CTAAGAAAGTGCTTGGGCTTCAT GGTTTGTGGCAATAATGGCTTTC	KX218428.1
β-actin	β-actin Fw β-actin Rv	GACTTCGAGCAGGAGATGG CAAGAAGGATGGCTGGAACA	[29]

2.7. Statistical analysis

All data are given as Mean ± SE. One-way analysis of variance and Duncan's multiple range test [38] were used to find out the difference among various treatments. Statistical significance was accepted at $P < 0.05$ level.

3. Results

3.1. Cumulative mortality of rohu

Rohu fry were fed with test diets containing 0.5% seeds (D1) and leaves (D2) of *A. aspera* and control diet (D3) for initial 80 days and then challenged with *A. hydrophila*. After bacterial challenge, the mortality of fish was recorded for 10 days at 12 h interval. First mortality was recorded in D2 diet fed rohu within 12 h of challenge with bacterial pathogen. The mortality rates were 2, 4 and 10% in D1, D2 and D3 diets fed groups, respectively after 24 h of challenge. In control diet fed group, 50% fish died within five days of challenge. The cumulative mortality rate was significantly ($P < 0.05$) higher in control diet fed rohu compared to the seeds and leaves supplemented diets fed fish (Fig. 1). The cumulative mortality rates were 28, 48 and 76% in D1, D2 and D3 diets fed rohu, respectively.

3.2. Average weight

Plant supplemented diet showed a positive impact on the growth of rohu fry. The average weight was significantly ($P < 0.05$) higher in rohu fed with 0.5% seeds supplemented diet (D1) compared to other two feeding regimes. The average weight of

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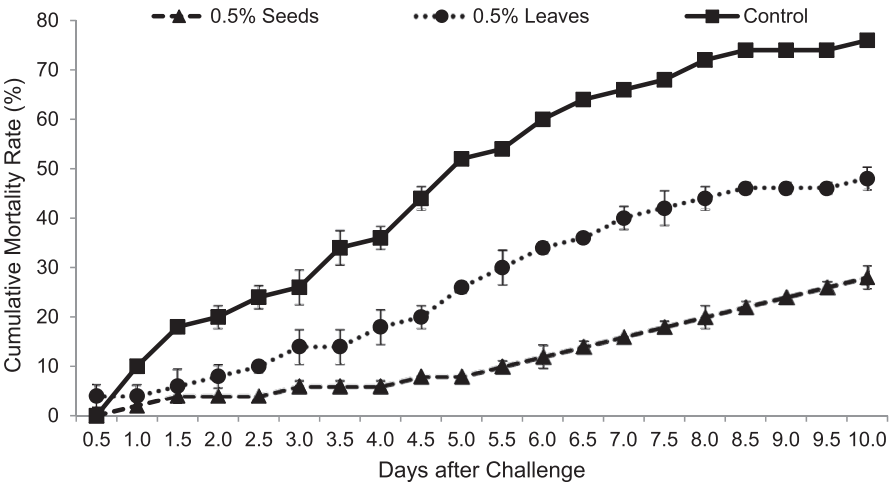

Fig. 1. Cumulative mortality of *L. rohita* fed with three different diets and challenged with *A. Hydrophila*.

D1 diet fed rohu was 6.5 and 12.5% higher compared to the D2 and D3 diets fed fish, respectively (Table 3).

3.3. Myeloperoxidase and nitric oxide synthase assays

Myeloperoxidase activity was significantly ($P < 0.05$) higher in D1 and D2 diets fed rohu compared to the control diet fed fish. There was no significant ($P > 0.05$) difference in myeloperoxidase activity between the former two groups. Nitric oxide synthase levels in hepatopancreas and kidney were significantly ($P < 0.05$) higher in D1 diet fed rohu compared to others. The level was 5−7 folds higher in hepatopancreas compared to the kidney of rohu fed with same diet (Table 4).

3.4. Oxidation of tissue lipids and proteins

TBARS levels were significantly ($P < 0.05$) lower in hepatopancreas and kidney of D1 diet fed rohu compared to others. Similarly, carbonyl protein level was significantly ($P < 0.05$) lower in D1 diet fed rohu compared to others. Highest TBARS and carbonyl protein levels were found in D3 diet fed rohu. The levels

Table 3. Initial and final average weights of *L. rohita* fed with three different diets and challenged with *A. hydrophila*. Mean ± SE (25 fish hapa^{-1}, 3 hapas treatment^{-1}; 25 × 3 = 75 in each treatment) sharing different letters in the same column are significantly ($P < 0.05$) different.

Parameters	(D1) 0.5% Seeds	(D2) 0.5% Leaves	(D3) Control
Initial weight (g)	1.9 ± 0.08a	1.9 ± 0.08a	1.9 ± 0.08a
Average weight (g)	25 ± 0.40a	23.50 ± 0.22b	22.22 ± 0.11c

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Table 4. Myeloperoxidase, nitric oxide synthase, thiobarbituric acid reactive substances (TBARS) and carbonyl protein levels found in *L. rohita* fed with three different diets and challenged with *A. hydrophila*. Mean ± SE (6 fish hapa^{-1}, 3 hapas treatment^{-1}; 6 × 3 = 18 in each treatment) sharing different letters in the same column are significantly ($P < 0.05$) different.

Parameters	0.5% Seeds (D1)	0.5% Leaves (D2)	Control (D3)
Serum			
Myeloperoxidase (O.D. at 450 nm)	0.92 ± 0.04a	0.91 ± 0.02a	0.62 ± 0.03b
Hepatopancreas			
Nitric oxide synthase (μmol mg tissue^{-1})	20.82 ± 0.14a	15.25 ± 0.26b	15.36 ± 0.20b
TBARS (mmol MDA mg protein^{-1})	0.98 ± 0.02c	1.32 ± 0.04b	2.94 ± 0.03a
Carbonyl protein (nmol mg protein^{-1})	7.80 ± 0.04c	8.56 ± 3.16b	16.71 ± 5.05a
Head kidney			
Nitric oxide synthase (μmol mg tissue^{-1})	4.30 ± 0.02a	2.74 ± 0.02b	1.78 ± 0.02c
TBARS (mmol MDA mg protein^{-1})	0.75 ± 0.01c	1.97 ± 0.05b	3.25 ± 0.05a
Carbonyl protein (nmol mg protein^{-1})	5.48 ± 0.19c	7.78 ± 0.27b	17.99 ± 0.28a

were higher in hepatopancreas compared to the kidney of rohu cultured in the same feeding regime (Table 4).

3.5. Gene expression

Expression of various immune-related genes supported the physiological study. In hepatopancreas of seeds (D1) supplemented diet fed rohu, the expressions of lysozyme C and G were significantly ($P < 0.05$) higher compared to others. This treatment was followed by D2 diet fed rohu and minimum level was found in control diet (D3) fed fish (Fig. 2). The expression of lysozyme C was higher compared to lysozyme G in the hepatopancreas of rohu cultured in the same feeding regime. Lysozyme G levels were significantly ($P < 0.05$) higher in kidney of rohu fed with D1 and D2 diets compared to the D3 diet fed fish (Fig. 3). There was no change in lysozyme C level in plant supplemented diets fed rohu compared to the control diet fed fish. In hepatopancreas of D1 diet fed rohu, the expression of TNF-α was significantly ($P < 0.05$) higher compared to others. This treatment was followed by D2 and D3 diets fed rohu (Fig. 4). In kidney, the expression of TNF-α was significantly ($P < 0.05$) higher in D1 and D2 diets fed rohu compared to D3. The expression of TNF-α was 2−6 folds higher in hepatopancreas compared to kidney of rohu cultured in same feeding regime. There was up-regulation ($P < 0.05$) of IL-10 in hepatopancreas of rohu fed with D1 diet compared to others (Fig. 5). This treatment was followed by D2 diet fed rohu. In kidney, IL-10 was up-regulated ($P < 0.05$) only in D1 diet fed rohu and it was down-regulated in D2 diet fed fish compared to the control diet fed fish. In hepatopancreas of D1 diet fed rohu, IL-1β was up-regulated ($P < 0.05$) compared to others (Fig. 6). Whereas, in kidney significantly ($P < 0.05$) higher

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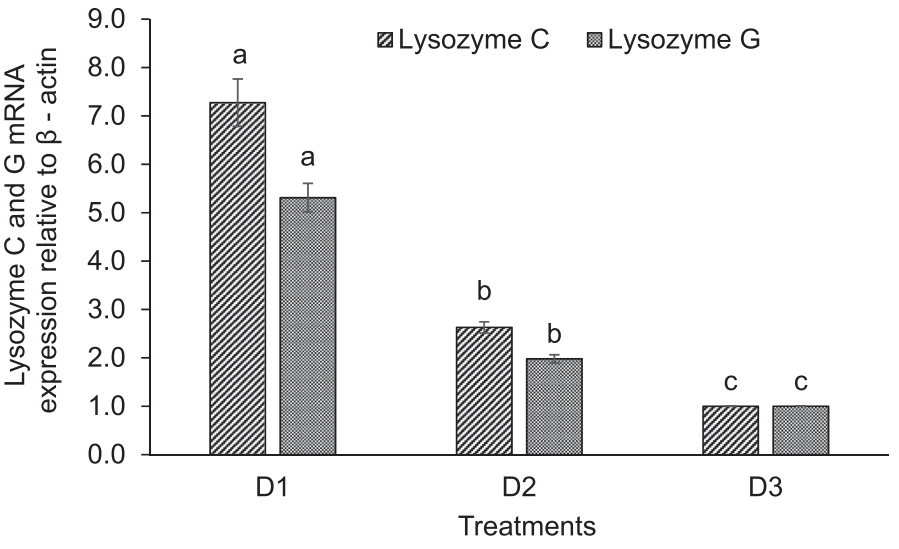

Fig. 2. Expression of lysozyme C and lysozyme G in hepatopancreas of *L. rohita* fed with three different diets and challenged with *A. hydrophila*. The relative expression of the target gene lysozyme C/G was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($P < 0.05$) different (2 fish hapa^{-1}, 3 hapas treatment^{-1}; $2 \times 3 = 6$).

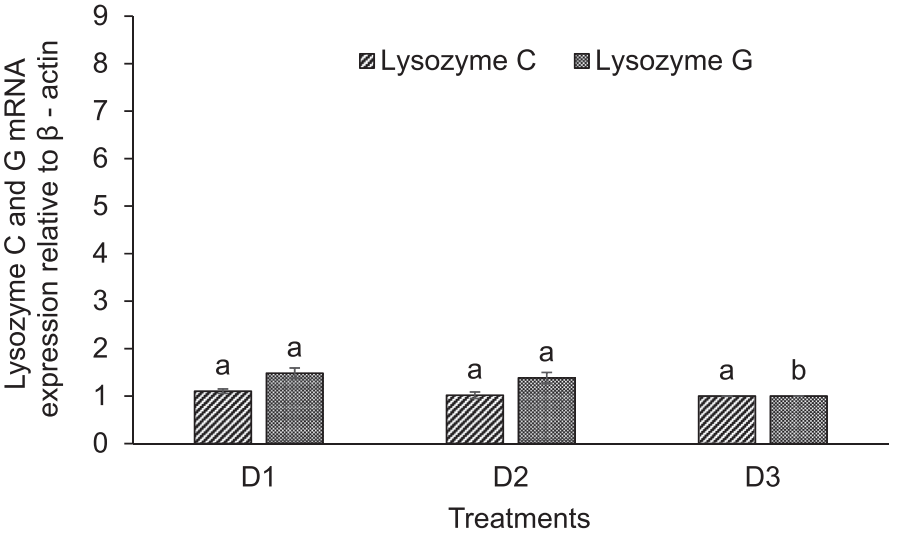

Fig. 3. Expression of lysozyme C and lysozyme G in kidney of *L. rohita* fed with three different diets and challenged with *A. hydrophila*. The relative expression of the target gene lysozyme C/G was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($P < 0.05$) different (2 fish hapa^{-1}, 3 hapas treatment^{-1}; $2 \times 3 = 6$).

expression was found in D2 diet fed fish compared to others. Similar trend was also found with TLR-4. There was up-regulation ($P < 0.05$) of TLR-4 in hepatopancreas and kidney of rohu fed with D1 and D2 diets, respectively compared to D3 diet fed fish (Fig. 7).

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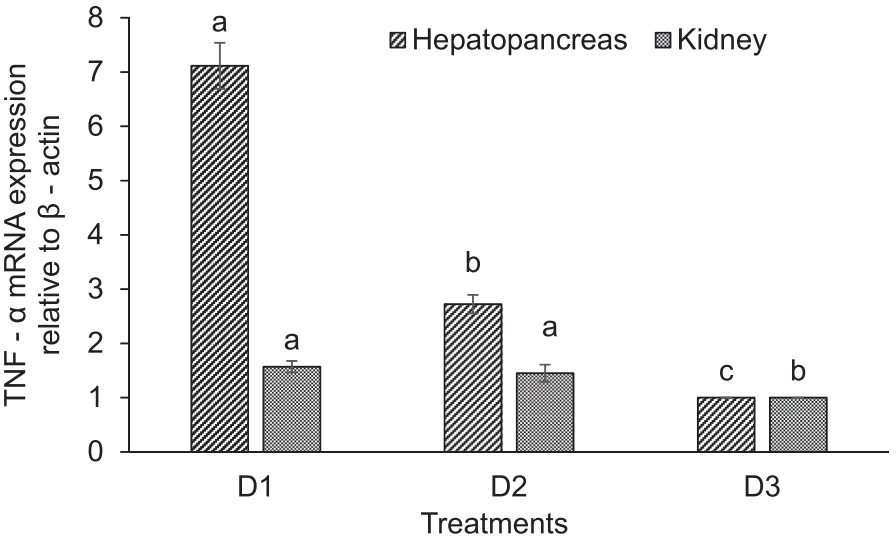

Fig. 4. Expression of TNF-α in hepatopancreas and kidney of *L. rohita* fed with three different diets and challenged with *A. hydrophila*. The relative expression of the target gene TNF-α was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($P < 0.05$) different (2 fish hapa^{-1}, 3 hapas treatment^{-1}; $2 \times 3 = 6$).

4. Discussion

The feeding of rohu fry with *A. aspera* seeds and leaves enriched diets showed very positive effect even in the pond conditions. The mortality rate was highest in the control diet fed fish. The cumulative mortality rate of fish was 28–48% reduced in

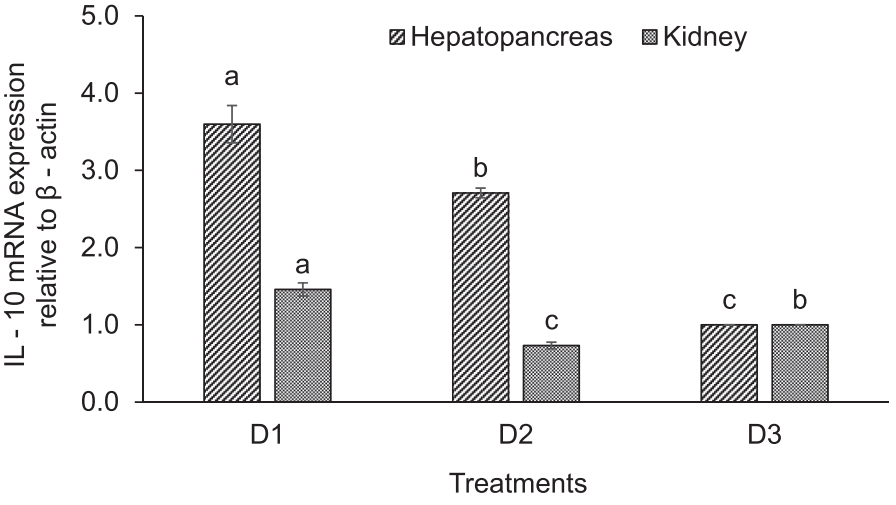

Fig. 5. Expression of IL-10 in hepatopancreas and kidney of *L. rohita* fed with three different diets and challenged with *A. hydrophila*. The relative expression of the target gene IL-10 was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($P < 0.05$) different (2 fish hapa^{-1}, 3 hapas treatment^{-1}; $2 \times 3 = 6$).

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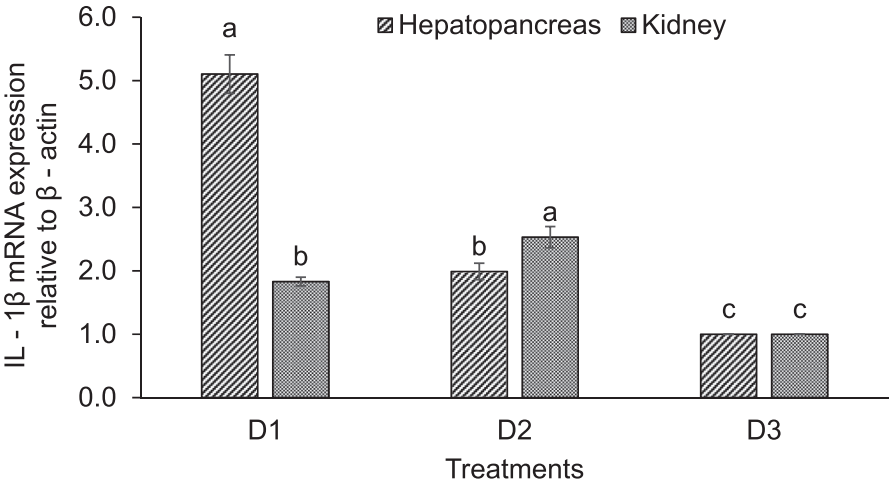

Fig. 6. Expression of IL-1β in hepatopancreas and kidney of *L. rohita* fed with three different diets and challenged with *A. hydrophila*. The relative expression of the target gene IL-1β was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($P < 0.05$) different (2 fish hapa^{-1}, 3 hapas treatment^{-1}; $2 \times 3 = 6$).

enriched diet fed rohu. Like earlier laboratory based study, best result was obtained in the seeds supplemented diets fed rohu compared to the other feeding regimes. This is the first report showing immunostimulatory and disease resistance properties of leaves of *A. aspera*. In laboratory experiments, early larvae and fingerlings of rohu were fed with seeds supplemented diets and then challenged with *A.*

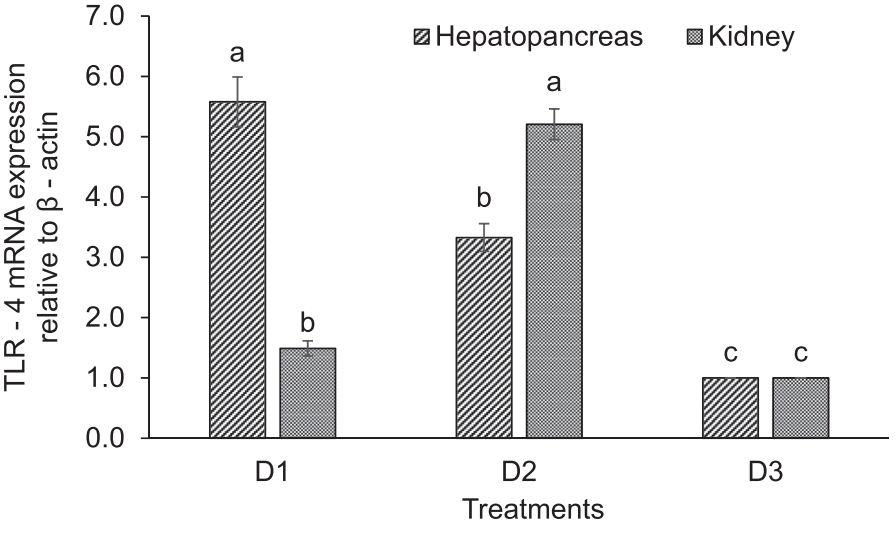

Fig. 7. Expression of TLR-4 in hepatopancreas and kidney of *L. rohita* fed with three different diets and challenged with *A. hydrophila*. The relative expression of the target gene TLR-4 was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($P < 0.05$) different (2 fish hapa^{-1}, 3 hapas treatment^{-1}; $2 \times 3 = 6$).

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hydrophila. Significantly lower mortality was found in seeds supplemented diets fed rohu compared to the control one [10, 30]. This is clear from the study that *A. aspera* provides protection against bacterial pathogen to all age groups of rohu in control laboratory conditions as well as in natural pond.

Like earlier study conducted in the laboratory conditions, growth stimulatory property of plant supplemented diet was observed in rohu fry cultured in pond. The average weight was 6.5−12.5% higher in rohu fed with 0.5% seeds supplemented diet compared to other two feeding regimes in the present study. Enhanced growth of fish was also found in leaves supplemented diet fed fish compared to the control one in the pond study. Giri et al. [6] found that supplementation of leaves of other plant, the guava leaves at 0.5% level enhanced the growth of rohu compared to the control diet fed fish. The growth of fish is an indicator of health status that influences the other physiological conditions. In a laboratory study, common carp larvae fed with 0.5% seed supplemented diet showed higher average weight compared to the fish fed with control diet [20]. Enhanced growth and survival are the basic requirements of successful aquaculture. In the present study, these two primary requisites are achieved in the pond conditions. The study of immunological and oxidative stress parameters and gene expression supported these two basic observations.

Higher levels of myeloperoxidase and nitric oxide synthase showed the efficient immune system of rohu in the present study. This pond study confirmed the earlier laboratory experiments. Enrichment of diets with plant ingredients improved the efficiency of the immune system. Seeds are superior to leaves. A tissue-specific physiological activity was recorded in the present study as nitric oxide synthase level was always higher in hepatopancreas compared to the kidney of rohu cultured in the same feeding regime. Myeloperoxidase and nitric oxide synthase are indicators of the immunological status of the fish. Myeloperoxidase shows antimicrobial activity. It occurs abundantly in neutrophil granulocytes; elevated level of myeloperoxidase helps in the destruction and elimination of invading pathogens from the host body [39, 40]. Nitric oxide synthase catalyzes the production of cellular signalling molecule nitric oxide that plays vital role in defence mechanism of fish [41]. In the present study, the elevated levels of myeloperoxidase and nitric oxide synthase indicated the improved defence system of rohu fed with plant supplemented diets. Supplementation of seeds of *A. aspera* enhanced the nitric oxide synthase level in catla [42]. The presence of long-chain polyunsaturated fatty acids such as linolenic and oleic acids in the seeds [20] may be associated with the immunostimulatory properties of rohu challenged with bacterial pathogen in the present study.

Feeding of fish with enriched diets stimulated the immune system and as well as reduced the stress in fish [14, 24, 26]. This was evident from the lower levels of TBARS and carbonyl protein in hepatopancreas and kidney of enriched diets fed fish compared to the control fish in the present study. The study also showed that

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hepatopancreas was more sensitive to stress compared to kidney as TBARS and carbonyl protein levels were highest in the former regardless of feeding regime. Seeds showed better performance in stress reduction compared to the leaves; still the latter was better compared to the control treatment. Elevated levels of TBARS and carbonyl protein are indicators of oxidation of tissue lipid and protein, respectively. Lipid peroxidation is a well-established mechanism of oxidative damage caused by reactive oxygen species [43]. Lipid peroxidation is the process of oxidative degradation of PUFA and its occurrence in biological membranes causes impaired membrane function, structural integrity and inactivation of several membrane-bound enzymes [44]. Lipid peroxidation may bring about protein damage by its end products, MDA and 4-hydroxynonenal [45]. Fish challenged with bacterial pathogen were prone to oxidation of lipid and protein. Dietary supplementation of plant ingredients reduced oxidative stress in rohu in the present study.

In the present study, lysozyme C, lysozyme G, TNF-α, IL-10, IL-1β and TLR-4 were up-regulated in hepatopancreas of enriched diets fed rohu compared to the control one after challenged with *A. hydrophila*. The expressions were higher in seeds supplemented diet fed rohu compared to the fish fed with leaves supplemented diet. In kidney of same rohu, most of these genes were up-regulated compared to the control diet fed fish, except lysozyme C and IL-10. IL-10 was down-regulated in leaves supplemented diet fed rohu compared to the control diet fed fish. In kidney, lysozyme G, TNF-α, IL-10 expressions were higher in the seeds supplemented diet fed rohu compared to the leaves supplemented diet fed fish, whereas, expressions of IL-1β and TLR-4 showed the opposite trend. The feeding of rohu fingerlings with other plant ingredient, ginger (root of the plant) showed up-regulation of IL-10, transforming growth factor-beta (TGF-β) in head kidney, intestine and hepatopancreas [8]. A significant difference was found in the expression pattern of IL-1β, TNF-α, lysozyme C and lysozyme G in *E. tarda* infected and un-infected rohu [29]. In rohu challenged with *A. hydrophila*, a significant up-regulation of IL-10 and down-regulation of IL-1β and TNF-α was reported in treated group compared to control [30]. This showed the response of these genes in presence of pathogen. In rohu fed with other plant leaves, the guava leaves supplemented diets, there were up-regulations of IL-1β, TNF-α in head-kidney, intestine and hepatopancreas, whereas, IL-10 was down-regulated [6]. Significantly higher expressions of lysozyme, TNF-α and IL-1β were observed in common carp fed with jujube *Ziziphus jujube* [1], ferula *Ferula assafoetida* [46] and loquat *Eriobotrya japonica* [47]. In another study, common carp fed with guava leaf powder enriched diets showed significant up-regulation of IL-1β while there was no change in the expression of TNF-α [7]. The expressions of NOD 1 and TLR-22 were higher in liver compared to kidney [48]. Tissue-specific expressions of various genes were reported in catla [42]. Similar results were also found in the pond experiment with rohu as the

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expressions of various genes were higher in hepatopancreas compared to kidney of fish cultured in the same feeding regime.

5. Conclusions

In conclusions, dietary supplementation of *A. aspera* seeds and leaves enhanced the growth of rohu, induced the immune system, reduced oxidative damage of tissue and protected fish from bacterial pathogen. There were up-regulations of most of the genes in enriched diets fed rohu compared to the control group. Seeds showed better performance compared to the leaves. The bioactive compounds present in seeds are beneficial for the fish health. This plant ingredient has immense prospect in the production of healthy fish in ponds.

Declarations

Author contribution statement

Rina Chakrabarti: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Neelesh Kumar: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jaigopal Sharma: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Samar Pal Singh: Performed the experiments; Analyzed and interpreted the data.

Amarjeet Singh, V Harikrishna: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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ORIGINAL ARTICLE

Effect of leaves and seeds of *Achyranthes aspera* as feed supplements on the immunological and stress parameters and related gene expressions of Asian catfish (*Clarias batrachus*)

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Abstract
The immunostimularory properties of *Achyranthes aspera* leaves and seeds supplemented feeds were evaluated in Asian catfish, *Clarias batrachus* (0.352±0.008 g). The experimental feeds contained 0.25% leaves (EFL1), 0.5% leaves (EFL2), 0.5% seeds (EFS) and 0% leaves or seeds (control feed; CF). In CF, leaves and seeds were absent. Fish were immunized with chicken-RBC after 60 days of feeding. The blood and tissue samples were collected on 7th, 14th and 21st days after immunization for various assays. The average weight of magur was significantly higher in EFS compared to the other treatments throughout the study period and EFL2 followed this group. Serum lysozyme level of fish was significantly higher in EFS on 7th and 14th days and in EFS and EFL2 on 21st day after immunization compared to the other treatments. Myeloperoxidase and nitric oxide synthase levels were always significantly higher in EFS diet fed fish compared to other treatments. The highest hemagglutination titer level was found in EFS throughout the study period. Thiobarbituric acid reactive substances (TBARS) and carbonyl protein levels were significantly lower in liver and kidney of enriched diets fed magur compared to CF treatment. TBARS and carbonyl protein levels were minimum in EFS diets fed fish. In EFS and EFL2 treatments, the expressions of *TNF-α*, *iNOS* and *NF-kB* were significantly higher compared to the CF group. *A. aspera* seeds and leaves showed significant immunostimulatory properties in Asian catfish fry.

Keywords *Achyranthes aspera* · *Clarias batrachus* · Lysozyme · Myeloperoxidase · *TNF-α* · *iNOS* and *NF-kB*

Introduction

The occurrence of infectious diseases has been affecting the aquaculture industry (Iwashita et al. 2015). In intensive aquaculture, the stressful condition weakened the immune system of fish and makes the organisms more susceptible to pathogen (Cerezuela et al. 2012; Giri et al. 2014). Therefore, it is most essential to improve the immunity of fish, so that the organism is able to overcome any stressful conditions prevailed in the aquatic environment. The non-specific and specific immune systems assist the fish to encounter pathogenic infection (Kumari and Sahoo 2005; Eslamloo et al. 2012). The non-specific immune response acts as a first line of defense; it represents a wide range of immune responses (Dalmo et al. 1997). Immunostimulants are a group of natural and synthetic compounds that increase the non-specific cellular and humoral defense responses. The boosting of immune system through medicinal plants is an eco-friendly and cost-effective approach. These plants are used as both chemotherapeutics and feed additives (Dawood et al. 2018). The immunostimulatory property of the plant is dose-dependent and the route of administration (*viz.*, injection, immersion, oral etc.) also plays important role (Awad and Awaad 2017). The supplementation of plant-based immunostimulant in diets improves the immune system of fish (Sharma et al. 2019, 2021).

Nitric oxide synthases (NOSs) are a family of enzymes responsible for catalyzing the synthesis of cellular signaling molecule nitric oxide (NO) from L-arginine. The inducible form (iNOS) is expressed in macrophages, Kupffer

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cells, neutrophils, fibroblasts vascular smooth muscle and endothelial cells in response to pathogens (Eddy 2005). NO is a key mediator of innate (non-specific) host defense mechanism and involved in a variety of responses to infection and tissue damage. In addition, NOS activates cyclic guanosine monophosphate (cGMP), which induces mitochondrial KATP channel opening (Mattila and Thomas 2014; Maksin-Matveev et al. 2015). The tumor necrosis factor-α (*TNF-α*) is a proinflammatory cytokine and also one of the early immune genes which is expressed at early stage of infection in fish. It has an important role in regulation of inflammation. *TNF-α* triggers the expression of various immune genes (*IL-1β, IL-8, COX-2, IL-17C* etc.) which participate in inflammation (Rashmeei et al. 2020; Shekarabi et al. 2021). The *NF-kB* pathway was also activated due to stimulatory effects of *TNF-α* (Zhang et al. 2012; Hong et al. 2013). The TLR signaling pathway via activation of *NF-kB* gene, induces proinflammatory cytokines such as interleukin, type I interferon molecules, tumor necrosis factor molecules that provide direct defense response as well as alert adaptive immune cells against pathogens (Iwasaki and Medzhitov 2004; Kawai and Akira 2010). The study of some immunological parameters *viz*., lysozyme, myeloperoxidase, nitric oxide synthase and expression patterns of immune-related genes help to understand the capacity of fish to overcome the environmental stress and diseases (Kumar et al. 2019; Sharma et al. 2022).

Achyranthes aspera L. (Amaranthaceae), prickly chaff flower is commonly found as a weed in India (Saini et al. 2016) and in different tropical countries. Various parts of the plant are used for the treatment of human diseases like, inflammation, diabetes, hypertension, pneumonia, diarrhea, dysentery, asthma etc. (Baraik et al. 2014). The immunostimulatory and disease resistance properties of seeds and leaves of *A. aspera* have been evaluated in carps like rohu *Labeo rohita*, catla *Catla catla*, and in common carp *Cyprinus carpio* (Rao and Chakrabarti 2005; Rao et al. 2006; Chakrabarti and Srivastava 2012; Chakrabarti et al. 2012; Sharma et al. 2019; Singh et al. 2019).

Asian catfish *Clarias batrachus* (Linn.), popularly called magur is a bottom dwelling, carnivore, freshwater fish. It belongs to the family Clariidae (Roy et al. 2019). It is one of the most widespread catfish genera in the world (Thomas et al. 2013). The fish is marketed in living condition; it has high nutritional value and consumer preference (Paul et al. 2015). *C. batrachus* has been considered as critically endangered due to its habitat destruction and over-exploitation (Binoy 2010). Many studies show the outbreak of diseases in magur, like ulcerative disease, furunculosis and fungal infection (Thomas et al. 2013; Chattopadhyay et al. 1992; Chauhan 2012; Sarkar and Rashid 2012). Therefore, it is essential to improve the immunity of this economically important fish using natural immunostimulants like, leaves and seeds of *A. aspera*. There is scarcity of literature in the field of evaluation of immunostimulation of Asian catfish using herbal ingredients.

The present investigation aims to study the effect of feeding of *Achyranthes aspera* leaves and seeds enriched diets in strengthening the immune system and reduction of oxidative stress (with special reference to lipid and protein oxidations) in Asian catfish *Clarias batrachus*.

Materials and methods

Preparation of feed, culture of fish and immunization

The feeds used for the study were, experimental feed containing 0.25% leaves (EFL1), experimental feed containing 0.5% leaves (EFL2), experimental feed containing 0.5% seeds (EFS) of *Achyranthes aspera* and control feed containing 0% leaves/seeds (CF). *A. aspera* plants were grown in the outdoor facility. Leaves and ripe seeds were collected, cleaned and ground. The powder of leaves and seeds were added in the experimental feeds. The dry fish powder, wheat flour, cod liver oil and vitamin-mineral premix were other ingredients used for the formulation of both control and experimental feeds (Table 1). The doses of seeds and leaves of *A. aspera* were selected based on the earlier study (Rao et al. 2006; Chakrabarti and Srivastava 2012; Singh et al. 2020).

Clarias batrachus were obtained from a fish farm and randomly distributed in twelve tanks at a stocking density of 12 fish/tank (60 L). Fish were fed with control feed prior to the start of the experiment (Kumar et al. 2019). After 7 days of acclimation, fish were looking healthy and were accepting food properly; then the feeding trial started. The fry (0.352 ± 0.008 g) were fed with experimental and control feeds. Three replicates were used for each feeding scheme (36 fish/treatment). A separate group of fish was fed with control feed and they served as sham control during immunization. The feeding rate was 5% of the body weight of the fish. The excess feed was siphoned after 1 h of feeding, oven dried and measured. The guidelines of Animal Ethics Committee, Department of Zoology, University of Delhi (DU/ZOOL/IAEC-R/2015/08) was followed for the culture of fish and sampling.

The chicken blood was collected in Alsever's solution (1:3) and stored at 4°C for 24 h. The settled chicken (c-RBC) was washed thrice with phosphate buffer saline (1X PBS, pH 7.4). Then 80 mL of 1X PBS (pH 7.4) was mixed with 20 mL of c-RBC. This solution (20%, v/v) was used for the immunization of fish. After 60 days of feeding, the fish increased in size. Fish from all culture tanks were collected and anaesthetized with tricaine methanesulfonate (MS222,

Table 1 Formulation of fish diets and evaluation of their proximate composition

Ingredient (g/kg diet)	Diets[1]			
	CF	EFL1	EFL2	EFS
Feed composition				
Fishmeal	651.6	651.6	651.6	651.6
Wheat flour	334.4	331.9	329.4	329.4
Cod liver oil	10.0	10.0	10.0	10.0
[2]Vitamin and mineral complex	4.0	4.0	4.0	4.0
Leaves powder	-	2.5	5.00	-
Seeds powder	-	-	-	5.0
Proximate composition (g/100 g on dry matter basis)				
Moisture	7.14	11.09	10.03	9.43
Crude protein	36.96	37.05	36.98	37.02
Crude fat	8.64	9.24	7.82	8.31
Total carbohydrates	40.06	34.47	37.05	37.21
Ash	7.20	8.15	8.12	8.03
Crude fibre	4.1	6.8	6.5	4.4
[3]Energy (kcal/100 g)	385.84	369.24	366.50	371.71

[1]CF = Control feed, EFL1 = 0.25% leaves supplemented feed, EFL2 = 0.5% leaves supplemented feed,

EFS = 0.5% seeds supplemented feed

[2]Supradyan multivitamin tablets with minerals and trace elements contains (as mg/kg in diets): = Vitamin A (as acetate) 12; Cholecalciferol 0.1; Thiamine mononitrate, 40; Riboflavine 40; Pyridoxine hydrochloride, 12; Cyanocobalamin, 0.06; Nicotinamide, 400; Calcium pantothenate 65.20; Ascorbic acid 600; α-Tocopheryl acetate, 100; Biotin, 1.00. Minerals: Tribasic calcium phosphate, 516; Magnesium oxide, 240; Dried ferrous sulphate, 128.16; Manganse sulphate monohydrate 8.12; Total phosphorus, 103.20. Trace elements: Copper sulphate pentahydrate 13.56; Zinc sulphate, 8.80; Sodium molybdate dihydrate, 1.00; Sodium borate 3.52

[3]Energy (kcal/kg) = [(Crude protein g/kg × 4) + (Crude lipid g/kg × 9) + (Total Carbohydrate g/kg × 4)]

Sigma, USA) at the concentration of 25 mg/L of water (Carter et al. 2011). Then the weight of individual fish was measured and fish were immunized (the day of immunization, 0 day). The fish was injected intraperitoneally with chicken-RBC (50 µL/fish) using 26G × ½ syringe (0.45 × 13 mm). In earlier study, c-RBC was used for the immunization of fish (Rao et al. 2004) and same protocol was followed in the present study. Five control diet fed fish were injected with phosphate buffer saline (PBS) to check the effect of handling stress and they served as the sham control. Fish were introduced into the respective tank after immunization. Then magur were collected on 7th, 14th and 21st days after immunization. Twelve fish per treatment were sampled on each day. Water temperature (25.6 - 26.5°C), pH (7.5 - 7.67) and dissolved oxygen levels (5.10 - 6.25 mg/L) of culture tanks were recorded at weekly interval using HACH (USA) water quality analyzer. The duration of the study was 81 days.

The weight of all fish (36 fish/treatment) was measured on the day of immunization (0 day) and on 7th, 14th and 21st days (12 fish/treatment) after immunization using electronic balance (Shimadzu, AUX220, Japan).

Specific growth rate (SGR) of fish was calculated using the formula:

$$\text{SGR}\ (\%) = \frac{(\text{In final weight of fish} - \text{In initial weight of fish})}{\text{Duration of experiment}} \times 100$$

Feed conversion ratio (FCR) was calculated using the formula:

$$\text{FCR} = \frac{\text{Dry weight of food consumed by individual fish during experiment}}{\text{Wet weight gain of individual fish}}$$

Collection of samples and assays

On 7th day after immunization, fish were collected (12 fish/treatment), anaesthetized with MS222 and samples were collected for various assays. The blood sample was collected from the caudal vein of individual fish using sterilized syringe (1 mL) fitted with 26G × ½ "(0.45 × 13 mm) needle (Rao et al. 2004). The syringe and needle were rinsed with 2.7% ethylenediaminetetraacetic acid (EDTA) solution. The blood samples were allowed to clot in a sterilized microcentrifuge tube (1.5 mL) and stored in a refrigerator (4°C) overnight. After 12 h sample was centrifuged at 400 × *g* for 10 min at 4°C and serum was collected in sterile tube and stored at -20°C for the assay of various immunological enzymes. The liver and kidney were collected from each treatment (4 fish/treatment) and stored at -80°C for various enzymatic assays. Similarly, tissues of 4 fish per treatment were kept in TRIzol® reagent (Thermo Scientific, USA) for gene expression study. Like 7th day, samples were also collected on 14th and 21st days of immunization from each tank.

The Microplate Reader (Synergy H1 Hybrid Reader, BioTek, USA) was used for the assay of various enzymes. The serum lysozyme was measured using *Micrococcus lysodeikticus* (20 mg/100 mL sodium acetate buffer 0.02M., pH 5.5) solution (Siwicki 1989). Serum (10 µL) was mixed with 100 µL of solution and the initial absorbance was recorded at 450 nm. The final absorbance was measured after 1 h incubation. The lysozyme level was presented as mg/mL.

Myeloperoxidase levels of fish in various culture conditions were measured (Quade and Roth 1997). In each well of a microplate, 10 µL serum, 90 µL Hank's balanced salt solution and 35 µL of TMB/H_2O_2 were added, properly mixed and allow to incubate. After 2 min of incubation, 35 µL of H_2SO_4 was added to stop the reaction. Hanks' Balanced Salt Solution (HBSS) was used as blank. The standard curve

was prepared with 1,3,5-trinitrobenzene (TNB). The enzyme activity was estimated at 450 nm.

The hemagglutination assay was performed to measure the antigen-specific antibody response (Singh 1983; Das et al. 2000). Chicken blood (c-RBC) was collected in Alsever's solution (1:3) and stored at 4°C for 24 h. Chicken-RBC was washed thrice with PBS (pH 7.4) and centrifuged, then mixed in the PBS to make 2% (v/v) solution. A 50 µL serum was serially diluted with PBS in a 96-well round bottom ELISA plate. A 50 µL of c-RBC (2%) was added to each well, incubated for 60 min at room temperature (25°C), and then overnight at 4°C. The reciprocal of the highest dilution that represented agglutination was considered as the hemagglutination antibody titer.

The nitric oxide synthase levels of liver and kidney of magur was assayed (Lee et al. 2003). Tissue (100 mg) was homogenized in 1x PBS (pH 7.4) and centrifuged at 10,000 × *g* at 4°C for 15 min. The supernatant (100 µL) was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance was recorded at 540 nm. Distilled water was used as blank. The standard curve was prepared with sodium nitrite. The concentration of nitric oxide was expressed in µM/ mg tissue.

The measurement of thiobarbituric acid reactive substances (TBARS) indicates the tissue lipid oxidation. The liver/kidney was homogenized in KCl solution (1 g in 900 µL). The assay mixture was composed of the following: 25 µL homogenate, sodium dodecyl sulphate (25 µL), acetic acid (187.5 µL), thiobarbituric acid (187.5 µL) and distilled water (75 µL). After 1 h of incubation in water bath (95°C), the sample was brought into room temperature and a mixture of distilled water (125 µL) and *n*-butanol-pyridine (625 µL) was added to the sample. The whole reaction mixture was shaken vigorously and centrifuged. Then TBARS level (µmol malondialdehyde/mg tissue) of the sample was measured (532 nm). The standard curve was prepared with 1, 1, 3, 3-tetramethoxy propane (Ohkawa et al. 1979).

The carbonyl protein levels in liver and kidney were assayed (Lenz et al. 1989). The tissue (100 mg) was homogenized in 1 mL of 50 mmol potassium phosphate buffer (containing ethylenediaminetetraacetic acid and phenylmethylsulfonyl fluoride). The homogenate (250 µL) was mixed with 0.5 mL of trichloro acetic acid (10%) and centrifuged at 13000 x *g* for 5 min. The supernatant was removed and the pellet was mixed with 1 mL of 10 mM dinitrophenyl hydrazine. The mixture was kept at room temperature for 1 h. and centrifuged at 13000 x *g* for 5 min. The pellet was washed thrice with 1 mL of ethanol-butylacetate (1/1, v/v). The pellet was completely dissolved with 6 M guanidine hydrochloride and again centrifuged at 13000 x *g* for 5 min. The absorbance of supernatant was recorded at 370 nm. The carbonyl protein was expressed as nmol/mg protein. The molar extinction coefficient was 22×10^3/M/cm. Total protein content was estimated (750 nm) using bovine serum albumin as standard (Lowry et al. 1951). Three replicates were used for each assay.

Relative mRNA expression study

The liver and kidney of magur were kept in TRIzol reagent (Invitrogen, ThermoFisher Scientific, USA) at 4°C for the study of relative mRNA expressions of different genes *viz*., tumor necrosis factor-α (*TNF-α*), nitric oxide synthase (*iNOS*), nuclear factor-kB (*NF-kB*). The RNA was extracted from the tissue following manufacturer's protocol. Briefly, the preserved sample (100 mg) was taken out and was incubated at 25°C for 15 min after proper mixing. Then 200 µL chloroform (Sigma-Aldrich, USA) was added to the sample and was vigorously shaken for 30 sec and incubated at 25°C for 10 min. After incubation, the sample was centrifuged at 12000 x *g* at 4°C for 15 min. The upper aqueous layer was transferred into a 1.5 mL RNase free microcentrifuge tube containing 1 µL of glycogen and 500 µL 2-propanol/ isopropanol (Sigma-Aldrich) was added gently to the sample and incubated at 25°C for 10 min. The sample was centrifuged at 12000 x *g* at 4°C for 10 min and supernatant was removed. Pellet was mixed with 1 mL of 75% ethanol (Merck, USA) and centrifuged at 7500 x *g* at 4°C for 5 min. The supernatant was removed and the pellet was air dried till it became transparent. Finally, it was mixed with 40 µL of deionized, diethylpyrocarbonate (DEPC)-treated water (ThermoFisher Scientific, USA) and incubated at 58°C in an oven for 10 min and stored at -20°C for further use.

The NanoDrop spectrophotometer (Thermo Scientific, USA) was used to check the purity of RNA (260:280). The quality of RNA was tested in gel electrophoresis (agarose 1%). The RNA (1 µg) was first treated with 1U of DNase to avoid contamination of DNA and then used for cDNA preparation through reverse transcription with random primers. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Scientific) was used for the study. The cDNA synthesis confirmation was checked with housekeeping gene (*β-actin*) through PCR-amplification.

The relative quantification of various genes was performed with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, ThermoFisher Scientific). The real-time PCR (Applied Biosystems, QuantStudio 6 Flex, USA) was used for the estimation. The primers for specific genes were designed using NCBI tool (Table 2). The efficiency and specificity of primers were also checked using a serial dilution of cDNA and constructing a standard curve with correlation coefficient. The reaction mixture (10 µL) consisted of cDNA (1 µL, 1:3), 2 × PowerUp™ SYBR™ Green PCR Master Mix (5 µL), 2.5 µmol forward

Table 2 The primers of the target genes and their sequences used for qPCR analysis

Target gene primers	Forward/ reverse	Nucleotide sequence (5' - 3')	T_m (^{0}C)	Product length (bp)	Accession number
TNF-α	Forward reverse	TCTCAGGTCAATACAACCCGC GAGGCCTTTGCGGAAAATCTTG	60 ^{0}C	125	KM593875
iNOS	Forward reverse	ATGGGCACTGAGATTGGAGC CTTCGTCCTTCCACAGCGAT	55 ^{0}C	121	KT180212
NF-kB	Forward reverse	CTCGGTGAAGCGAGGAAGAG CATTCCGGCTCCGACTCTC	60 ^{0}C	124	MG571500
β-actin	Forward reverse	GAGCACCCTGTCCTGCTTAC GTACAGGGACAGCACAGCC	60 ^{0}C	132	EU527190

TNF-α: Tumour necrosis factor α; *iNOS: Inducible nitric oxide synthase*; *NF-kB: Nuclear factor kappa B*; *β-actin: Beta-actin*

and reverse primers (0.25 µL each) and nuclease free water (3.5 µL). The conditions of real-time PCR were as follows: 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. All samples were run in duplicate and a non-template control (NTC) was also run without cDNA. The specificity of qPCR was checked through melt curve analysis. The conditions for melt curve analysis are as follows: 95°C for 15 sec, 60°C for 1 min, and at 95°C for 15 sec. After completing the programme, the threshold cycle (C_T) value of each well was recorded and analysis was performed using standard $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001). It was considered that primers were amplified with 100% efficiency. The *β-actin* was used as a housekeeping gene and also used for normalization of target genes. The data of treatment groups were compared to the control group.

Statistical analysis

The data for growth parameters, various enzymes and mRNA expressions are provided as means ± standard error. The Kolmogorov-Smirnov test was used to ascertain the normality of the data of average weight of fish and of various parameters in different treatments. The results indicated normality in the data ($p > 0.05$), except for one parameter hemagglutination titer, which resulted in significance value $p < 0.05$ in all treatment categories. The one way ANOVA analysis was performed among four different treatments and also for three different days of sampling (7, 14 and 21) for each parameter in the treatment groups. The non-parametric Kruskal-wallis test was applied to study the differences in hemagglutination titer among different treatments and different days. Further, Tukey HSD post-hoc analysis confirmed the pair-wise significance among different treatments and different days of individual treatment for each parameter. Statistical analysis was performed using SPSS 26.0 version (South Asia (P) Ltd., Bangalore, India) software. The significance level was accepted as $p < 0.05$.

Results

Growth performance

The average weight of magur was significantly higher in EFS compared to other three treatments throughout the experimental period (Table 3). Highest growth of fish was found on 21st day after immunization regardless of feeding schemes. The SGR was significantly higher in EFS

Table 3 Average weight, specific growth rate and feed conversion ratio found in Asian catfish (*Clarias batrachus*) cultured under different feeding regime. Means with different superscripts in the same row are significantly different (n=3)

Samplings	Experimental diets			
	CF	EFL1	EFL2	EFS
Final average weight (g)				
0th Day	2.23 ± 0.14^c	2.37 ± 0.13^c	3.08 ± 0.17^b	3.88 ± 0.20^a
7th Day	3.01 ± 0.11^d	3.68 ± 0.18^c	4.35 ± 0.16^b	4.95 ± 0.14^a
14th Day	3.69 ± 0.12^d	4.15 ± 0.09^c	5.03 ± 0.23^b	7.08 ± 0.21^a
21st Day	4.10 ± 0.09^c	5.83 ± 0.30^b	5.91 ± 0.22^b	7.79 ± 0.30^a
Specific growth rate (%)				
0th Day	3.07 ± 0.11^c	3.17 ± 0.09^c	3.61 ± 0.09^b	4.00 ± 0.09^a
7th Day	4.35 ± 0.63^c	6.30 ± 0.37^a	4.98 ± 0.53^b	3.52 ± 0.52^d
14th Day	2.90 ± 0.04^b	1.75 ± 0.23^d	2.07 ± 0.14^c	5.10 ± 0.05^a
21st Day	1.52 ± 0.09^c	4.84 ± 0.25^a	2.30 ± 0.07^b	1.37 ± 0.08^c
Feed conversion ratio (FCR)				
0th Day	0.46 ± 0.04^a	0.42 ± 0.03^a	0.31 ± 0.02^b	0.24 ± 0.02^c
7th Day	0.83 ± 0.14^b	0.51 ± 0.04^d	0.69 ± 0.09^c	1.05 ± 0.18^a
14th Day	1.25 ± 0.02^c	2.22 ± 0.29^a	1.81 ± 0.13^b	0.65 ± 0.01^d
21st Day	2.51 ± 0.16^b	0.70 ± 0.04^d	1.60 ± 0.06^c	2.80 ± 0.16^a

CF = Control feed, EFL1 = 0.25% leaves supplemented feed, EFL2 = 0.5% leaves supplemented feed, EFS = 0.5% seeds supplemented feed

compared to other treatments on the day of immunization and on 14th day after immunization. The FCR was significantly lower in EFS compared to other treatments on the day of immunization and on 14th day after immunization.

Biochemical assays

Serum lysozyme, myeloperoxidase and hemagglutination titer

Serum lysozyme, myeloperoxidase and hemagglutination titer levels of magur cultured in four treatments were estimated on three different days after immunization (Table 4). The serum lysozyme level was significantly higher in EFS compared to other treatments on 7th and 14th days after immunization. The levels were significantly higher in EFS and EFL2 compared to other two feeding regimes on 21st day after immunization. The myeloperoxidase level was significantly higher EFS compared to other treatments in all days of sampling. The hemagglutination titer level was maximum in EFS throughout the study period.

The days of immunization also influenced these parameters. Serum lysozyme and myeloperoxidase levels were significantly higher on 21st day after immunization regardless of treatments, except myeloperoxidase level in the control diet fed (CF) magur. In the CF, myeloperoxidase level was maximum on 14th day after immunization. In all these treatments, highest hemagglutination titer level was found on 14th day after immunization.

Nitric oxide synthase, thiobarbituric acid reactive substances and carbonyl protein

The nitric oxide synthase level in liver of EFS diet fed magur was significantly higher compared to other treatments throughout the study period (Table 5). The nitric oxide synthase level was significantly higher in kidney of EFS diet fed magur. The TBARS level was significantly lower in liver of EFS diet fed fish compared to the fish fed other diets in all days of sampling. Similar trend was also found in kidney. In both liver and kidney of EFS diet fed magur, the carbonyl protein levels were significantly lower compared to other treatments throughout the study period.

The effect of days of immunization was also found in this study. The nitric oxide synthase levels in liver and kidney increased gradually with the days of immunization. Highest nitric oxide synthase level was found on 21st day after immunization in all these treatments. TBARS and carbonyl protein showed the opposite trends. In liver and kidney, TBARS levels gradually decreased with the days of immunization regardless of treatments. Similar trend was found with carbonyl protein. In all these feeding schemes, carbonyl levels were significantly higher on 7th day and lower on 21st day after immunization compared to other days of sampling.

Relative mRNA expression study

In liver and kidney of magur, *TNF-α, iNOS* and *NF-kB* genes expressions were significantly higher in EFS compared to other treatment throughout the study period (Fig. 1). Treatment EFL2 followed the EFS. In EFL1, *NF-kB* was down-regulated in kidney on 7th and 14th days after immunization compared to the control diet fed fish. There were

Table 4 Serum lysozyme (mg/mL), myeloperoxidase (O.D. at 450 nm) and hemagglutination titer levels found in Asian catfish (*Clarias batrachus*). Means with different superscripts in the same row are significantly different (n=3)

Parameters	Experimental diets			
	CF	EFL1	EFL2	EFS
7 th day				
Serum lysozyme	57.98 ± 3.09^{d}	63.03 ± 1.17^{c}	80.04 ± 3.59^{b}	88.21 ± 3.21^{a}
Myeloperoxidase	1.48 ± 0.01^{d}	1.61 ± 0.04^{c}	1.79 ± 0.02^{b}	1.93 ± 0.04^{a}
Hemagglutination	4.5 ± 1.26^{d}	5 ± 1.00^{c}	7 ± 1.00^{b}	10 ± 1.15^{a}
14 th day				
Serum lysozyme	50.29 ± 8.49^{d}	74.14 ± 5.55^{c}	203.93 ± 1.88^{b}	218.93 ± 8.44^{a}
Myeloperoxidase	1.57 ± 0.01^{d}	1.78 ± 0.02^{c}	1.93 ± 0.03^{b}	2.23 ± 0.04^{a}
Hemagglutination	10 ± 2.00^{c}	10 ± 2.00^{c}	14 ± 2.00^{b}	24 ± 4.62^{a}
21st day				
Serum lysozyme	97.86 ± 3.80^{c}	129.29 ± 8.01^{b}	279.05 ± 9.30^{a}	282.50 ± 5.96^{a}
Myeloperoxidase	1.25 ± 0.02^{d}	1.85 ± 0.04^{c}	2.17 ± 0.03^{b}	2.52 ± 0.05^{a}
Hemagglutination	6 ± 1.15^{b}	7 ± 1.00^{b}	10 ± 2.00^{a}	14 ± 2.00^{a}

CF = Control feed, EFL1 = 0.25% leaves supplemented feed, EFL2 = 0.5% leaves supplemented feed, EFS = 0.5% seeds supplemented feed

Table 5 Nitric oxide synthase, NOS (µmol/mg tissue), thiobarbituric acid reactive substances, TBARS (µmol MDA/mg protein) and carbonyl protein (nmol/mg protein) levels found in Asian catfish (*Clarias batrachus*). Means with different superscripts in the same row are significantly different (n=3)

Tissues	Parameters	Experimental diets			
		CF	EFL1	EFL2	EFS
7th day					
Liver	NOS	0.68 ± 0.04^{d}	1.10 ± 0.01^{c}	1.72 ± 0.03^{b}	2.38 ± 0.04^{a}
	TBARS	9.30 ± 0.27^{a}	4.70 ± 0.08^{b}	2.54 ± 0.14^{c}	1.30 ± 0.02^{d}
	Carbonyl protein	21.55 ± 0.43^{a}	9.85 ± 0.22^{b}	6.69 ± 0.22^{c}	4.69 ± 0.07^{d}
Kidney	NOS	0.11 ± 0.02^{d}	0.45 ± 0.03^{c}	0.96 ± 0.02^{b}	1.13 ± 0.05^{a}
	TBARS	11.34 ± 0.33^{a}	6.91 ± 0.06^{b}	4.11 ± 0.07^{c}	1.87 ± 0.08^{d}
	Carbonyl protein	21.02 ± 1.35^{a}	11.17 ± 0.18^{b}	7.55 ± 0.18^{c}	3.40 ± 0.12^{d}
14 th day					
Liver	NOS	0.94 ± 0.03^{d}	1.77 ± 0.01^{c}	2.39 ± 0.02^{b}	3.06 ± 0.04^{a}
	TBARS	7.02 ± 0.18^{a}	4.06 ± 0.09^{b}	1.97 ± 0.04^{c}	1.16 ± 0.04^{d}
	Carbonyl protein	14.43 ± 0.27^{a}	7.96 ± 0.33^{b}	5.32 ± 0.07^{c}	3.92 ± 0.11^{d}
Kidney	NOS	0.27 ± 0.03^{d}	0.74 ± 0.02^{c}	1.15 ± 0.03^{b}	1.37 ± 0.04^{a}
	TBARS	9.58 ± 0.10^{a}	5.05 ± 0.09^{b}	2.78 ± 0.03^{c}	1.27 ± 0.02^{d}
	Carbonyl protein	17.96 ± 0.28^{a}	7.60 ± 0.22^{b}	4.72 ± 0.11^{c}	2.22 ± 0.11^{d}
21st day					
Liver	NOS	1.53 ± 0.03^{d}	2.47 ± 0.04^{c}	3.15 ± 0.03^{b}	3.76 ± 0.04a
	TBARS	5.92 ± 0.21^{a}	3.72 ± 0.07^{b}	1.62 ± 0.05^{c}	0.85 ± 0.03^{d}
	Carbonyl protein	12.27 ± 0.21^{a}	6.04 ± 0.25^{b}	2.38 ± 0.21^{c}	1.13 ± 0.14^{d}
Experimental diets	NOS	0.42 ± 0.03^{d}	0.94 ± 0.02^{c}	1.31 ± 0.02^{b}	1.57 ± 0.02^{a}
	TBARS	8.51 ± 0.19^{a}	4.10 ± 0.05^{b}	2.14 ± 0.07^{c}	0.93 ± 0.04^{d}
	Carbonyl protein	14.70 ± 0.29^{a}	5.82 ± 0.07^{b}	3.48 ± 0.14^{c}	1.12 ± 0.05^{d}

CF = Control feed, EFL1 = 0.25% leaves supplemented feed, EFL2 = 0.5% leaves supplemented feed, EFS = 0.5% seeds supplemented feed

up-regulations of all these genes in EFL1 on 21st day after immunization.

Discussion

In magur, the effect of *A. aspera* seeds and leaves supplemented diets were recorded. The supplementation of leaves and seeds at 0.5% level (EFL2 and EFS) enhanced the growth of magur compared to the control (CF) diet fed fish. The enhanced growth was also found in rohu fed with *A. aspera* seeds and leaves (Sharma et al. 2019; Srivastava and Chakrabarti 2012) and guava *Psidium guajava* leaves (Giri et al. 2015) supplemented diets. The chemical composition study showed the nutritional value of seeds and leaves. The seeds contain all essential amino acids, fatty acids, different oleonic acid and ecdysterone (Varuna et al. 2010). The presence of ecdysterone influenced the growth of fish (Chakrabarti et al. 2012). All essential amino acids were present in the seeds and leaves. The amino acid contents were higher in seeds compared to leaves (Kumar et al. 2021). Various secondary metabolites like, alkaloids, flavonoids, saponins, steroids and terpenoids were reported in leaves (Umamaheswari et al. 2012) and their presence influenced the performance of magur.

The fatty acid profiles of seeds and leaves influenced the immune system of enriched diets fed fish. The monounsaturated fatty acids (MUFA) and n-6 polyunsaturated (PUFA) contents were higher in seeds compared to leaves, whereas n-3 PUFA contents were higher in leaves compared to the seeds (Kumar et al. 2021). The higher lysozyme levels were found in EFS and EFL2 diets fed fish compared to the other diets fed fish throughout the study period. Lysozyme modulates host immune system against infections (Ragland and Criss 2017). The myeloperoxidase level was always maximum in EFS. The lysosomal protein myeloperoxidase plays significant role in non-specific immune system (Hampton and Orrenius 1998). Minimum hemagglutination titer levels were observed in EFL1 and CF. Significantly higher levels of nitric oxide synthase were recorded in liver and kidney of EFS diet fed magur compared to others. The EFL2 diet fed magur followed this treatment. In earlier study, higher levels of lysozyme, myeloperoxidase and nitric oxide synthase were found in seeds and leaves supplemented diets fed catla and rohu (Sharma et al. 2019; Chakrabarti et al. 2014). The presence of long-chain polyunsaturated fatty acids such as linolenic and oleic acids in the seeds may be associated with the immunostimulatory properties (Chakrabarti et al. 2012).

The increased levels of TBARS and carbonyl protein in the tissue are indicators of oxidative damage (Parvez and

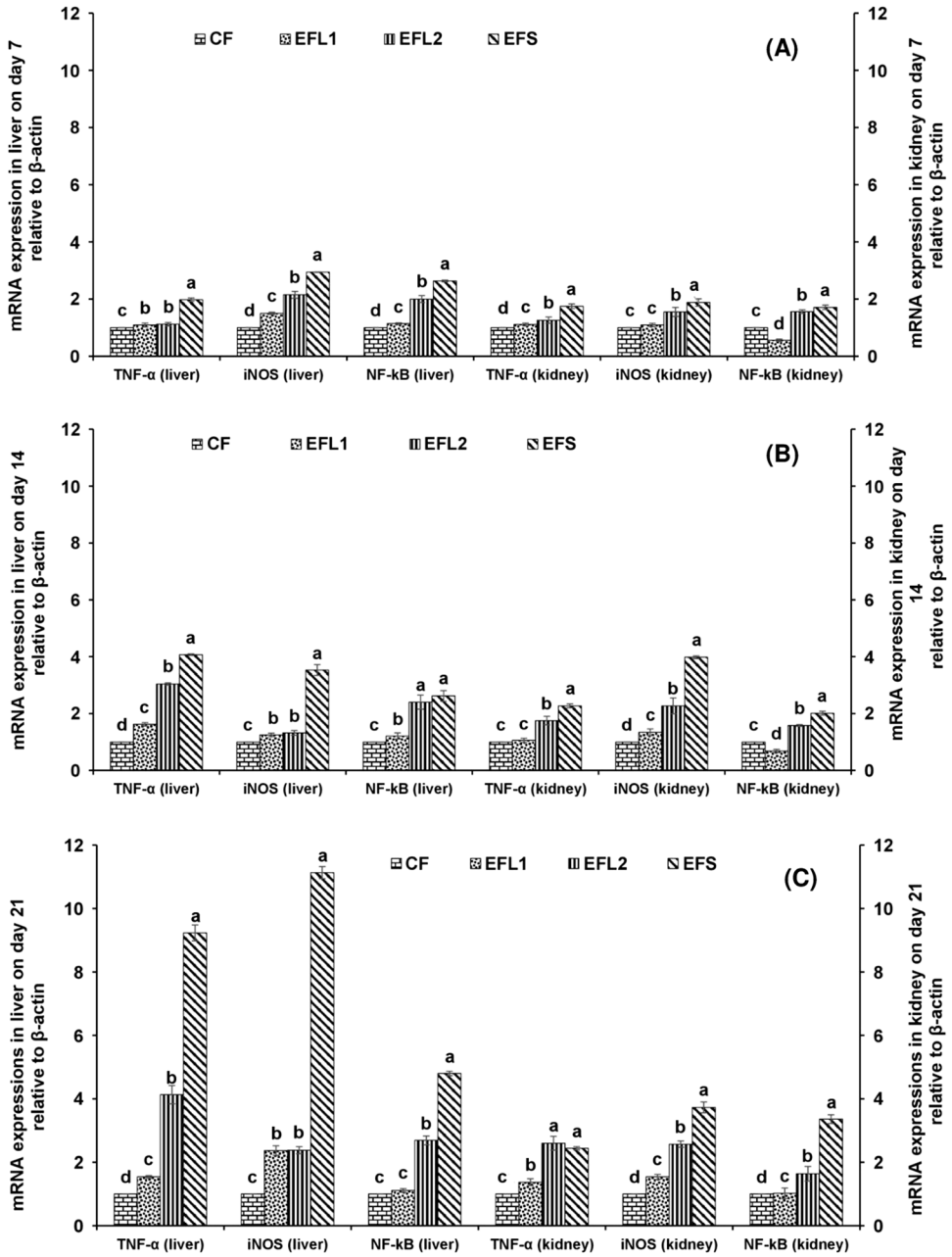

Fig. 1 Expression of *TNF-α*, *iNOS*, *NF-kB* in liver and kidney of four different diets fed Asian catfish (*Clarias batrachus*) on (A) 7th day, (B) 14 th day and (C) 21 st day after immunization. The relative expressions of the target genes were normalized to the expression of *β-actin* (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($p < 0.05$) different (n=3)

Raisuddin 2006). TBARS and carbonyl protein contents were always minimum in EFS diet fed magur. The presence of vitamin C in the seeds reduced the oxidative stress in the enriched diet fed fish (Kumar et al. 2021). Similar results were found in *A. aspera* seeds and leaves supplemented diets fed rohu (Sharma et al. 2019). The seeds supplemented diet gave protection against oxidative damage to UV-B radiated fish (Singh et al. 2013a; Singh et al. 2013b; Sharma et al. 2015).

Higher expressions of *TNF-α*, *iNOS* and *NF-kB* were found in liver and kidney of EFS and EFL2 diets fed magur compared to the control diet fed fish. The expressions were higher in EFS compared to EFL2. Higher expression of *TNF-α* was observed in seeds and leaves enriched diets fed rohu (Sharma et al. 2019). The up-regulation of *TNF-α* was found in guava leaves supplemented diet fed rohu (Giri et al. 2015). *TNF-α* supernatants treated endothelial cells promoted respiratory burst activity and migration of leucocytes in carps (Forlenza et al. 2009). The exposure of rohu at early stage to bisphenol-A caused oxidative stress and suppress *NF-κB* signaling pathway leading to immunosuppression (Faheem et al. 2020).

Conclusion

Achyranthes aspera seeds and leaves enriched diets showed immunostimulatory properties in fish. The seeds and leaves should be supplemented at 0.5% level in fish diets and seeds showed better result compared to leaves.

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Availability of data and material The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution RC, JGS, PM: designed the study; NK, RC, JGS: cultured the fish and analyzed samples; RC, JGS, PM: prepared the manuscript; PM, RC: performed statistical analysis; NK, PM, RC: prepared graphs and tables.

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Declarations

Ethics approval All experiments were performed following the guidelines and regulations with approval from the ethical committee of Institutional Animal Ethics Committee, IAEC, University of Delhi, Delhi, approval number DU/ZOOL/IAEC-R/2015/08 was followed for the culture of fish and sample analysis.

Consent to participate Not applicable.

Consent for publication All contributing authors have been asked to give consent to publication at the point of acceptance, to confirm that they have approved the final version of the manuscript and have made all required statements and declarations.

Conflict of interest No conflict of interest among authors.

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Evaluation of nutritional value of prickly chaff flower (*Achyranthes aspera*) as fish feed ingredient

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ABSTRACT

The nutritional value of seeds and leaves of *Achyranthes aspera* as fish feed ingredients was evaluated. The protein and lipid contents were higher in seeds and ash content was higher in leaves. In seeds, total 18 essential and non-essential amino acids were present; total 17 amino acids were found in leaves, cysteine was absent. The essential and non-essential amino acid contents were higher in seeds compared to leaves except, leucine, tryptophan, valine, alanine and tyrosine. Taurine content was higher in leaves. Higher saturated, monounsaturated and *n*-6 polyunsaturated fatty acids (*n*-6 PUFA) contents were observed in seeds, whereas, *n*-3 PUFA level was higher in leaves. In seeds, oleic and linoleic acid levels were 1863.34±9.25 and 3342.05±8.05 mg/100 g, respectively; α-linolenic acids level in leaves was 1058.07±3.71 mg/100 g. Vitamins A, D_2, E, B_1, B_2, B_6, B_{12} and C were present in seeds; vitamins A, D_2, E, B_2 and B_{12} were found in leaves. In seeds and leaves, four macro, nine trace and four ultra-trace minerals were found and contents were higher in leaves, except zinc. In seeds and leaves, iron contents were 76.82±4.15 and 293.73±11.40 µg/g, respectively. Thus, seeds and leaves are rich sources of nutrients.

Keywords: α-linolenic acid, *Achyranthes aspera*, Amino acids, Linoleic acid, Minerals, Vitamins

Achyranthes aspera, the prickly chaff flower (Amerantheceae), occupies a significant position in Ayurveda, Unani and folk medicines of India. The herb grows profusely in the tropical climate of Asia, South America and Africa. In India, the plant grows throughout the year. The plant has several medicinal applications, viz. for the treatment of diabetes, dysentery, fever, hypertension, asthma (Girach-Aminuddin and Khan 1992, Tang 1992, Liersch 1992). The roots are applied for the treatment of diarrhoea and cold in children; leaves are effective to treat asthma and seeds have emetic and hydrophobic properties (Bishit and Sandhu 1990, Borthakur and Goswami 1995, Singh 1995). The aqueous extract of the plant stimulates immune system of mice (Vasudeva *et al*. 2002). Even the roots, leaves and seeds of the plant improve the immune system of fishes and provide protection against bacterial pathogens (Rao *et al*. 2004, Rao *et al*. 2006, Rao and Chakrabarti 2005, Chakrabarti and Rao 2006, Chakrabarti and Rao 2012, Chakrabarti *et al*. 2012, Kumar *et al*. 2019, Sharma *et al*. 2019, Singh *et al*. 2019). The seeds protect fishes from harmful UV-B irradiation (Singh *et al*. 2013a, Singh *et al*. 2013b). Feeding of fish with *A. aspera* seeds and leaves enriched feeds always enhances the growth of fishes.

The scattered information are available on the composition of various parts of the plant. In seeds of *A. aspera*, two glycosides of oleanolic acid and two homogeneous saponins, saponin A and B are found (Hariharan and Rangaswami 1970). The seeds contain amino acids, fatty acids, different oleonic acid, saponins and ecdysterone (Varuna *et al*. 2010). The calorific value of seeds is 3.92/g (Goyal *et al*. 2007). In petroleum ether fraction of seeds, linolenic, oleic, palmitic and stearic acids are found; ecdysterone has been reported in the alcohol and aqueous alcohol extracts of the seeds (Chakrabarti *et al*. 2012). In root, stem and leaves extracts alkaloids, saponin, tannin, steroid reducing sugars, glycosides, phenolic compounds and protein are present (Dhale and Bhoi 2013). In leaves, 13 chemical compounds are isolated through HPLC and alkaloids are the dominant one (Meles *et al*. 2017). The proximate and mineral compositions of roots and leaves are documented (Joy *et al*. 2017, Rana *et al*. 2019). Review of literature shows the pharmatological importance of the plant and most of these studies are conducted in isolation. The present investigation has been designed to evaluate the nutritional value of seeds and leaves of *Achyranthes aspera* as fish feed ingredient. A holistic approach has been taken for their proper neutraceutical applications.

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MATERIALS AND METHODS

Biochemical composition of leaves and seeds: A. aspera were cultivated in a specialized nursery in the outdoor facility (Fig. 1a, b). The mature seeds and leaves were collected. After air-drying, ground and powder was stored at 4°C for various assays. The proximate composition of leaves and seeds were analyzed following standard methods (AOAC 2000). Three replicates were used for each assay. Moisture content was estimated after drying the sample in an oven at 105°C. Oven dried sample was kept at 550°C for 8 h for the determination of ash contents of leaves and seeds. The crude protein content was analyzed using an automated Micro-Kjeldahl apparatus (N × 6.25). Crude lipid contents of leaves and seeds were assayed gravimetrically (Folch *et al*. 1957).

Fig. 1a. *Achyranthes aspera* with juicy leaves and young flowers. The plants were grown in the outdoor facility.

Fig. 1b. *Achyranthes aspera* with maturing seeds. The plants were grown in the outdoor facility.

Amino acids: Hitachi L-8900 Automatic Amino Acid Analyser (Tokyo, Japan) was used for estimation of amino acids present in the leaves and seeds. The first step of estimation was hydrolysis of the sample. The dried powder of leaves and seeds were hydrolysed with HCl (6 N) for estimation of all amino acids, except methionine, cysteine and tryptophan. The hydrolysis was performed at 110°C for 22 h. Methionine and cysteine were treated with performic acid and hydrobromic acid (48%); methanesulfonic acid (4N) and 3-(2-aminoethyl) indole was used for tryptophan estimation. In the second step, the hydrolysed sample was dried in a nitrogen evaporator and after drying, HCl (0.02 N) was added in the sample. The protein content of sample was 0.5 mg/mL. Finally, the sample was kept in the Auto sampler and 20 µL sample was injected for analysis. All amino acids were monitored at 570 nm except, proline and hydroxyproline; these were measured at 440 nm. Amino acids were quantified with Standards solution (WPCI, Japan).

Fatty acids: The leaves and seeds were analyzed for fatty acid profile study with Gas Chromatograph (GC)-Flame Ionization Detector, Clarus 580 (PerkinElmer, Waltham, USA). Total lipid was extracted from leaves and seeds samples (Folch *et al*. 1957). The fatty acid methyl esters, FAME (1 mg/mL) was prepared from the extracted lipid by transesterification using sulphuric acid in methanol at 50°C for 16 h (Christie 2003). After extraction and purification of FAME (Tocher and Harvie 1988), 1 mL sample was kept in a glass vial of Auto sampler of GC. In GC column (60 m ZB-wax, Phenomenex, UK), the sample was separated and quantified. The injector temperature was 260°C and nitrogen was used as a carrier gas (2 mL/min). The following conditions were set for oven ramping: initial setting at 150°C and hold for 3 min, then raising of temperature @ 2°C/min to 180°C hold for 2 min, again raising of temperature to 220°C @ 1°C/min and hold for 15 min. The data were collected from pre-installed programme software (TotalChrom Workstation Ver6.3, PerkinElmer, USA). Standard (Sigma-Aldrich, USA) was used for the identification of fatty acids.

Vitamins: Vitamin contents of leaves and seeds were analyzed with UHPLC (Thermo Fisher Scientific, USA) with C18 column (3 µM, 150×4.6 mm). The fat soluble vitamins were analyzed (Qian and Sheng 1998). In an extraction tube (10 mL), 4 mL of hexane was added to 1 g sample and flushed with nitrogen gas; then centrifuged at 1,500× g for 5 min. Supernatant (1 mL) was transferred in a vial (10 mL) and evaporated under nitrogen evaporator. The residue was dissolved in *n*-butanol (0.3 mL) and injected to the UHPLC. Mobile phase used for fat soluble vitamins was methanol and the flow rate was 1 mL/min. The vitamin levels were recorded at 290 nm.

The water soluble vitamins were analyzed (Sami *et al*. 2014). The dry sample (2 g) was hydrolyzed with 0.1 N H_2SO_4 at 121°C for 30 min for the estimation of thiamine (B_1), riboflavin (B_2), pyridoxine (B_6) and cobalamin (B_{12}). After bringing at room temperature, 2.5 M sodium acetate was mixed with the hydrolyzed sample and pH of the sample was 4.5. The enzyme takadiastase (50 mg) was added to the sample and incubated at 35°C for 12 h. Then the sample was filtered with Whatman filter paper (no. 4) and distilled water was added to make the volume 50 mL. Sample (2 g) was treated with a solution of metaphosphoric acid (0.3 M) and acetic acid (1.4 M) for the estimation of vitamin C. The sample was centrifuged at 9000 × g for 15 min and filtered with Whatman filter paper. Mobile phase used for water soluble vitamins was a mixture of 0.1 M potassium acetate (33 mL, pH 4.9) and 67 mL acetonitrile with water

(1:1); the flow rate was 1 mL/min. The absorbance was recorded at 254 nm at 25°C. The micropore (0.45 µm) syringe filter was used to filter the samples before injection; then placed into the UHPLC vial. Then 20 µL sample was injected automatically in UHPLC system.

Minerals: The ICP-MS (Inductively Coupled Plasma Mass Spectrometer, Agilent 7900, USA) was used for the study of mineral composition of leaves and seeds. The following conditions were used for the operation of the equipment - Nebulizer and auxillary gas flow were 1 L/min; plasma gas flow was 15 L/min, helium gas flow inside the reaction cell was 0.2 mL/min. Forward and reflected powers were set at 1500 W and 45 W, respectively. Analyzer vacuum pressure was maintained at 6×10^{-5} Pascal. In a closed digestion vessel, 150 mg sample (leaves/seeds powder) was taken and mixed with 8 mL of suprapure (69%) nitric acid (Merck, USA); then the sample was kept in a Microwave (Multiwave PRO; Anton Paar, Austria) for digestion. The digestion programme in the Microwave was as follows - first ramping at 1200 W for 15 min and then 30 min holding at 1200 W. The digested sample after cooling was taken in a separated tube and the volume was made 40 mL with Milli-Q water. The sample was filtered through syringe filter (0.2 µm, Thermo Scientific, USA) and the filtrate was collected in a glass vial. A 20 µL sample was injected automatically in the ICP-MS. Standard solution (Agilent Technologies, USA) for each mineral was diluted with Milli-Q water (containing 1% HNO_3) to make concentrations of 20, 40, 60, 80, 100, 250, 500, 1000 µg/L. The calibration (standard) curve was prepared. The Milli-Q water served as blank.

Statistical analysis: Data were expressed as mean ±SEM with a significance value using t-test statistic to compare the differences between leaves and seeds. Significance level was accepted at $P<0.05$.

RESULTS AND DISCUSSION

Biochemical analysis: Protein and lipid contents were significantly higher in seeds of *A. aspera* compared to the leaves. Significantly higher ash content was found in leaves compared to the seeds (Table 1).

A marked variation was found between the composition of seeds and leaves. In the present assay, plants are grown in a well planned nursery and used for various biochemical studies. So the results are reproducible–this is the advantages of the present investigation over the assays conducted with wild collections. It was interesting to see that protein and lipid levels were higher in seeds compared to the leaves, whereas maximum ash content was found in leaves. In Bangladesh, plants were collected from wild source and proximate composition of the leaves was assayed. The protein, lipid, ash and carbohydrate contents were 18.13±1.67, 1.88±0.20, 21.43±0.33 and 39.91±1.85 mg/100 g (DW), respectively in the leaves (Rana *et al.* 2019). In the present investigation, protein and lipid contents were higher and ash content was lower in nursery grown leaves compared to the wild collection in Bangladesh. Joy *et al*. (2017) reported the protein, lipids and ash levels as 25.76±3.71, 4.89±0.41 and 3.73±0.04 g/100 g, respectively in raw leaves. Therefore, a variation in the biochemical composition of leaves was recorded in different studies. The composition may vary depending on the environmental conditions, where the plant was grown.

Table 1. Proximate composition of *A. aspera* seeds and leaves (dry matter g/100 g)

Parameter	Seeds	Leaves
Protein	29.68±0.27[a]	23.05±0.05[b]
Lipid	4.36±0.01[a]	2.50±0.08[b]
Ash	2.74±0.05[b]	15.47±0.12[a]

Means (±SEM) sharing different letters in the same row are significantly ($P<0.05$) different (n=3).

Amino acids composition: Among essential amino acids, histidine, isoleucine, lysine, methionine and arginine were significantly higher in seeds compared to leaves. Leucine, tryptophan and valine levels were significantly higher in leaves compared to the seeds. The phenylalanine and threonine contents of leaves and seeds were not significantly different. All non-essential amino acids (except, alanine and tyrosine) contents were significantly higher in seeds compared to leaves. Alanine content was significantly higher in leaves compared to the seeds. The tyrosine level of leaves and seeds was not significantly different. Cysteine was absent in leaves (Table 2). Among free amino acids, phosphoserine and glutamine were present in seeds and absent in the leaves; α-amino adipic acid and hydroxylysine present in leaves were absent in seeds. Taurine was found in both leaves and seeds and the amount was significantly higher in leaves compared to the seeds. β-alanine and hydroxyproline levels were significantly higher in seeds compared to leaves, whereas β-amino isobutyric acid and γ-amino-n-butyric acid levels were significantly higher in leaves compared to the seeds. Total essential, non-essential and free amino acids contents were higher in seeds compared to the leaves (Table 2).

Essential and non-essential amino acids compositions were same in both seeds and leaves, except cysteine was absent in the leaves; the amount of individual amino acid was higher in seeds compared to leaves except, leucine, tryptophan and valine. These amino acids were higher in leaves. It was interesting that free amino acid taurine content was higher in leaves compared to the seeds of the plant. The amino acids like, leucine, isoleucine, phenylalanine and valine contents of *A. aspera* seeds were similar to Bengal gram; methionine and cysteine contents in seeds were higher than most pulses (Goyal *et al*. 2007). The present investigation confirmed the earlier findings of amino acid profile of seeds.

Fatty acid profile: The comparative study of fatty acid profiles of seeds and leaves showed that total saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) were around 2 and 9-fold higher in seeds compared to the leaves (Table 3). Among SFAs, carproic, caprylic, capric

Table 2. Amino acid profile of *A. aspera* (g/100 g dry weight)

Amino acid	Seeds	Leaves
Essential amino acids		
Histidine (His)	0.92±0.01[a]	0.55±0.03[b]
Isoleucine (Ile)	1.38±0.01[a]	1.31±0.07[b]
Leucine (Leu)	2.06±0.01[b]	2.21±0.13[a]
Lysine (Lys)	1.73±0.04[a]	1.33±0.07[b]
Methionine (Met)	0.61±0.02[a]	0.50±0.04[b]
Phenylalanine (Phe)	1.54±0.09[a]	1.52±0.08[a]
Threonine (Thr)	1.35±0.01[a]	1.34±0.05[a]
Tryptophan (Trp)	0.17±0.17[b]	0.71±0.00[a]
Valine (Val)	1.53±0.01[b]	1.80±0.11[a]
Arginine (Arg)	3.08±0.57[a]	1.83±0.06[b]
Σ Essential amino acids	14.37±1.92[a]	13.11±0.64[a]
Non-essential amino acids		
Alanine (Ala)	1.17±0.02[b]	1.54±0.08[a]
Aspartic acid (Asp)	3.03±0.02[a]	2.67±0.14[b]
Cysteine (Cys)	0.48±0.08[a]	–
Glutamate (Glu)	7.22±0.01[a]	3.44±0.19[b]
Glycine (Gly)	2.83±0.02[a]	1.61±0.09[b]
Proline (Pro)	1.35±0.05[a]	1.18±0.06[b]
Serine (Ser)	1.59±0.01[a]	1.16±0.03[b]
Tyrosine (Tyr)	1.33±0.05[a]	1.33±0.06[a]
Σ Non-essential amino acids	18.99±0.66[a]	12.93±1.34[b]
Free amino acids		
Phosphoserine (p-ser)	0.13±0.02[a]	–
Taurine (Tau)	0.08±0.01[b]	0.13±0.01[a]
Phospho ethanolamine (PEA)	0.04±0.01[a]	0.04±0.02[a]
Glutamine ($GluNH_2$)	0.01±0.00[a]	–
Cystathionine (Cysthi)	0.30±0.01[b]	0.32±0.05[a]
β-Alanine (β-Ala)	0.26±0.01[a]	0.18±0.01[b]
β-Amino isobutyric acid (β-AiBA)	0.17±0.04[b]	0.19±0.12[a]
γ-Animo-n-butyric acid (γ-ABA)	0.13±0.07[b]	0.19±0.01[a]
1-Methylhistidine (1-Mehis)	0.09±0.01[a]	0.11±0.02[a]
Hydroxyproline (Hypro)	1.48±0.04[a]	0.10±0.01[b]
α-Amino adipic acid (α-AAA)	–	0.01±0.00[a]
Hydroxylysine (Hylys)	–	0.04±0.01[a]
Σ Free amino acids	2.67±0.23[a]	1.31±0.53[b]

Means (± SEM) sharing different letters in the same row are significantly (P< 0.05) different (n=3).

Table 3. Fatty acid profile of *A. aspera* seeds and leaves (mg/100 g)

Fatty acid	Formula	Seeds	Leaves
Saturated fatty acids			
Caproic acid	6:0	7.88±0.02[a]	–
Caprylic acid	8:0	18.05±0.16[a]	–
Capric acid	10:0	25.56±0.29[a]	–
Lauric acid	12:0	5.50±0.37[a]	–
Myristic acid	14:0	22.82±0.16[a]	6.81±0.32[b]
Palmitic acid	16:0	1780.41±8.14[a]	910.71±2.32[b]
Stearic acid	18:0	1.97±0.20[b]	4.59±0.83[a]
Arachidic acid	20:0	–	6.24±0.18[a]
Lignoceric acid	24:0	22.07±1.37[a]	10.92±0.81[b]
Σ SFA		1884.28±7.84[a]	939.27±3.66[b]
Monounsaturated fatty acids			
Palmitoleic acid	16:1 n-7	–	45.83±0.83
Oleic acid	18:1 n-9	1863.34±9.25[a]	164.81±0.62[b]
Gondoic acid	20:1 n-9	104.25±0.88[a]	–
Erucic acid	22:1 n-9	61.42±0.11[a]	13.94±0.80b
Σ MUFA		2029.01±8.33[a]	224.58±1.69[b]
n-6 Polyunsaturated fatty acids			
Linoleic acid	18:2 n-6	3342.05±8.05[a]	604.87±2.58[b]
Σ n-6 PUFA		3342.05±8.05[a]	604.87±2.58[b]
n-3 Polyunsaturated fatty acids			
α-Linolenic acid	18:3 n-3	29.66±0.50[b]	1058.07±3.71[a]
Σ n-3 PUFA		29.66±0.50[b]	1058.07±3.71[a]

Means (±SEM) sharing different letters in the same row are significantly (P < 0.05) different (n = 3).

and lauric acids were present in seeds, absent in leaves. Arachidic acid was present in leaves and absent in the seeds. Palmitic acid was the dominant SFA in both seeds and leaves and maximum amount was present in the former one. Palmitoleic and gonodoic acids were absent in seeds and leaves, respectively. In seeds, oleic acid content was 11-fold higher compared to the leaves. Higher levels of linoleic acid, the *n*-6 polyunsaturated fatty acid (*n*-6 PUFA) and α-linolenic acid, the *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) were found in seeds and leaves, respectively. In seeds, linoleic acid content was 3342.05±8.05 mg/100 g and in leaves α-linolenic acid level was 1058.07±3.71 mg/100 g.

Higher saturated, monounsaturated and *n*-6 PUFA contents were observed in seeds, whereas, *n*-3 PUFA level was higher in the leaves. Daulatabad and Ankalgi (1985) recorded the fatty acid composition of seeds. They reported that oleic (22.6%) and linoleic acids (49.4%) were dominant unsaturated fatty acids and palmitic (18.6%) acid was the major constituent of the saturated fatty acids; lauric (0.4%), myristic (1.2%), stearic (4.4%), arachidic (1.6%) and behenic (1.8%) acids were present in small amount in the seeds. The fatty acid profile of seeds of the present investigation is comparable with the previous data. In the present assay, oleic, linoleic and palmitic acids contents were 25.57, 45.87 and 24.44%, respectively. The arachidic and behenic acids were absent in seeds. In the present study, the arachidic acid was found in the leaves.

Vitamin contents: The retinol (vitamin A) content was same in seeds and leaves (Table 4). Vitamin D_2 (ergocalciferol) and vitamin E (tocopherol) levels were significantly higher in leaves compared to the seeds. Among water soluble vitamins, thiamine (B_1) and pyridoxine (B_6) were absent in leaves and present in the seeds; riboflavin (B_2) and cobalamin (B_{12}) contents were significantly higher in seeds compared to leaves. Vitamin C was present in seeds and absent in the leaves.

There were some differences in the vitamin composition between seeds and leaves, like, vitamin D_2 and vitamin E were higher in leaves compared to seeds; vitamins B_1 and B_{12} were higher in seeds. The absence of vitamins B_1, B_6 and C in leaves was conspicuous. Fatima *et al*. (2014) measured various vitamins in the whole plant collected from

Table 4. Vitamin contents of seeds and leaves of *A. aspera* (mg/100 g, dry weight)

Vitamin	Seeds	Leaves
Fat soluble		
Retinol (A)	0.26±0.08[a]	0.26±0.09[a]
Ergocalciferol (D_2)	1.49±0.01[b]	69.53±0.53[a]
Tocopherol (E)	0.62±0.07[b]	34.92±0.40[a]
Water soluble		
Thiamine (B_1)	2.16±0.15[a]	–
Riboflavin (B_2)	137.43±6.17[a]	25.81±2.75[b]
Pyridoxine (B_6)	2.93±0.19[a]	–
Cobalamin (B_{12})	79.83±8.40[a]	1.34±0.06[b]
Ascorbic acid (C)	166.40±3.41[a]	–

Means (±SEM) sharing different letters in the same row are significantly (P<0.05) different (n=3).

the local market. In the whole plant, vitamins B_1, B_2, B_3 and B_6 contents were 0.27, 0.28, 0.58 and 0.27 mg/100 g, respectively and vitamin B_9 content was 39 µg/100 g. These values were lower compared to the present study.

Mineral composition: All macro, trace and ultra-trace minerals contents (except, zinc) were significantly higher in leaves compared to seeds (Table 5). Zinc content was significantly higher in the latter compared to former one. In leaves, sodium, potassium, calcium and magnesium levels were 0.15±0.01, 27.50±0.32, 2.10±0.12 and 5.70±0.17 mg/100 g, respectively. Iron contents were 76.82±4.15 and 296.73±11.40 µg/100 g of seeds and leaves, respectively. Significantly higher level of cobalt (0.23±0.04 µg/g) was found in leaves compared to the seeds.

Like ash, most of the minerals (except zinc) were higher in the leaves compared to the seeds in the present assay. In the whole plant, different minerals, viz. calcium, sodium, potassium, magnesium, iron, copper, zinc etc. were observed (Fatima *et al*. 2014). Total 14 minerals were reported in the leaves of the plant; Ca and K were absent (Joy *et al*. 2017). The present assay showed presence of 17 minerals (four macro, nine trace and four ultra-trace minerals) in both leaves and seeds.

In conclusion, the present investigation helps to understand the nutritional and pharmaceutical values of the seeds and leaves of *A. aspera*. Seeds and leaves are rich sources for amino acids, fatty acids, vitamins and minerals and have ample scope for application in fish feed industry.

Table 5. Minerals composition of *A. aspera* seeds and leaves (dry powder)

Mineral	Seeds	Leaves
Macro minerals (*mg/g*)		
Sodium (Na)	0.06±0.01[b]	0.15±0.01[a]
Potassium (K)	6.35±0.04[b]	27.50±0.32[a]
Calcium (Ca)	0.17±0.01[b]	2.10±0.12[a]
Magnesium (Mg)	2.18±0.01[b]	5.70±0.17[a]
Trace minerals (*µg/g*)		
Molybdenum (Mo)	0.28±0.02[b]	0.66±0.10[a]
Manganese (Mn)	30.20±0.63[b]	43.09±1.58[a]
Aluminium (Al)	41.07±4.16[b]	333.36±16.62[a]
Iron (Fe)	76.82±4.15[b]	296.73±11.39[a]
Zinc (Zn)	41.77±0.18[a]	36.05±2.18[b]
Copper (Cu)	7.67±0.19[b]	9.49±0.57[a]
Strontium (Sr)	3.39±0.26[b]	58.10±1.54[a]
Cadmium (Cd)	0.10±0.04[b]	0.62±0.34[a]
Lead (Pb)	0.21±0.02[b]	1.32±0.06[a]
Ultra-trace minerals (*µg/g*)		
Chromium (Cr)	2.18±0.38[b]	8.43±1.39[a]
Cobalt (Co)	0.09±0.01[b]	0.23±0.04[a]
Nickel (Ni)	1.35±0.44[b]	3.03±0.23[a]
Tin (Sn)	0.18±0.04[b]	0.38±0.03[a]

Means (±SEM) sharing different letters in the same row are significantly (P<0.05) different (n=3).

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Evaluation of immunostimulatory properties of prickly chaff flower *Achyranthes aspera* in rohu *Labeo rohita* fry in pond conditions

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ABSTRACT

The immunostimulatory properties of seeds and leaves of *Achyranthes aspera* (Linn.) was evaluated in rohu *Labeo rohita* in the pond conditions. Rohu fry (1.9 ± 0.08 g) were introduced in nine hapas (25 hapa^{-1}) set inside a pond. Two test diets enriched with 0.5% seeds (D1) and leaves (D2) of *A. aspera* and control diet (D3) were fed for 60 days. Then fish were immunized with chicken-RBC and blood and tissue samples were collected on days-7, 14 and 21 after immunization. The average weight was significantly ($p < .05$) higher in D1 diet fed rohu compared to other two feeding regimes. Specific growth rate and feed conversion ratio were maximum and minimum in D1 diet fed rohu. Serum lysozyme, myeloperoxidase and nitric oxide synthase levels were significantly ($p < .05$) higher in D1 diet fed rohu compared to others. This group was followed by D2 diet fed rohu. Significantly ($p < .05$) lower thiobarbituric acid reactive substances (TBARS) and carbonyl protein levels were found in D1 fed rohu compared to others. TBARS and carbonyl protein levels were also lower in D2 diet fed rohu compared to the control one. There were significant ($p < .05$) up-regulation of lysozyme C, lysozyme G and tumor necrosis factor-α (TNF-α) in hepatopancreas of rohu fed with D1 diet compared to others. This group was followed by the D2 diet fed rohu. The expression of lysozyme C was higher compared to lysozyme G regardless of feeding regimes. In enriched diets fed rohu, the expression of interleukin 10 (IL-10) was significantly ($p < .05$) lower compared to the control fish. Toll like receptor 4 (TLR-4) was significantly ($p < .05$) higher in D1 and D2 diets fed rohu compared to other two feeding regimes on day-7 and day-21 after immunization, respectively. Seeds and leaves enhanced the growth, induced the immune system of rohu and gave protection against oxidative stress in pond conditions. Seeds are more efficient compared to the leaves. The information generated from the field study has practical utility.

1. Introduction

The management of disease and health care are two important aspects of aquaculture. Diseases are the major cause of economic loss to the aquaculture industry and control of disease is an utmost need for sustainable production. The use of immunostimulants is a useful preventive measure to control the disease of cultured species. It reduces the impact of environmental stress on fish and improves the health conditions. The immunostimulant activates the immune system in a non-specific way and thus provides resistance against a variety of pathogens. The administration of immunostimulants via food is an effective method because of fast delivery and simple entry route. Recently, there is a growing interest on the application of plant ingredients as immunostimulants. These are biocompatible, biodegradable, cost-effective and environment-friendly. Many studies in laboratory conditions show the positive impact of plant ingredients in different fish species. In tilapia *Oreochromis mossambicus*, the immunostimulatory effect of leaf extract of miers, *Tinospora cordifolia* (Sudhakaran et al., 2006) and *Solanum trilobatum* (Divyagnaneswari et al., 2008) has been recorded. The supplementation of mango, *Magnifera indica* kernel (Sahu et al., 2007), guava *Psidium guajava* leaves (Giri et al., 2015) and ginger *Zingiber officinale* in the diets improves the immune system of rohu *Labeo rohita*. This is essential to test the immunostimulatory properties of the plant ingredients in the pond conditions to make the useful application of the valuable natural products.

Achyranthes aspera (Linn.), an herb commonly known as the prickly

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chaff flower (family: Amaranthaceae) is found throughout India. The plant has application as Ayurvedik Medicine. Among the different parts of the plant, seeds and roots show immunostimularory activity in fish cultured in laboratory conditions. *A. aspera* enhanced antigen-specific antibody, serum globulin levels, anti-trypsin activity etc. in carps (Vasudeva and Chakrabarti, 2005; Chakrabarti and Rao, 2006; Chakrabarti et al., 2012). The seeds supplemented diet protects the fish against UV-B irradiation (Singh et al., 2013a,b, 2015; Sharma et al., 2015). The presence of ecdysterone, various amino acids and two essential fatty acids linolenic acid and oleic acid in seeds of *Achyranthes aspera* shows positive impact on fish (Goyal et al., 2007; Chakrabarti et al., 2012).

Labeo rohita, rohu (family: Cyprinidae) is an herbivore carp and remains in the mid-column region of the water. This species has been intensively used in composite culture and has huge consumers' preference. Various health problems like, ulcer, dropsy, saprolegniasis, argulosis etc. are reported in carps (ICAR (Indian Council of Agricultural Research), 2013). Therefore, there is an urgent need to control the diseases in the natural ponds. The study of non-specific immunological parameters viz. serum lysozyme, myeloperoxidase, nitric oxide synthase etc. helps to understand the health status of the cultured species. The elevated levels of these parameters are indicators of healthy immune system of fish. The expressions of various immune related genes viz. toll-like receptors (TLRs), pro-inflammatory TNF-α, anti-inflammatory interleukin 10 (IL-10) etc. give a clear picture of the immune system of fish (Belvin and Anderson, 1996; Ellis, 2001; Aoki and Hirono, 2006; Magnadottir, 2006). The elevated levels of thiobarbituric acid reactive substances and carbonyl protein are indicators of stress in fish; these are produced after oxidation of lipids and proteins, respectively in tissues. All these information are essential to control the diseases in aquaculture. The present investigation aims to evaluate the immunostimulatory properties of seeds and leaves of *Achyranthes aspera* in rohu *Labeo rohita* in the pond conditions. The growth enhancing property of the plant has also evaluated in the present study.

2. Materials and methods

2.1. Culture of fish and feeding

Indian major carp rohu fry (1.9 ± 0.08 g) were procured from Chatterjee Brothers' Fish Farm, Mogra, West Bengal. Rohu fry were introduced in hapas (25 hapa^{-1}) set inside the pond (54.5 m × 30.5 m × 2.25 m) with bamboo stick (Fig. 1). The pond is located at Rohtak Centre, Central Institute of Fisheries Education (Indian Council of Agricultural Research), Haryana. Each hapa (2.0 m × 1.5 m × 1.5 m) consisted of nylon net; the top of the hapa was covered with a net to avoid the escape of fish. Two test diets enriched with 0.5% seeds (diet 1, D1) and leaves (diet 2, D2) of *Achyranthes aspera* and control diet (diet 3, D3) without plant ingredients were prepared (Table 1) using Twin-screw Extruder (Basic Technology, Kolkata, India). Three replicates were used for each feeding regime. Fish were fed @ 5% of body weight once daily at 9.00 a.m. for 60 days.

Fig. 1. Culture of *Labeo rohita* in hapas in a pond of CIFE Rohtak Centre.

Table 1
Composition of experimental and control diets.

Ingredients	Diets		
	D1	D2	D3
Composition (g kg^{-1})			
Fish powder	482.76	482.76	482.76
Wheat flour	488.24	488.24	493.24
Cod liver oil	20.00	20.00	20.00
Vitamin & mineral pre-mix	4.00	4.00	4.00
Leaves	–	5.00	–
Seeds	5.00	–	–
Proximate composition (g 100 g^{-1})			
Crude protein	36.71	35.67	33.58
Crude fat	8.31	7.82	8.64
Carbohydrates	37.52	38.36	43.45
Ash	8.03	8.12	7.20
Moisture	9.43	10.03	7.14
Crude fibre	0.44	0.65	0.41
Energy (kcal 100 g^{-1})	371.7	366.5	385.8

D1 = diet 1, D2 = diet 2 and D3 = diet.

2.2. Immunization of fish

The chicken blood (c-RBC) was collected in Alsever's solution (1:3) and stored at 4 °C for 24 h. Then c-RBC was washed three times with phosphate buffer saline (PBS, pH 7.4). c-RBC (20 ml) was properly mixed with 80 ml of PBS (pH 7.4). Fish were (25 fish hapa^{-1}, 3 replicates and 3 feeding regimes; 3 × 3 = 9 hapas) anaesthetized with MS-222 (Sigma, USA). Rohu were injected intraperitoneally with 60 µl of 20% (v/v) chicken-RBC (using syringe with 26G × ½ needle, Hindustan Syringes & Medical Devices Ltd.) for immunization. One group of fish was injected with PBS buffer only (sham control). Then fish were released in the respective hapa.

2.3. Average weight, specific growth rate and feed conversion ratio

The average weight of individual fish was recorded during immunization (day-0) and on each day (8 fish hapa^{-1}) of sampling, i.e. on days-7, 14 and 21. The specific growth rate (SGR) and feed conversion ratio (FCR) were calculated.

$$SGR = 100 \times (I_n W_t - I_n W_i)/t$$

where, W_i and W_t were the initial and final body weights and t was the time in days.

FCR = Dry weight food consumed by individual fish during experiment/ Wet weight gain of individual fish.

2.4. Biochemical assays

Fish were sampled on days-7, 14 and 21 after immunization. On each sampling day, fish from hapas (8 fish hapa^{-1}) were collected and anaesthetized with MS-222. The weight of individual fish was measured; then blood and tissue (hepatopancreas) samples were collected for various estimations and gene expression study. Blood sample was collected from individual rohu using Dispo van 2 ml syringe from the caudal vein. The sample was transferred in serological tube and allowed to clot at 4 °C overnight. The serum was then spun down at 400 × *g* for 10 min and stored in sterile tube at −20 °C until used for various assays. Tissue samples were immediately kept at −80 °C for further use. Three replicates were used for each feeding regime.

Serum lysozyme activity was estimated following the method of Siwicki (1989). *Micrococcus lysodeikticus* solution (20 mg 100 ml^{-1} of

0.02 M sodium acetate buffer, pH 5.5) was mixed with 10 µl of serum. The absorbance was recorded at 450 nm with Microplate reader (Synergy H1 Hybrid Reader, Biotek, USA). The activity was expressed in mg ml^{-1}. The Hen's egg lysozyme (Sigma) was used as standard.

Myeloperoxidase content was measured with Hank's balanced salt solution (HBSS, without Ca^{2+} or Mg^{2+}). HBSS (90 µl) was added in each well of 96-well microplate containing 10 µl of serum (Quade and Roth, 1997); 20 mM 3,3′,5,5′ tetramethylbenzidine hydrochloride (35 µl, Genei, India) and 5 mM H_2O_2 were added to each well. The assay mixture was incubated for 2 min; then 35 µl of 4 M H_2SO_4 was added to stop the reaction. The optical density was measured at 450 nm.

Nitric oxide synthase level of hepatopancreas was measured (Lee et al., 2003). The tissue was homogenized and supernatant (100 µl) was mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid); the mixture was incubated 10 min at room temperature. The absorbance was recorded at 540 nm and the activity was expressed as µmol mg $tissue^{-1}$. Sodium nitrite was used as standard.

The thiobarbituric acid reactive substances (TBARS) level in hepatopancreas was assayed (Ohkawa et al., 1979) to study the level of lipid peroxidation. Hepatopancreas (100 mg) was homogenized in chilled KCl (0.9 ml). The sample was incubated for 1 h at 100 °C in acid medium containing 0.45% sodium dodecyl sulphate and 0.6% thiobarbituric acid. After cooling, the sample was centrifuged at 800 ×*g* at 25 °C for 15 min. The optical density of supernatant was measured at 532 nm and expressed as mmol malondialdehyde mg $protein^{-1}$. The standard curve was prepared with 1,1,3,3-tetramethoxy propane.

The carbonyl protein level was assayed to study the oxidation of proteins (Lenz et al., 1989). Hepatopancreas (100 mg) was homogenized in 1 ml of chilled potassium phosphate buffer (50 mM, pH 7.0). Homogenate (250 µl) was mixed with 0.5 ml of 10% trichloroacetic acid; then centrifuged at 13,000 ×*g* for 5 min. Pellet was mixed with 1 ml of dinitrophenyl hydrazine (10 mM) and was incubated at room temperature for 1 h. The mixture was centrifuged at 13,000 ×*g* for 5 min and the pellet was washed three times with 1 ml of ethanol-butyl acetate (1:1, v/v). Pellet was dissolved in 1.5 ml of 6 M guanidine hydrochloride and centrifuged at 13,000 ×*g* for 5 min. The absorbance of supernatant was recorded at 370 nm. The activity was expressed as nanomoles of carbonyl protein mg $protein^{-1}$ in the guanidine hydrochloride solution. The molar extinction coefficient 22 × 10^3 M^{-1} cm^{-1} was used for quantification of carbonyl protein.

2.5. Isolation of RNA, preparation of cDNA and gene expression study

In hepatopancreas, the relative mRNA expressions of lysozyme C, lysozyme G, interleukin 10 (IL-10), tumor necrosis factor-α (TNF-α) and toll like receptor-4 (TLR-4) were studied. The total RNA was extracted using TRIzol reagent (Ambion, Life Technologies, USA) following the protocol of manufacturer. The total RNA concentration was measured with Nanodrop spectrophotometer (Thermo Scientific, USA) at 260 and 280 nm. The purity of RNA was checked and used for qRT-PCR analysis. RNA (1 µg) was treated with 1 U of DNase I (Amplification grade 1 kit, Sigma-Aldrich, USA) to remove any genomic DNA contamination. The quality of DNase treated RNA was checked with agarose gel (1%) electrophoresis. DNase treated RNA was reversed transcribed to cDNA using High-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) following the protocol of manufacturer. The OD of cDNA at 260/280 was 1.8. β-actin PCR-amplification using cDNA as a template also confirmed the high-quality of cDNA.

Gene expression was quantified with qRT-PCR using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) in Quant Studio 6 Flex system (Applied Biosystems). Primers were designed using NCBI online primer design tool (Table 2). Primers efficiency was evaluated with standard curve and melt curve analysis using QuantStudio 6 Flex Real Time PCR system software v1.1 (Supplementary Table S1; Figs. S1 and S2). Samples were run in duplicate with each reference gene and non-template control was also run. The components of 10 µl reaction were as follows: 1 µl cDNA (1:3), 5 µl of 2× PowerUp™ SYBR™ Green PCR Master Mix (Applied Biosystems), 0.25 µl of each primer (2.5 µmol Fw and Rv) and 3.5 µl of nuclease-free water. The cycling conditions were: pre-denaturation at 95 °C for 10 min followed by either 40 cycles of 95 °C for 15 s and 60 °C for 1 min (primer Tm 60 °C) or 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 1 min (primer Tm < 60 °C). The specificity of reaction was estimated using melt curve analysis at 95 °C for 15 s, 60 °C for 1 min and at 95 °C for 15 s. The analysis was performed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The β-actin was used as positive control and for sample normalization (Singh et al., 2016; Sharma et al., 2017; Anand et al., 2018). The data of treatment groups were compared to the control group.

Table 2
Target genes and sequences of primers used for qPCR analysis.

Target gene	Primer	Primer sequence (5′-3′)	Accession number/ reference
Lysozyme C	Lyso C Fw	CGATGATGGCACTCCAGGT	EF203085.1
	Lyso C Rv	CATGCTTTCAGTCCTTCGGC	
Lysozyme G	Lyso G Fw	CAATGGCTTTGGCCTCATGC	KC934746.1
	Lyso G Rv	CACGTGGGAAACTTTGCTCTG	
TNF-α	TNF-α Fw	GGCGGCTTGAAAGTAGTGGA	FN543477.1
	TNF- α Rv	TATGCAGAACGTCGTGGTCC	
IL-10	IL-10 Fw	GCTCAGTGCAGAAGAGTCGAC	Banerjee et al. (2015)
	IL-10 Rv	CCCGCTTGAGATCCTGAAATATA	
TLR-4	TLR-4 Fw	CTAAGAAAGTGCTTGGGCTTCAT	KX218428.1
	TLR-4 Rv	GGTTTGTGGCAATAATGGCTTTC	
β-actin	β-actin Fw	GACTTCGAGCAGGAGATGG	Mohanty and Sahoo (2010)
	β-actin Rv	CAAGAAGGATGGCTGGAACA	

2.6. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) and Duncan's multiple range test (Montgomery, 1984). Statistical significance was accepted at $p < .05$ level.

3. Results

3.1. Average weight, specific growth rate and feed conversion ratio

Plant supplemented diet showed a positive impact on the growth of rohu. The average weight was significantly ($p < .05$) higher in rohu fed with 0.5% seeds supplemented diet (D1) compared to other two feeding regimes in all four days of sampling (Fig. 2). There was no significant ($p > .05$) difference in average weight of D2 and D3 diets

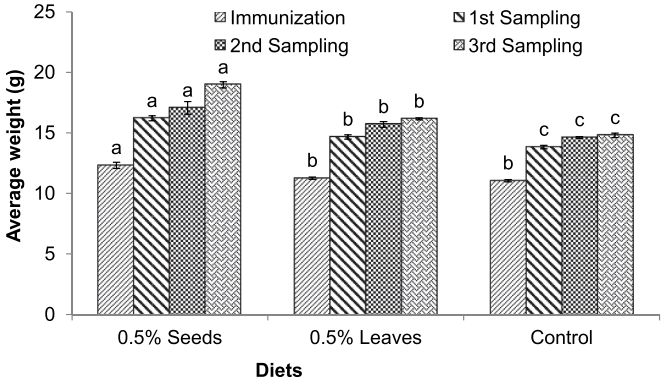

Fig. 2. Average weight of *Labeo rohita* cultured in three different feeding regimes. Vertical bars with different superscripts are significantly ($p < .05$) different (n = 3).

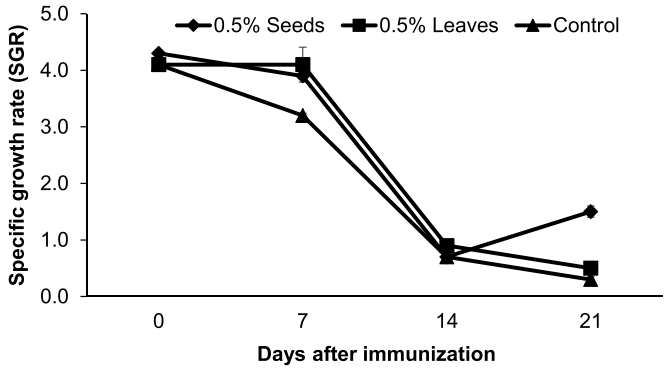

Fig. 3. Specific growth rate of *Labeo rohita* cultured in three different feeding regimes (n = 3).

Table 3
Feed conversion ratio (FCR) of *Labeo rohita* culture under three different feeding regimes and immunized with c-RBC.

Sampling	Feed conversion ratio		
	0.5% Seeds (D1)	0.5% Leaves (D2)	Control (D3)
Immunization day	0.50 ± 0.01	0.55 ± 0.01	0.56 ± 0.01
First sampling	0.44 ± 0.01	0.49 ± 0.01	0.52 ± 0.01
Second sampling	0.47 ± 0.02	0.52 ± 0.00	0.56 ± 0.01
Third sampling	0.46 ± 0.01	0.56 ± 0.00	0.61 ± 0.01

fed rohu at the time of immunization (0-day). Minimum average weight was found in D3 diet fed fish on days-7, 14 and 21 after immunization. The average weight of fish increased gradually with the duration of the culture period regardless of feeding regimes. A similar trend was also observed with specific growth rate. The highest SGR was found in D1 diet fed rohu in all sampling days. SGR showed a decreasing trend with age of fish in all these treatments (Fig. 3). Feed conversion ratio (FCR) was significantly ($p < .05$) lower in D1 diet fed rohu compared to the other feeding regimes throughout the study period (Table 3).

3.2. Biochemical assays

Blood and tissue samples collected on days-7, 14 and 21 after immunization showed the immunostimulatory properties of seeds and leaves of *Achyranthes aspera*. Serum lysozyme activity was significantly ($p < .05$) higher in rohu fed with D1 diet compared to other two feeding regimes. The activity was minimum in D3 diet fed fish throughout the study period. Highest activity was recorded on day-7 after immunization compared to the other days of sampling regardless of feeding regimes (Table 4). Similar trend was found with myeloperoxidase activity. Myeloperoxidase activity was significantly ($p < .05$) higher in D1 diet fed rohu compared to the fish cultured in other two feeding regimes. Highest activity was recorded on day-7 after immunization and then the activity gradually reduced regardless of treatments.

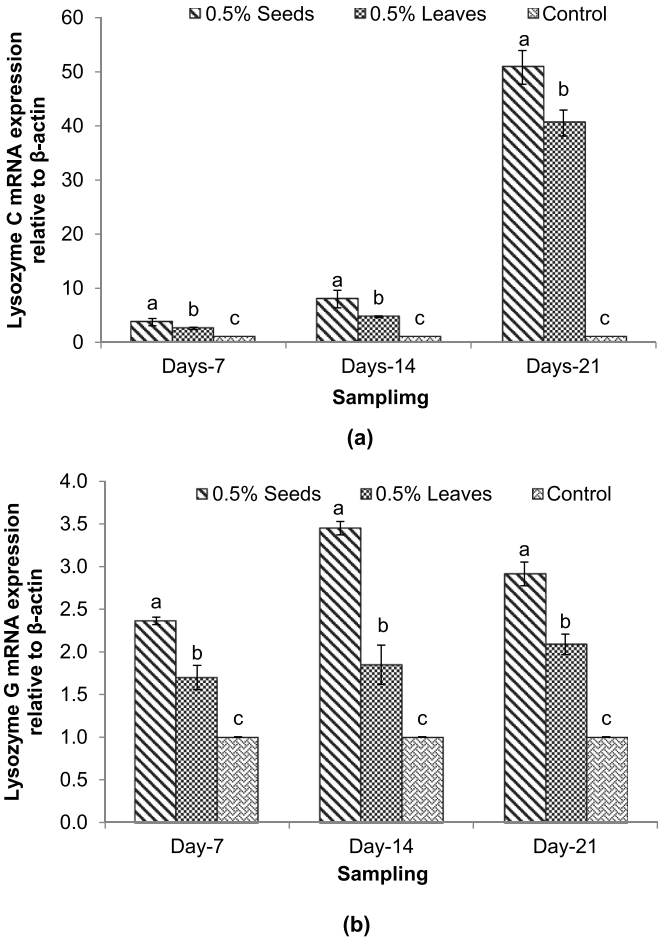

Fig. 4. Expression of (a) lysozyme C and (b) lysozyme G in hepatopancreas of *Labeo rohita* cultured in three different feeding regimes. The relative expression of the target gene lysozyme C/G was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($p < .05$) different (n = 3).

Table 4
Serum lysozyme, myeloperoxidase, nitric oxide synthase, thiobarbituric acid reactive substances (TBARS) and carbonyl protein levels found in *Labeo rohita* in different days after immunization. Means with different superscripts in the same column are significantly ($p < .05$) different (n = 3).

Treatments	Lysozyme (mg ml^{-1})	Myeloperoxidase (OD at 450 nm)	Nitric oxide synthase (μmol mg tissue^{-1})	TBARS (mmol MDA mg protein^{-1})	Carbonyl protein (nmol mg protein^{-1})
First sampling					
D1	105.54 ± 1.0a	1.56 ± 0.04a	4.66 ± 0.07a	0.30 ± 0.02c	2.18 ± 0.07c
D2	99.29 ± 1.71b	1.10 ± 0.06b	0.60 ± 0.02b	0.42 ± 0.05b	2.56 ± 0.07b
D3	95.08 ± 0.46c	0.97 ± 0.03c	0.36 ± 0.01c	0.76 ± 0.03a	3.18 ± 0.05a
Second sampling					
D1	94.29 ± 2.67a	1.31 ± 0.08a	10.17 ± 0.17a	1.26 ± 0.04c	3.44 ± 0.04c
D2	85.71 ± 2.18b	0.93 ± 0.09b	9.40 ± 0.15b	1.75 ± 0.16b	5.18 ± 0.19b
D3	32.38 ± 6.05c	0.75 ± 0.06c	8.45 ± 0.11c	2.51 ± 0.14a	6.48 ± 0.08a
Third sampling					
D1	87.00 ± 3.81a	1.21 ± 0.04a	2.93 ± 0.01a	1.44 ± 0.10c	5.57 ± 0.09c
D2	70.86 ± 0.71b	1.00 ± 0.08b	1.20 ± 0.02b	3.04 ± 0.06b	9.04 ± 0.11b
D3	36.19 ± 2.45c	0.75 ± 0.05c	0.72 ± 0.02c	6.23 ± 0.07a	13.60 ± 0.08a

D1 = diet 1, D2 = diet 2, D3 = diet 3.

Nitric oxide synthase level in hepatopancreas was also significantly ($p < .05$) higher in D1 diet fed rohu compared to other two feeding regimes. Highest activity was recorded on day-14 after immunization compared to day-7 and day-21 after immunization regardless of treatments. Significantly ($p < .05$) lower TBARS level was found in seed-enriched diet (D1) fed rohu compared to others. Carbonyl protein level was significantly ($p < .05$) lower in D1 diet fed rohu compared to the fish cultured in other feeding regimes. Minimum TBARS and carbonyl protein levels were observed on day-7 after immunization and then the level gradually increased with the duration of culture period in all these three feeding regimes. Highest TBARS and carbonyl protein were recorded in D3 fed rohu (Table 4).

3.3. Gene expression

Expression of various immune-related genes supported the physiological study. There were significant ($p < .05$) up-regulation of lysozyme C and lysozyme G in hepatopancreas of rohu fed with D1 diet compared to the fish culture in other feeding regimes (Fig. 4a, b). Highest lysozyme C expression was found on day-21 after immunization in seeds and leaves enriched diets fed rohu compared to fish cultured in same feeding regimes on days-7 and 14. The expression of lysozyme C was higher compared to lysozyme G in the hepatopancreas of rohu regardless of feeding regimes. The expression of TNF-α was significantly ($p < .05$) higher in D1 diet fed rohu compared to other two feeding regimes (Fig. 5) throughout the study period. This group was followed by the D2 diet fed rohu. The activity was highest on day-7 after immunization and then gradually decreased in seeds and leaves enriched diets fed rohu. In enriched diets fed rohu, the expressions of IL-10 were significantly ($p < .05$) lower compared to the control diet fed fish (Fig. 6). The expression of TLR-4 was significantly ($p < .05$) higher in D1 diet fed rohu compared to other two feeding regimes on day-7 after immunization. Then the expression was down-regulated in this feeding regime. In D2 diet fed rohu, the expression was up-regulated on day-21 after immunization (Fig. 7).

4. Discussion

Enrichment of diets with seeds and leaves of *A. aspera* enhanced the growth of rohu. The average weight and SGR were significantly ($p < .05$) higher in seeds supplemented diet (D1) fed rohu before and after immunization. SGR showed a gradual decreasing trend with the days of culture. Average weight was lowest in control diet fed fish. FCR value confirmed that diet was utilized efficiently in enriched diets fed

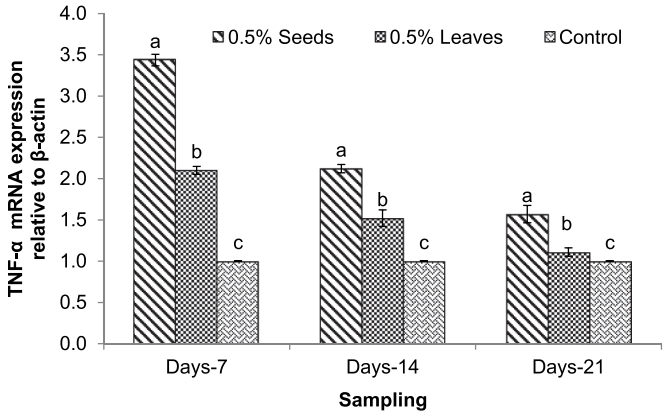

Fig. 5. Expression of TNF-α in hepatopancreas of *Labeo rohita* cultured in three different feeding regimes. The relative expression of the target gene TNF-α was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($p < .05$) different (n = 3).

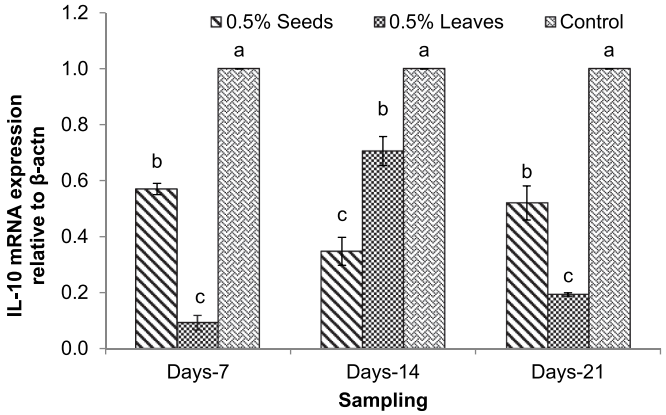

Fig. 6. Expression of IL-10 in hepatopancreas of *Labeo rohita* cultured in three different feeding regimes. The relative expression of the target gene IL-10 was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($p < .05$) different (n = 3).

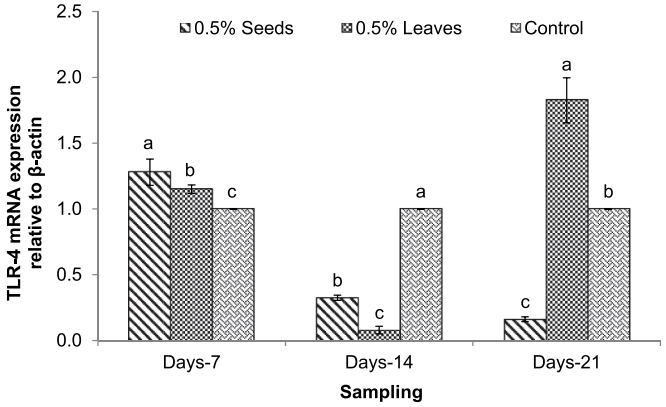

Fig. 7. Expression of TLR-4 in hepatopancreas of *Labeo rohita* cultured in three different feeding regimes. The relative expression of the target gene TLR-4 was normalized to the expression of β-actin (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly ($p < .05$) different (n = 3).

rohu. In laboratory study, supplementations of seeds of *A. aspera* and guava leaves enhanced growth of rohu (Srivastava and Chakrabarti, 2012; Giri et al., 2015). The present pond study confirmed the earlier laboratory experiments. The growth of fish is an indicator of health status that influences the physiological conditions.

The elevated levels of serum lysozyme, myeloperoxidase and nitric oxide synthase in rohu fed with seeds and leaves enriched diets showed the immunostimulatory properties of the plant ingredients even in the pond conditions. These enzymes are indicators of strong defence system of fish. Lysozyme protects the fish from bacterial pathogen as this cationic enzyme is capable to lyse certain Gram-positive and Gram-negative bacteria (Alexander and Ingram, 1992; Saurabh and Sahoo, 2008). Myeloperoxidase has antimicrobial activity and higher level of myeloperoxidase helps in the destruction and elimination of invading pathogens from the host body (Yano, 1992; Dalmo et al., 1997). Nitric oxide synthase catalyzes the production of cellular signalling molecule nitric oxide and this plays vital role in defence mechanism of fish (Rombout et al., 2005). Higher levels of these enzymes were found in seeds supplemented diet fed rohu compared to the leaves enriched diet fed fish. Leaves also showed promising results. This is first report of immunistimulatory properties of leaves of *A. aspera* in pond conditions. Feeding of *Catla catla catla* with *A. aspera* enriched diets increased the serum lysozyme and nitric oxide synthase levels in fish (Chakrabarti

et al., 2014). In earlier study Chakrabarti et al. (2012) showed the presence of linolenic acid (42%) and oleic acid (33%) in the seeds of *A. aspera* along with ecdysterone (3.4–3.65). Seeds are also rich source of various amino acids viz. leucine, isoleucine, phenylalanine, valine etc. (Goyal et al., 2007). The presence of long-chain polyunsaturated fatty acids may be associated with the immunostimulatory properties; amino acids and ecdysterone may play significant role in growth enhancement of the cultured species.

The feeding of rohu with enriched diets reduced the stress compared to the control diet fed one after immunization. The enrichment of diets prevented the oxidation of lipids and proteins in hepatopancreas of immunized rohu. Significantly ($p < .05$) lower levels of TBARS and carbonyl proteins were found in seeds and leaves enriched diets fed rohu compared to the control diet fed fish. The malondialdehyde (MDA) is the major oxidation product and increase in MDA level is a key indicator of lipid peroxidation (Freeman and Crapo, 1981; Bohr et al., 2004). Lipid peroxidation is a well-established mechanism of oxidative damage and reactive oxygen species are responsible for this (Devasena et al., 2001). Singh et al. (2013a) reported that supplementation of *A. aspera* seeds in diets reduced the TBARS level in UV-B irradiated rohu. Carbonyl proteins induction is used as a biomarker of oxidative stress in fish (Parvez and Raisuddin, 2005). Feeding of juvenile jian carp, *Cyprinus carpio* with graded levels of myo-inositol reduced the oxidation of lipids and proteins (Jiang et al., 2010). The oxidation of protein also affected protein metabolism (Dabrowski and Guderley, 2002). In rohu, supplementation of plant ingredients helped in the reduction of oxidative stress in the present study.

Expression of various immune-related genes confirmed the physiological study. In hepatopancreas, lysozyme C, lysozyme G and pro-inflamatory gene TNF-α expression were up-regulated in seeds supplemented diet fed rohu and this group was followed by leaves supplemented diet fed fish. Lysozyme C expression was higher compared to lysozyme G. In the present study, up-regulation of lysozyme genes might be responsible for increased serum lysozyme level. In earlier study, increased expressions of lysozyme C and lysozyme G were found in Japanese flounder *Paralichthys olivaceus* (Hikima et al., 1997, 2001). There was down-regulation of ant-inflammatory gene IL-10 in enriched diets fed rohu compared to the control diet fed fish. TLR-4 expression was higher in D1 and D2 diets fed rohu on day-7 and day-21 after immunization, respectively. In laboratory study, in catla fed with *A. aspera* seed-enriched diet, an inverse relationship was found between the expression of TNF-α and IL-10 in kidney (Chakrabarti et al., 2014). There were up-regulations of IL-1β, TNF-α in head-kidney, intestine and hepatopancreas, whereas, IL-10 was down-regulated in rohu fed with guava leaves supplemented diets (Giri et al., 2015). The feeding of ginger showed up-regulation of IL-10 and transforming growth factor-beta (TGF-β) in rohu (Sukumaran et al., 2016).

5. Conclusions

This was confirmed from the present pond study that like laboratory experiments, both seeds and leaves of *A. aspera* enhanced the growth of rohu, induced the immune system and prevented tissue from oxidative damage. Though seeds performed better compared to the leaves still leaves showed promising results. The information generated from the field study has aquacultural application.

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Conflict of interest

There is no conflict of interest.

Authors' Contributions

RC, JGS: designed the study; NK, VH, RC, JGS: cultured the fish and analysed samples; SPS, AS: helped during sampling and analysis of samples; RC, JGS: wrote the manuscript; NK, SPS: prepared graphs and tables.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.02.065.

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ORIGINAL PAPERS

Evaluation of UV–B protective properties of leaves and seeds of *Achyranthes aspera* in Asian catfish *Clarias batrachus* (Linn.)

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Abstract

The ultraviolet–B (UV–B) radiation is harmful to the aquatic organisms. The UV–B protective properties of leaves and seeds of herb *Achyranthes aspera* were evaluated in *Clarias batrachus*. Fish were fed with four diets—EFL1, EFL2 containing 0.25 and 0.5% leaves, EFS containing 0.5% seeds and control, CF. After 83 days of feeding, fish were exposed to UV–B (157 μW/cm^2) for 7 days at the rate of 15 min/day. One batch of fish in each treatment was kept unexposed. Significantly higher final weight was found in EFS followed by EFL2 and EFL1 treatments. It was higher in unexposed fish compared to the exposed ones. Among exposed fish, significantly higher lysozyme was found in EFS and myeloperoxidase in EFS and EFL2 compared to others. Nitric oxide synthase and superoxide dismutase levels were significantly higher in liver and head kidney of EFS diet fed fish compared to others. Thiobarbituric acid reactive substances (TBARS) and carbonyl protein levels were minimum in EFS followed by EFL2. The independent sample *t*-test showed that nitric oxide synthase was significantly higher and myeloperoxidase and TBARS levels were significantly lower in unexposed group compared to the exposed fish in respective treatment. There were up-regulations of *TNF-α*, *iNOS*, *NF-kB, BAX, Cytochrome c, SOD-c, Caspase 3, Caspase 9, BCL2* in liver and head kidney of leaves and seeds incorporated diets fed fish compared to control. Supplementation of *A. aspera* seeds and leaves at 0.5% level in diets gave UV–B protection to the fish.

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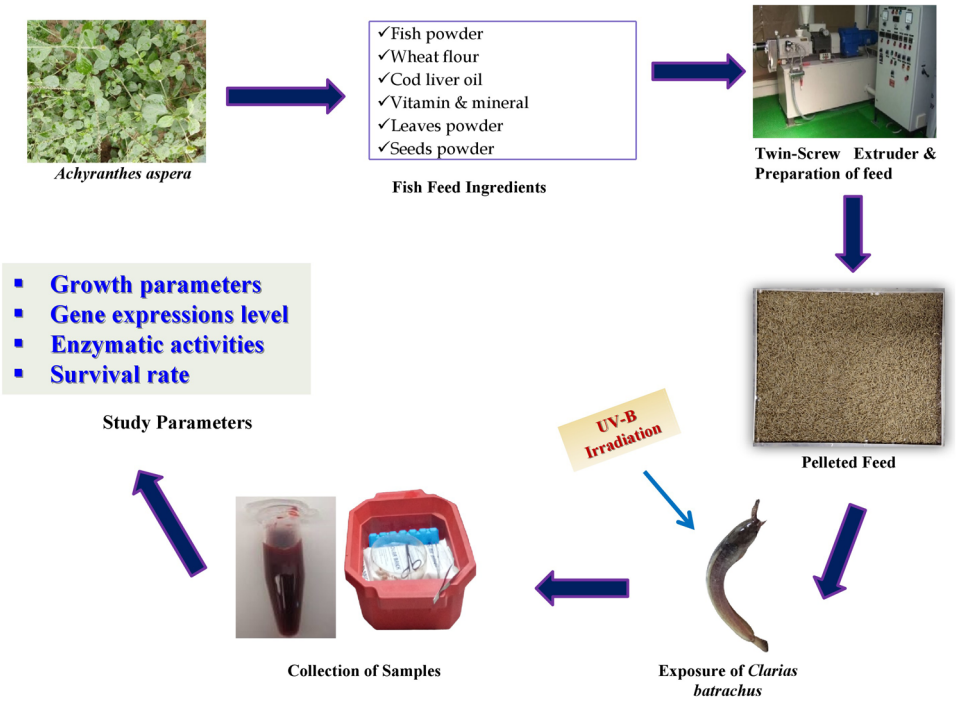
Graphical abstract



Keywords Ultraviolet–B radiation · *Clarias batrachus* · Lysozyme · Myeloperoxidase · *TNF-α* · *Caspase 3*

1 Introduction

The significant stratospheric ozone layer depletion results into enhanced ultraviolet–B (UV–B) radiation to the biosphere [1]. It is a potential threat to the aquatic organisms including fish. UV radiation may penetrate up to a depth of 10 m in freshwater bodies containing less dissolved organic matter [2]. The rate of penetration of UV–B radiation depends on certain factors like, the depth of the water column, presence of dissolved organic carbon and inorganic particulate matter [3, 4]. In shallow and transparent water bodies the penetration rate of UV–B is higher compared to the turbid one [5, 6]. The natural water bodies in India are receiving bright sunlight especially during summer. The intensity of UV–B radiation has been recorded as 236.2 μW cm^{-2} in the month of June in Lake Naini, Delhi, India [7]. The harmful exposure of UV–B radiation affects the aquatic organisms in many ways viz., it affects their behavior, physiology, immune system, reproduction, growth, etc. [8–13]. Thereby, it reduces the aquatic production. Various studies show that UV–B radiation is most detrimental to the early development stages of fishes [14–16]. It is interesting to see that many fishes are also highly sensitive to UV radiation at later development stages [17–21]. The exposure of Atlantic salmon *Salmo salar* juveniles, subadult three-spined stickleback *Gasterosteus aculeatus* and juvenile sea chub *Graus nigra* to UV radiation reduces the growth of fishes and causes loss in body conditions [17, 22, 23]. The exposure to UV–B may reduce the appetite, change the feeding behavior and catabolism in fish [23–25]. In chronic stress, fish probably allocate energy for repairing UV–B-induced DNA damage instead of using the energy for digestion [17, 23]. DNA damage has been observed in UV–B exposed catla *Catla catla* larvae compared to the unexposed control fish [7, 26]. A direct relationship was found between the dose of UV–B and DNA fragmentation in the muscle of catla. DNA damage can be caused directly by exposure to UV radiation or indirectly through the production of reactive oxygen species (ROS). It results into apoptosis or cellular necrosis in many organisms [27, 28].

The enhanced tissue glutamic oxaloacetic transminase (GOT), glutamate pyruvate transminase (GPT), thiobarbituric acid reactive substance (TBARS) and heat shock protein 70 (Hsp 70) are found in UV–B-induced catla larvae [16, 26]. The UV–B irradiation severely affected the immune system of rohu *Labeo rohita* larvae [29] and therefore, the protective mechanism is affected. It suppresses the cellular component (like non-specific cytotoxic cells, NCC) of innate immune system [30–33]. In teleosts, the NCC are the natural defense against infection and diseases, these are like

mammalian natural killer (NK) cells [33]. The UV exposure also suppresses the humoral components of the immune system. In rainbow trout, *Oncorhyncus mykiss* 4 h exposure to UV–B results in a significant decrease in lysozyme level [32], in Atlantic salmon reduced complement bacteriolytic activity and immunoglobulin M (IgM) level are observed after 54 days of exposure [17, 34]. Lysozyme provides an essential defense against Gram-positive bacteria and activates both phagocytes and complements activity [35].

In UV exposed common carp *Cyprinus carpio*, lesions and fungal infection in the dorsal skin are observed on the third week of exposure [31]. These lesions and infection are associated with reduced IgM levels compared to healthy fish. The reduced IgM level may be an indicator of the impairment in the immune function of the fish. Actually, UV radiation induced suppression/modulation of the immune system affected the disease resistance properties in fish [6] resulting in enhanced disease incidences. Like fungal infection in rainbow trout and lahontan cutthroat trout *Oncorhyncus clarkii henshawi* [36] and in guppy *Poecilia reticulata* [21], trematode parasites *Diplostomum spathaceum* and bacteria *Yersinia ruckeri* infections in juvenile rainbow trout [37]. The exposure of UV–B reduces the feed consumption rate and growth of seabream *Sparus aurata* and affects the immune system of fish [38].

Asian catfish *Clarias batrachus* (Linn.), popularly called magur is the member of the Order Siluriformes, Family Clariidae. It is widely distributed and has consumers' preferences for its taste and nutritional quality. The presence of accessory respiratory organ has made them unique and the fish frequently comes to the surface for gulping air. *C. batrachus* are usually cultured in small and shallow water body at high stocking density [39]. The high density culture results into immune system suppression and chances of diseases in fish [40, 41]. The occurrence of ulcerative lesions on the body has been reported in the farmed fish [42]. The 16S rRNA study has confirmed that *Aeromonas caviae*—like bacterium is the causative agent for this. The occurrence of various diseases like bacterial, viral, fungal and parasitic infections is recorded in various developmental stages of *C. batrachus* [43]. The environmental stress may be one of the main factors associated with the outbreak of diseases in this economically important cultivable fish. As the fish is grown in the shallow water at high stocking density and has the habit to come to the water surface frequently, they are more prone to the UV–B exposure. This UV–B exposure is one of the causative factors for stress in fish that results into impaired immune system [6].

Earlier study shows the harmful effect of UV–B in carps (family: Cyprinidae) like, catla and rohu [7, 29, 44]. Therefore, it is essential to evaluate the impact of UV–B on the immune system of the economically important cultivable catfish, magur. It is also essential to find out the remedial measures of UV–B induced damage in fishes to control the disease and this will help in the sustainable aquaculture development. The UV–B protective capacity of vitamin C and seeds of *Achyranthes aspera* are evaluated in carps [26, 29]. The study of immunological parameters helps to understand the defense mechanisms of the fishes. Immunostimulant enhances the immunocompetence in fishes and thereby, protects the fishes from various pathogens. The non-specific defense mechanisms plays significant role in fish [45]. Review of literature shows that so far no study has been conducted to evaluate the impact of UV–B radiation in magur.

The expressions of various immune related genes viz. toll-like receptors (*TLRs*), pro-inflammatory *TNF-α*, anti-inflammatory interleukin 10 (*IL 10*), etc., give a clear picture of the immune system of fish [46–49]. The generation of inducible nitric oxide synthase (*iNOS*)-dependent nitric oxide (NO) may be a tool of fighting against pathogens. NO plays significant role in the induction and/or suppression of apoptotic phenomena [50, 51]. It may protect cells against oxidative stress [52]. In aquatic organisms, environmental stressor may induce abnormal apoptosis [53]. It plays critical role in the regulation of host response during infection [54]. The endoproteases *Caspase 3* plays vital role in apoptotic processes [55]. The oral administration of herbal immunostimulator may be useful to reduce the harmful effect of UV–B in fish.

The herb prickly chaff flower *Achyranthes aspera* is the member of Amaranthaceae family. Various secondary metabolic products are found in different parts of *A. aspera*, like, in the leaves p-benzoquinone, nerol, asarone, hydroquinone compounds are present [56], seeds contain D-glucuronic acid, 10-tricosanone, 4-tritriacontanone, 10-octacosanone, three oleonolic acid glycosides, linolenic and oleic acids [57–61]. In the whole plant, ecdysterone, betaine, achyranthine compounds are found [56, 59]. In a comprehensive study, Kumar et al. [62] have reported the biochemical compositions of seeds and leaves of *A. aspera*. The seeds and leaves are rich sources of amino acids, polyunsaturated fatty acids (PUFA), minerals and vitamins. The immunostimulatory properties of the seeds of the plant are well studied in various carps like, catla, rohu and common carp [61, 63–70].

The present study aims to evaluate the effect of oral administration of seeds and leaves of *A. aspera* on growth, immunostimulation, oxidative stress reduction and modulation of expressions of specific genes related to immune system and cell apoptosis in UV–B-exposed *Clarias batrachus*. The UV–B exposed experimental diets fed magur are compared with control diet fed exposed magur, as well as experimental diets fed unexposed fish. This is the first study to see the effect of leaves supplemented diets in UV–B irradiated catfish. The information generated from the study may be useful to develop an optimum culture technique that

minimizes the occurrence of diseases in the important cultivable fish.

2 Materials and methods

2.1 Formulation of feed and fish culture

Fish feeds were formulated using dry fish powder, wheat flour, cod liver oil and vitamin–mineral premixes (Table 1). In experimental feeds leaves and seeds of *Achyranthes aspera* were incorporated and doses of plant ingredients were selected based on the earlier study [69]. Three experimental feeds were—EFL1, containing 0.25% leaves, EFL2, containing 0.5% leaves and EFS containing 0.5% seeds. The control feed (CF) was without plant ingredients.

The magur *Clarias batrachus* fry were obtained from a fish farm having induced breeding facility. Fry were randomly distributed in twenty four aquaria. The stocking density was 10 fish/aquarium (20 L). Fish were fed with control feed prior to the start of the experiment. After 7 days of acclimation, the fry (352.4 ± 8.0 mg) were fed ad libitum with either control feed (CF) or experimental feed (EFL1/EFL2/EFS) supplemented with leaves/ seeds of *A. aspera*. There were six replicates for each feeding scheme (6 replicates × 4 treatments). Fish were fed twice daily at 9.00 a.m. and 5.00 p.m. The excess food and excreta were siphoned regularly. Water quality parameters like, temperature, pH and dissolved oxygen contents were monitored using HACH (USA) water quality analyzer attached with specific probes at weekly intervals. Water temperature, pH and dissolved oxygen levels ranged from 25.6 to 26.5 °C, 7.5–7.67 and 5.10–6.25 mg/L throughout the study period in different culture systems.

The guidelines of the University of Delhi Institutional Animal Ethics Committee, IAEC (DU/ZOOL/IAEC-R/2015/08) were followed for the culture of magur and their sampling.

Table 1 Formulation of fish diets and evaluation of their proximate composition

Ingredient (g/kg diet)	Diets			
	CF	EFL1	EFL2	EFS
Feed composition				
Fishmeal	651.6	651.6	651.6	651.6
Wheat flour	334.4	331.9	329.4	329.4
Cod liver oil	10.0	10.0	10.0	10.0
Vitamin and mineral complex	4.0	4.0	4.0	4.0
Leaves powder	–	2.5	5.00	–
Seeds powder	–	–	–	5.0
Proximate composition (g/100 g on dry matter basis)				
Moisture	7.14	11.09	10.03	9.43
Crude protein	36.96	37.05	36.98	37.02
Crude fat	8.64	9.24	7.82	8.31
Total carbohydrates	40.06	34.47	37.05	37.21
Ash	7.20	8.15	8.12	8.03
Crude fiber	4.1	6.8	6.5	4.4
Energy (kcal/100 g)	385.84	369.24	366.50	371.71

2.2 Exposure of fish to UV–B and various assays

After 83 days of feeding, magur kept in three replicates of each feeding scheme (30 fish/treatment) were exposed to UV–B irradiation. The other three replicates of the same feeding scheme remained unexposed to UV–B (served as positive control). The Philips tube light (TL 20/12 RS, Holland) was served as source of UV–B (280–320 nm). The pre–burned (100 h) tubes were fixed above the aquaria (one tube/aquarium) and to avoid outside light and reflectance between aquaria itself, the aquaria were covered with black plastic sheets. The dose of UV–B was 157.11 ± 0.31 μW/cm^2. The duration of exposure was 15 min/day and it was continued for 7 days. The spectral output of the tubes, as defined by the manufacturer has maximum emission at 313 nm, with negligible energy above 320 [71]. UV–B tubes were covered with cellulose acetate, which absorbs wavelength of < 280 nm. The Sunphotometer, Microtops II of Solar Light Company (USA) was used for the measurement of UV–B irradiation. Experimental and control batches of magur were kept under fluorescent lamps (Philips 20 W) without UV components. The photoperiod was as follows: dark 12 h: light 12 h. The dose of UV–B radiation and exposure duration in the present study were selected on the basis of earlier study [14]. They used three exposure durations of 5, 10 and 15 min. In the present study, 15-min exposure was used to evaluate the UV–B protective properties of the diets at highest level.

Fish were harvested and euthanized with MS-222 (Sigma, USA). The number of fish in each tank was counted and the final weight of individual fish was recorded. The blood sample was collected from the caudal vein of individual fish with needles fitted in a syringe (1 mL) that was rinsed with ethylenediaminetetraacetic acid (2.7%). The sample was kept at 4 °C overnight for clotting, then the sample was centrifuged at 400×*g* (4 °C for 10 min) and serum was stored at − 20 °C for various assays. The liver and head kidney were collected from individual magur and stored at − 80 °C for various enzymatic assays. The liver and head kidney of four fish per treatment were kept in TRIzol reagent (Thermo Scientific, USA) for gene expression study.

The specific growth rate (SGR) of fish was calculated using the formula:

$$\mathrm{SGR} = \left(\ln W_t - \ln W_i\right)/t \times 100$$

where, W_i and W_t were the initial and final body weight and t, the time in days.

2.3 Assays

The enzymatic assays were performed with Synergy H1 Hybrid Multi-mode Reader (BioTek, USA). Three replicates were used for each assay. The serum lysozyme was estimated following the method of Siwicki [72]. In brief, 10 µL serum was mixed with 100 µL *Micrococcus luteus* solution. The initial absorbance and the final absorbance (after 1 h of incubation) were recorded at 450 nm. The serum lysozyme activity was expressed as mg/mL.

The myeloperoxidase contents were assayed following the method of Quade and Roth [73]. The reaction mixture was composed of serum (10 µL), Hank's balanced salt solution (90 µL) and tetramethyl benzidine/hydrogen peroxide (35 µL). After proper mixing, it was incubated for 2 min and then sulfuric acid (35 µL) was added to stop the reaction. The myeloperoxidase activity was recorded at 450 nm.

The chicken blood (c-RBC) was used for the hemagglutination assay. In Alsever's solution, chicken blood was collected (1:3) and stored in a refrigerator (4 °C) for 24 h. It was washed thrice with PBS (pH 7.4), centrifuged and mixed in the PBS to make 2% (v/v) solution. In a 96-well round bottom ELISA plate, a 50 µL serum was serially diluted with PBS. Then 50 µL of c–RBC (2%) was added to each well, incubated for 60 min at 25 °C, and then overnight at 4 °C. The reciprocal of the highest dilution that represented agglutination was considered as the hemagglutination antibody titer.

The tissue nitric oxide synthase level was measured following the protocol of Lee et al. [74]. In 1-mL phosphate-buffer saline solution (pH 7.4), the tissue was blended and then centrifuged at 4 °C for 15 min (10,000×*g*). The supernatant was used for the assay. In each well of 96-well microplate, 100 µL supernatant was poured and mixed with 100 µL Griess reagent. The mixture was incubated for 10 min. The nitric oxide synthase activity was measured at 540 nm and expressed as µmol/mg tissue.

The thiobarbituric acid reactive substances (TBARS) assay indicates the tissue lipid oxidation [75]. The tissue sample was washed in chilled 0.9% NaCl and then homogenized in chilled potassium chloride (KCl) solution (1 g in 900 µL). In a micro-centrifuge tube, 25 µL homogenate was taken. Then sodium dodecyl sulfate (25 µL), 20% acetic acid (187.5 µL), 0.8% thiobarbituric acid (187.5 µL) and 75 µL of distilled water were added to it and mixed properly. The whole mixture was kept at 95 °C in a water bath for 1 h. After cooling the sample, distilled water (125 µL) and a mixture of *n*–butanol:pyridine (625 µL) were added to it. The whole reaction mixture was shaken vigorously, centrifuged at 800×*g* for 10 min at 25 °C and organic layer was taken for the assay. The TBARS level was measured at 532 nm and expressed as mmol MDA/mg tissue.

Tissue carbonyl protein level was estimated [76]. In 1 mL of 50 mmol potassium phosphate buffer, 100 mg tissue was homogenized. The ethylenediaminetetraacetic acid and phenylmethylsulfonyl fluoride were present in the buffer. In a microcentrifuge tube, 250 µL homogenate and 500 µL 10% trichloroacetic acid were taken and centrifuged (13,000×*g*) at 25 °C for 5 min. Pellet was collected, mixed with 1 mL (10 mmol) 2,4–dinitrophenylhydrazine (DNPH) and incubated for 1 h at 25 °C. After incubation, mixture was centrifuged (13,000×*g*) at 25 °C for 5 min. The pellet was obtained and washed with 1 mL ethanol:butylacetate mixture (1:1), then supernatant was discarded. The procedure of washing was repeated thrice. Final pellet was dissolved in 1.5-mL 6 M guanidine hydrochloride and centrifuged at 13,000×*g* at 25 °C for 5 min. The supernatant was used for the estimation of carbonyl protein at 370 nm and the content was expressed as nmol/mg protein. The molar extinction coefficient was 22×10^3/M/cm.

The superoxide dismutase (SOD) was measured in tissues [77]. In 1 mL of chilled 0.1 M sodium phosphate buffer, 100 mg tissue was blended and centrifuged at 10,500×*g* for 20 min at 4 °C. The supernatant was used for estimation of superoxide dismutase. The reaction mixture was prepared with 1.2 mL of 0.052 M sodium pyrophosphate buffer, 200 µL of post mitochondrial supernatant (PMS), 100 µL of 186 mM phenazine methosulphate, 300 µL of 300 mM nitroblue tetrazolium and 1 mL of distilled water. In the reaction mixture, 200 µL of 780 mM nicotinamide adenine dinucleotide, NADH was added and incubated for 90 s. Then 1 mL of glacial acetic acid was added to stop the reaction. The superoxide dismutase level was measured at 560 nm and expressed as units/mg protein. Total protein content was measured with Folin-Ciocalteu reagent [78]. The bovine serum albumin was used as standard and absorbance was recorded at 750 nm. Three replicates were used for all these assays.

2.4 Relative mRNA expression

The liver and head kidney of magur kept in TRIzol® Reagent were used for the study of relative mRNA expressions for different genes viz., *tumor necrosis factor-α* (*TNF-α*), *inducible nitric oxide synthase* (*iNOS*), *nuclear factor-kB* (*NF-kB*), *bcl-2-associated X protein* (*BAX*), *cytochrome complex* (*Cytochrome c*), *superoxide dismutase-c* (*SOD-c*), *Caspase 3, Caspase 9, B-cell lymphoma 2* (*BCL 2*). The RNA was extracted from the tissues. The NanoDrop Spectrophotometer of Thermo Scientific, USA was used to check the purity of RNA (260:280). The RNA quality was

tested in gel electrophoresis (agarose 1%). The RNA (1 µg) was first treated with 1U of DNase I (Sigma-Aldrich) to avoid contamination of DNA. The treated RNA was used for cDNA preparation through highcapacity cDNA reverse transcription (ThermoFisher Scientific) kit. The cDNA synthesis confirmation was checked with *β-actin* through PCR-amplification and gel electrophoresis.

The relative quantification of various genes was performed with PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific). The Applied Biosystems (USA) QuantStudio 6 Flex real-time PCR was used for the estimation. The primers for specific genes were designed using NCBI tool (Table 2). The efficiency and specificity of primers were also checked using a serial dilution of cDNA and constructing a standard curve with correlation coefficient. The reaction mixture (10 µL) consisted of cDNA (1 µL, 1:3), 2 × PowerUp™ SYBR™ Green PCR Master Mix (5 µL), 2.5 µmol forward and reverse primers (0.25 µL each) and nuclease free water (3.5 µL). The conditions of real-time PCR were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All samples were run in duplicates and a non-template control (NTC) was also run without cDNA. The specificity of qPCR was checked through melt curve analysis. The conditions for melt curve analysis are as follows: 95 °C for 15 s, 60 °C for 1 min, and at 95 °C for 15 s. After completing the programme, the threshold cycle (C_T) value of each well was recorded and analysis was performed using standard $2^{-\Delta\Delta C_T}$ method [79]. It was considered that primers were amplified with 100% efficiency. The *β-actin* was used as a housekeeping gene and also used for normalization of target genes. The data of treatment groups were compared to the control group.

2.5 Statistical analysis

The data for growth parameters, various enzymes and mRNA expressions are given as means ± standard error. The Kolmogorov–Smirnova test was performed to check the normality of the data/ parameters [80]. The significance value $p > 0.05$ indicates normality in all parameters except, hemagglutination titer that resulted in significance value $p < 0.05$ in all treatment categories. One-way Multi-variate analysis of variance (MANOVA) was performed to find out significant differences in the parameters among different treatments for the exposed and unexposed groups. The differences between two or more groups on multiple variables at once assessed with Wilks' lambda test [81]. To determine how the parameters differ in the various treatment groups, the Tests of Between-Subjects Effects was performed. Partial eta squared (η^2) is a way to measure and compare the effect size in the different variables [82]. Further, Tukey HSD post hoc analysis confirmed the pair-wise significance among different treatments and exposed and unexposed groups of individual treatment for each parameter [83]. The exposed and unexposed groups were compared using independent sample *t*-test. Statistical analysis was performed using SPSS 26.0 version [South Asia (P) Ltd., Bangalore, India] software. The significance level was accepted as $p < 0.05$.

Table 2 Target genes and sequences of primers used for qPCR analysis

Target gene	Forward/reverse	Sequence (5′–3′)	Product length	Accession number
TNF–α	Forward reverse	TCTCAGGTCAATACAACCCGC GAGGCCTTTGCGGAAAATCTTG	125	KM593875
NF–kB	Forward reverse	CTCGGTGAAGCGAGGAAGAG CATTCCGGCTCCGACTCTC	124	MG571500
iNOS	Forward reverse	ATGGGCACTGAGATTGGAGC CTTCGTCCTTCCACAGCGAT	121	KT180212
BAX	Forward reverse	GTAGTGTCGCGGAACGAACT TTTAGTCGGCTGGAGCGATG	150	KT003584
Cytochrome c	Forward reverse	TATACCCACCCCTTGCAGGA GGAGATGGCTGGCGGTTTTA	149	KT835312
SOD-C	Forward reverse	CATGGTGGACCACGTGATGA TGATTGAGTGAGGCCCAAGC	125	KF444052
Caspase 3	Forward reverse	TTGGAGGAACACACTGACCG AGGCAGACTCAAAGTCCAGC	137	KC921209
Caspase 9	Forward reverse	GAGGAAGTCGGGCTTTCACA AGGCTGTGTAGGGACAGGAA	128	KP261050
Bcl-2	Forward reverse	ATTACTGAAGACGCAGCCCC CGAGAGCAGGACGGTGTAAA	134	KC907874
β-actin	Forward reverse	GAGCACCCTGTCCTGCTTAC GTACAGGGACAGCACAGCC	132	EU527190

3 Results

3.1 Survival, final weight and specific growth rate of fish

The number of fish was recorded in all treatments after 90 days of culture. The survival rate of fish was not affected with UV–B exposure, all fish survived. Among UV–B-exposed fish, significantly higher final weight was found in 0.5% seed incorporated diet (EFS) fed magur compared to the other diets fed fish (Fig. 1). Minimum final weight was recorded in control diet (CF) fed fish. Specific growth rate (SGR) of magur was maximum and minimum in EFS and CF treatments, respectively. Similar trend was observed in unexposed fish. The final weight was higher in unexposed fish compared to the UV–B-exposed ones in the respective feeding regime.

3.2 Lysozyme, myeloperoxidase and hemagglutination titer

Among the exposed fish, serum lysozyme level was significantly higher in 0.5% seed incorporated diet EFS fed magur compared to the other diets fed fish (Table 3). The fish fed 0.5% leaves incorporated diet, EFL2 followed EFS diet fed fish. Similar trend was also found with unexposed fish. Myeloperoxidase levels were significantly higher in EFS and EFL2 diets fed exposed fish compared to other diets fed exposed fish. Among unexposed magur, highest myeloperoxidase level was found in EFS diet fed fish followed by EFL2, EFL1 treatments and minimum in CF. Highest hemagglutination titer level was found in EFS diet fed exposed fish and similar trend was found in the unexposed one. Myeloperoxidase level was significantly higher in exposed fish compared to the unexposed fish in the respective treatment.

3.3 Nitric oxide synthase, superoxide dismutase, thiobarbituric acid reactive substances and carbonyl protein

In liver of exposed fish, significantly higher nitric oxide synthase level was found in EFS diet fed magur compared to fish fed other diets (Table 4). This treatment was followed by EFL2, EFL1 and CF. Similar trend was found with unexposed fish. Significantly higher superoxide dismutase level was recorded in liver of 0.5% seeds incorporated diet fed magur compared to other treatments of both exposed and unexposed groups. TBARS level was significantly higher in CF diet fed magur compared to the experimental diets fed fish. It is applicable for both UV–B exposed and unexposed fish. An inverse relationship was found between the increasing dose of leaves in diets and TBARS levels in the liver of fish. Similarly carbonyl protein level in liver showed inverse relationship with the inclusion level of leaves in the diets of fish. It was significantly higher in the CF diet fed fish compared to the other treatments.

In head kidney of both exposed and unexposed fish, significantly higher nitric oxide synthase and superoxide dismutase levels were observed in EFS treatment compared to other treatments in respective exposure of fish (Table 5). Whereas, significantly higher TBARS and carbonyl protein levels were recorded in control diet (CF) fed UV–B

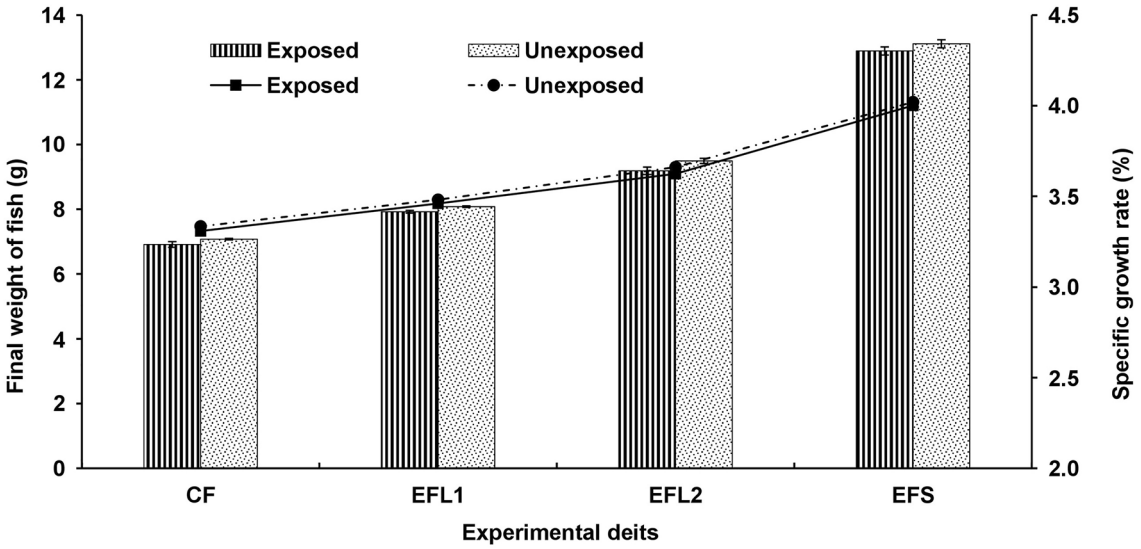


Fig. 1 Final body weight and specific growth rate of UV–B exposed and unexposed *Clarias batrachus* cultured in four different feeding regimes ($n = 3$)

Table 3 Serum lysozyme, myeloperoxidase and hemagglutination titer levels found in different diets fed and UV–B exposed and unexposed *Clarias batrachus*

Exposure	Experimental diets				*F*	df	*p*-value	Partial eta squared
	CF	EFL1	EFL2	EFS				
Serum lysozyme (mg/mL)								
Exposed	22.86 ± 1.01^{c}	40.32 ± 5.47^{c}	133.81 ± 5.66^{b}	200 ± 8.16^{a}	212.421	3	$p < 0.001$	0.982
Unexposed	21.91 ± 3.75^{c}	33.33 ± 2.99^{c}	128.57 ± 6.20^{b}	186.19 ± 3.70^{a}	329.268	3	$p < 0.001$	0.988
*p- value	0.814	0.350	0.556	0.174	–	6	–	–
Myeloperoxidase (OD at 450 nm)								
Exposed	1.922 ± 0.02^{b}	1.977 ± 0.04^{b}	2.88 ± 0.04^{a}	2.981 ± 0.04^{a}	254.671	3	$p < 0.001$	0.985
Unexposed	1.115 ± 0.06^{d}	1.423 ± 0.03^{c}	2.056 ± 0.04^{b}	2.338 ± 0.03^{a}	172.303	3	$p < 0.001$	0.977
*p-value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	6	–	–
Hemagglutination titer								
Exposed	12 ± 2.31^{b}	20 ± 5.66^{ab}	24 ± 4.62^{ab}	28 ± 4.00^{a}	3.182	3	0.063	0.443
Unexposed	10 ± 2.00^{b}	14 ± 2.83^{ab}	20 ± 4.00^{ab}	24 ± 4.62^{a}	3.412	3	0.053	0.460
*p- value	0.537	0.228	0.537	0.537	–	6	–	–

Means with different superscript in the same row are significantly different ($n = 3$). It indicates the differences among various treatments in both exposed and unexposed fish

*p-value indicates the comparison between exposed and unexposed groups using independent sample t-test

Table 4 Nitric oxide synthase, superoxide dismutase, TBARS and carbonyl protein levels found in liver of different diets fed and UV–B exposed and unexposed *Clarias batrachus*

Parameters	Experimental diets				*F*	df	*p*-value	Partial eta squared
	CF	EFL1	EFL2	EFS				
Nitric oxide synthase (μmol/mg tissue)								
Exposed	0.60 ± 0.02^{d}	1.78 ± 0.02^{c}	2.41 ± 0.03^{b}	3.09 ± 0.04^{a}	1350.163	3	$p < 0.001$	0.997
Unexposed	3.27 ± 0.03^{d}	4.43 ± 0.04^{c}	6.06 ± 0.18^{b}	7.00 ± 0.28^{a}	97.733	3	$p < 0.001$	0.961
*p-value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	6	–	–
Superoxide dismutase (unit/mg protein)								
Exposed	56.04 ± 1.55^{d}	75.23 ± 2.47^{c}	109.58 ± 3.27^{b}	168.60 ± 1.89^{a}	427.976	3	$p < 0.001$	0.991
Unexposed	56.30 ± 2.04^{d}	79.14 ± 1.04^{c}	115.16 ± 3.07^{b}	176.36 ± 2.68^{a}	484.638	3	$p < 0.001$	0.992
*p-value	0.926	0.195	0.260	0.130	–	6	–	–
TBARS (mM MDA/mg protein)								
Exposed	4.67 ± 0.06^{a}	2.70 ± 0.11^{b}	1.66 ± 0.02^{c}	1.11 ± 0.02^{d}	592.074	3	$p < 0.001$	0.993
Unexposed	2.55 ± 0.07^{a}	1.44 ± 0.04^{b}	0.69 ± 0.01^{c}	0.32 ± 0.04^{d}	511.388	3	$p < 0.001$	0.992
*p-value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	6	–	–
Carbonyl protein (nmol/mg protein)								
Exposed	10.46 ± 0.23^{a}	4.41 ± 0.11^{b}	2.58 ± 0.07^{c}	0.95 ± 0.06^{d}	934.450	3	$p < 0.001$	0.996
Unexposed	7.96 ± 0.17^{a}	3.63 ± 0.13^{b}	2.46 ± 0.09^{c}	0.91 ± 0.04^{d}	651.819	3	$p < 0.001$	0.994
*p-value	$p < 0.001$	0.003	0.339	0.579	–	6	–	–

Means with different superscript in the same row are significantly different ($n = 3$). It indicates the differences among various treatments in both exposed and unexposed fish

*p-value indicates the comparison between exposed and unexposed groups using independent sample t-test

exposed and unexposed fish compared to the experimental diets fed ones. An inverse relationship was found between the increasing dose of leaves in diets and TBARS and carbonyl protein levels in head kidney of magur.

The independent sample t-test was performed to compare the exposed and unexposed fish. It is interesting to see that nitric oxide synthase was significantly higher and TBARS level was significantly lower in liver of unexposed group

Table 5 Nitric oxide synthase, superoxide dismutase, TBARS and carbonyl protein levels found in head kidney of different diets fed and UV–B exposed and unexposed *Clarias batrachus*

Parameters	Experimental diets				*F*	df	*p*-value	Partial eta squared
	CF	EFL1	EFL2	EFS				
Nitric oxide synthase (μmol/mg tissue)								
Exposed	0.73 ± 0.03^{d}	1.11 ± 0.03^{c}	2.26 ± 0.03^{b}	2.46 ± 0.05^{a}	514.908	3	$p < 0.001$	0.992
Unexposed	1.77 ± 0.03^{d}	2.27 ± 0.03^{c}	4.03 ± 0.05^{b}	4.97 ± 0.04^{a}	1517.512	3	$p < 0.001$	0.997
*p-value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	6	–	–
Superoxide dismutase (unit/mg protein)								
Exposed	90.60 ± 6.84^{d}	109.40 ± 2.06^{c}	195.86 ± 3.92^{b}	287.01 ± 6.18^{a}	310.202	3	$p < 0.001$	0.987
Unexposed	61.74 ± 2.65^{d}	94.87 ± 0.70^{c}	136.47 ± 0.86^{b}	175.17 ± 2.33^{a}	712.669	3	$p < 0.001$	0.994
*p-value	0.008	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	6	–	–
TBARS (mM MDA/mg protein)								
Exposed	11.76 ± 0.22^{a}	6.89 ± 0.07^{b}	3.01 ± 0.08^{c}	1.29 ± 0.01^{d}	1387.058	3	$p < 0.001$	0.997
Unexposed	4.56 ± 0.14^{a}	3.13 ± 0.03^{b}	1.64 ± 0.04^{c}	0.433 ± 0.02^{d}	552.603	3	$p < 0.001$	0.993
*p-value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	6	–	–
Carbonyl protein (nmol/mg protein)								
Exposed	80.16 ± 2.32^{a}	46.44 ± 0.78^{b}	17.87 ± 0.32^{c}	10.81 ± 0.15^{d}	650.198	3	$p < 0.001$	0.994
Unexposed	10.47 ± 0.26^{a}	4.87 ± 0.11^{b}	2.43 ± 0.12^{c}	0.79 ± 0.12^{d}	658.102	3	$p < 0.001$	0.994
*p-value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	6	–	–

Means with different superscript in the same row are significantly different ($n = 3$). It indicates the differences among various treatments in both exposed and unexposed fish

*p-value indicates the comparison between exposed and unexposed groups using independent sample t-test

compared to the exposed fish in the respective treatment. Carbonyl protein levels in CF and EFL1 were significantly lower in liver of unexposed magur compared to the exposed fish in the respective treatment. There was no significant difference in carbonyl protein level in liver of EFL2 and EFS diets fed exposed and unexposed fish. In head kidney of unexposed fish, nitric oxide synthase level was significantly higher and superoxide dismutase, TBARS and carbonyl protein levels were significantly lower compared to the UV–B exposed fish in the respective treatment.

3.4 Relative mRNA expression

In liver and head kidney of both UV–B exposed and unexposed magur, there were up regulations of *TNF-α*, *iNOS*, *NF-kB, BAX, Cytochrome c, SOD-c, Caspase 3, Caspase 9, BCL2* in *A. aspera* leaves and seeds incorporated diets fed fish compared to the control diet fed fish. In liver, up regulation of *TNF-α*, *NF-kB*, *BAX*, *SOD-c, Caspase 3* and *Caspase 9* were recorded in 0.5% seeds supplemented diet (EFS) fed exposed magur compared to the other diets fed exposed ones (Fig. 2a). The expressions of *iNOS* and *BCL-2* were significantly higher in liver of 0.5% leaves and 0.5% seeds supplemented diets fed exposed fish compared to the other diets fed exposed ones.

In head kidney of UV–B-exposed 0.5% seeds supplemented diet fed fish (EFS), significantly higher expressions of *TNF-α*, *NF-kB*, *iNOS, BAX*, *SOD-c, Caspase 3*, *Caspase 9* and *BCL-2* were recorded compared to the other diets fed exposed fish (Fig. 2b). Among unexposed fish, significantly higher expressions of *TNF-α*, *BAX*, *SOD-c, Caspase 9* and *BCL-2* were found in EFS compared to other treatments.

The independent sample *t*-test showed that the expressions of all genes in liver (except *NF-kB* in EFL1 and EFL2, *iNOS* in EFL1 and EFS, *Caspase 9* in EFL2, *BCL-2* in EFL1) were significantly higher in UV–B exposed fish compared to the unexposed ones. Similarly, in head kidney significantly higher expressions were observed in exposed fish (except, *BAX* in EFL1 and EFL2, *Caspase 3* in EFL1, *Caspase 9* in EFL2 and *BCL-2* in EFL1 and EFL2) compared to the unexposed ones.

4 Discussion

The influence of oral administration of seeds and leaves of *A. aspera* on the UV–B exposed magur was recorded in the present study. A 7 days UV–B exposure affected the final weight of magur. Among the exposed fish, significantly higher final weight was recorded in 0.5% seeds incorporated diet (EFS) fed magur compared to other diets fed ones. Even the final weight was significantly higher in 0.5% (EFL2) and 0.25% (EFL1) leaves supplemented diets fed magur compared to the control diet (CF) fed fish after 7 days of UV–B

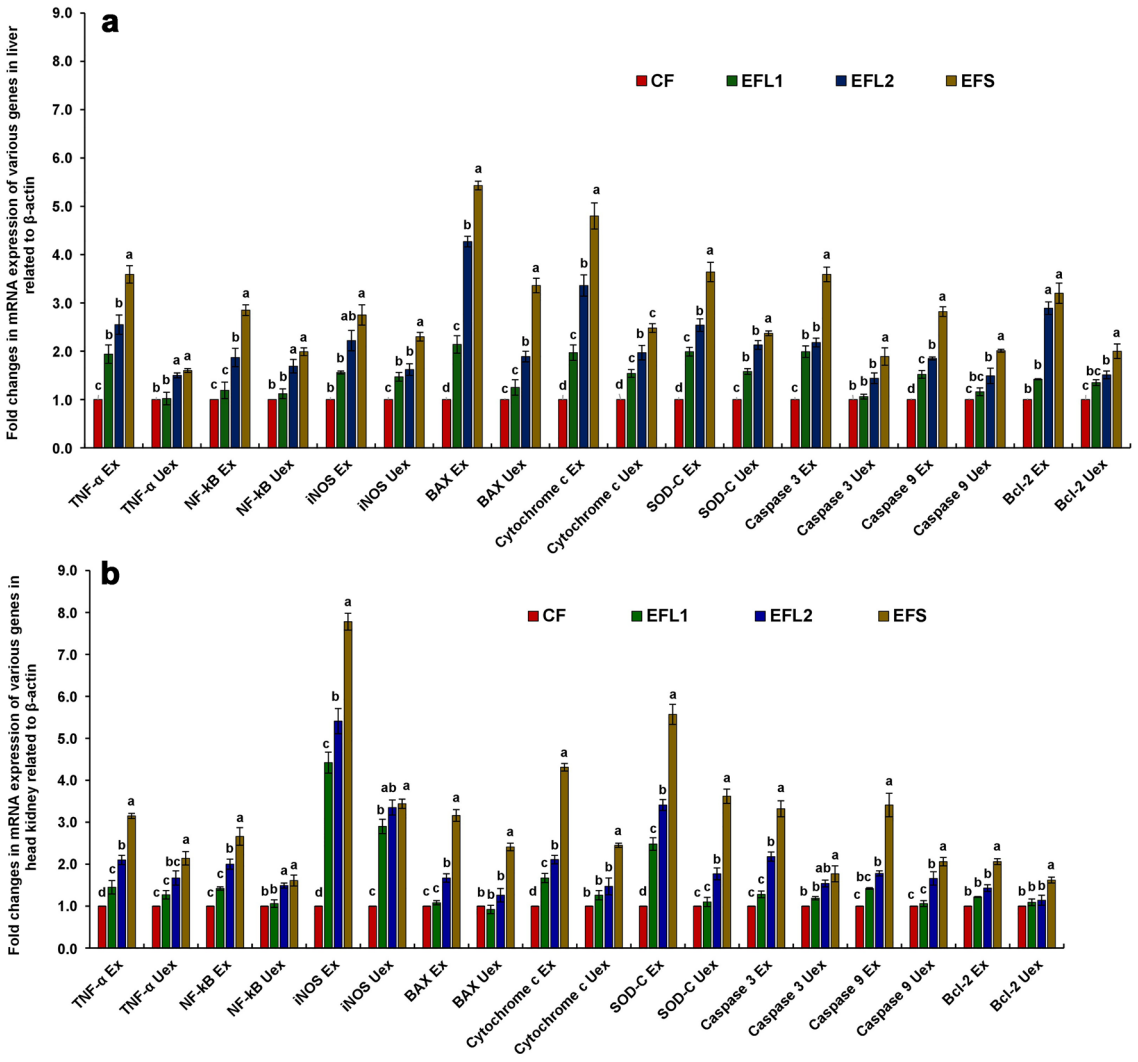


Fig. 2 Expression of *TNF-α*, *iNOS*, *NF-kB, BAX, Cytochrome c, SOD-c, Caspase 3, Caspase 9, BCL2* in (**a**) liver and (**b**) head kidney of four different diets fed *Clarias batrachus*. The relative expressions of the target genes were normalized to the expression of *β-actin* (internal control) and expressed as fold changes relative to the control diet fed group. Vertical bars with different superscripts are significantly different ($n=4$)

exposure. Similar higher growth was reported in UV–B exposed 0.5% *A. aspera* seeds supplemented diet fed rohu [44] and catla [16] compared to the control diet fed fishes. Enhanced growth was found in *Basella alba* leaf extract supplemented diets fed Nile tilapia *Oreochromis niloticus* [84] and in *Coriandrum sativum* extract incorporated diets fed rainbow trout *Oncorhynchus mykiss* [85]. The present study is the first report that shows positive impact of leaves of *A. aspera* in UV–B-exposed fish.

Feeding of magur with 0.5% seeds and 0.5% leaves incorporated diets stimulates their immune systems as enhanced serum lysozyme and myeloperoxidase levels were found in fish cultured in these two treatments compared to others. UV–B exposure enhanced the myeloperoxidase activity as higher values were found in exposed fish compared to the unexposed fish of respective treatment. The bactericidal activity of lysozyme helps to control the infection [86, 87]. The supplementation of herbal ingredients and their extracts enhanced lysozyme levels in various fishes, viz., the ethanolic extract of grass, *Cynodon dactylon* [88] in catla, leaf extract of *Azadirachta indica* in Asian seabass *Lates calcarifer* [89], *Euphorbia hirta* leaf extract in common carp [90]. Significantly higher levels of serum lysozyme and myeloperoxidase were reported in 0.5% *A. aspera* seeds enriched diet fed rohu after immunization with chicken–RBC [69]. In tilapia *Oreochromis mossambicus*, incorporation of *Citrus limon* peels oil [91] and night jasmine *Nyctanthes arbortristis* seeds [92] enhanced the myeloperoxidase level in fish. The hem containing enzyme myeloperoxidase plays significant role in the defense system of the organisms [93]. In the

present study, the hemagglutination titer level was maximum in EFS diet fed magur. In rainbow trout, the extract of green tea *Camellia sinensis* influenced the hemagglutination titer level [94]. Significantly higher levels of myeloperoxidase and hemagglutination titers were found in 0.5% seeds supplemented diets fed UV–B exposed rohu larvae [29].

The occurrence of nitric oxide synthase and antioxidant enzyme, superoxide dismutase levels in liver and head kidney of both exposed and unexposed fish were in the following order of EFS > EFL2 > EFL1 > CF and it showed the influence of seeds and leaves on magur. Earlier study showed that incorporation of seeds of *A. aspera* at 0.5% level increased nitric oxide level in UV–B exposed catla and rohu larvae [16, 44]. Higher level of nitric oxide levels in seeds and leaves supplemented diet fed magur indicated the strong immune system of fish. Nitric oxide synthase (NOS) are a family of enzymes that catalyze the production of nitric oxide (NO), the cellular signaling molecule that plays significant role in many biological processes. As a defense mechanism, the inducible isoform *iNOS* produces large amount of nitric oxide [95]. Unlike myeloperoxidase, nitric oxide synthase level in liver, and nitric oxide synthase level in head kidney were significantly higher in unexposed fish compared to the exposed fish in the respective treatment. Higher SOD activity was found in propiconazole exposed juvenile rainbow trout [96]. The treatment of common carp with the *Lactobacillus casei* induced lysozyme and SOD activities [97].

The thiobarbituric acid reactive substances (TBARS) and carbonyl protein are oxidative products of tissue lipid and protein and therefore, their higher concentrations showed stress in fish [98]. Higher levels of carbonyl groups indicate tissue damage [99]. The environmental pollutants induced oxidative stress in fish [100]. The positive impact of seeds and leaves of *A. aspera* were also reflected on the TBARS and carbonyl protein levels in liver and head kidney of both exposed and unexposed fish. The trends for TBARS and carbonyl protein were as follows: CF < EFL1 < EFL2 < EFS. TBARS levels were 1.16–2.55 folds and carbonyl protein levels were 1.35–2.37 folds higher in head kidney of UV–B irradiated magur compared to the liver of the same exposed fish. In unexposed fish, though TBARS level was 1.35–2.37 folds higher in head kidney of magur compared to the liver of fish, carbonyl protein levels were almost same in both tissues. Feeding of GIFT- tilapia with *Aloe vera* supplemented diet reduced the peroxidation of lipid [101].

The influence of plant ingredients was also visible on the gene expressions study. The up regulations of immune system and apoptosis related genes viz., *TNF-α*, *iNOS*, *NF-kB, BAX, Cytochrome c, SOD-c, Caspase 3, Caspase 9, BCL2* were documented in the leaves and seeds incorporated diets fed fish compared to the magur fed control diet in the present study. EFS treatment showed significantly higher expressions of most of the genes in both tissues, except *iNOS* and *BCL-2* in liver. Significantly higher expressions of these two genes were found in liver of EFS and EFL2 diets fed fish. The expressions of most of the genes (with few exceptions) were higher in the exposed magur compared to the unexposed fish of the respective treatment.

TNF-α is a pro-inflammatory cytokine and its up-regulation indicates inflammatory process [102]. Significantly higher expressions of *TNF-α* were found in guava *Psidium guajava* leaves and *A. aspera* seeds enriched diets fed rohu [69, 87]. The up-regulation of *TNF-α* was observed in UV–B exposed embryo/larvae of zebrafish *Danio rerio* [103, 104]. The treatment of common carp with the *Lactobacillus casei* up-regulated expression of *TNF-α* compared with control groups [97]. Nitric oxide synthase and *iNOS* are important immune-regulatory factors in fish. Like magur, feeding of common carp with *Rehmannia glutinosa* enhanced the expression of *iNOS* in different tissues like liver and spleen [105]. The exposure of rohu larvae to bisphenol caused oxidative stress and suppress *NF-κB* signaling pathway leading to immunosuppression [106]. In the present study, *NF-κB* was up-regulated in 0.5% leaves and seeds incorporated diets fed magur compared to the control and 0.25% leaves incorporated diets fed fish.

The apoptosis is a critical component in maintaining homeostasis and growth in normal physiological conditions, but some environments and feed stressors can induce abnormal apoptosis in aquatic animals [53]. The *Edwardsiella tarda* infection of murine macrophages caused up-regulation of antiapoptotic genes *Bcl2a1a*, *Bcl2a1b* [107]. The expression of *Caspase 3* was reported in *Enteromyxum scophthalmi* infected turbot, *Scophthalmus maximus* [108]. The up-regulations of apoptotic genes *Bax, BCL2* (apoptosis regulator), *Casp-3, Casp-9* were found in propiconazole exposed zebrafish embryo [109]. Scanning electron microscopic study showed that density of *iNOS* and *Caspase 3* + ve cells increased in rohu challenged with *Aeromonas hydrophila* [110] and these were the indicators of inflammatory responses and apoptosis in fish. The essential initiator *Caspase-9* plays an important role in mitochondria–mediated apoptosis signaling [111]. The exposure of common carp to paraquat activated expressions of *Caspase-3*, *Caspase-8* and *Caspase-9* [112]. The addition of teprenone in the diet of Chinese seabass *Lateolabrax maculatus* gave protection against hypoxia and oxidative stress generated apoptosis to fish [113]. The reduced expressions of pro-apoptotic signaling molecules like, *cytochrome C* (*Cyt-c*), *Caspase-9* and *Caspase-3* and enhance expression of *BCL-2* were found in fish. The up-regulation of *iNOS, SOD-C, TNF-α, Cytochrome c, Caspase 9* were recorded in seeds and leaves enriched diets fed magur after challenge with pathogen *A. hydrophila* [114]. In the present study, UV–B irradiation up-regulated the expressions of various genes

involved in apoptosis in magur. The expressions were lower in the UV–B unexposed magur. Enhanced expressions were recorded in seeds and leaves enriched diets fed fish (especially at 0.5% level) compared to the control diet fed fish. The presence of various amino acids, n-6 polyunsaturated fatty acids PUFA (linoleic acid) and n-3 PUFA (α–linolenic), vitamins and minerals improved the nutritional value of seeds and leaves [62].

5 Conclusions

UV–B radiation affected the physiology of magur. Incorporation of seeds and leaves at 0.5% level gave protection to the fish against harmful UV–B radiation. Proper care should be taken during the culture of this economically important catfish.

Author contribution RC, JGS, PM: designed the study; NK, RC, JGS: cultured the fish and analyzed samples; RC, JGS, PM, NK: prepared the manuscript; PM, RC: performed statistical analysis; NK, PM, RC: prepared graphs and tables.

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Availability of data and materials There are no additional data available.

Declarations

Conflict of interest No conflict of interest among authors.

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Freshwater Macrophytes: A Potential Source of Minerals and Fatty Acids for Fish, Poultry, and Livestock

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The freshwater macrophytes are abundant in tropical and subtropical climates. These macrophytes may be used as feed ingredients for fish and other animals. The nutritional value of twelve freshwater-cultured macrophytes was evaluated in the present study. Significantly higher crude protein (36.94–36.65%) and lipid (8.13–7.62%) were found in *Lemna minor* and *Spirodela polyrhiza*; ash content was significantly higher in *Hydrilla verticillata*, *Wolffia globosa,* and *Pistia stratiotes* (20.69–21.00%) compared with others. The highest levels of sodium, magnesium, chromium, and iron levels were recorded in *P. stratiotes*. *H. verticillata* was a rich source of copper, manganese, cobalt, and zinc; the contents of calcium, magnesium, strontium, and nickel were highest in *S. polyrhiza*. Selenium and potassium contents were higher in *Salvinia natans* and *W. globosa*, respectively. The n-6 and n-3 polyunsaturated fatty acids (PUFAs) contents were significantly higher in *W. globosa* and *Ipomoea aquatica*, respectively compared with others. Linoleic and α-linolenic acids were dominant n-6 and n-3 PUFAs. The highest value (4.04) of n-3/n-6 was found in *I. aquatica.* The ratio ranged from 0.61 to 2.46 in other macrophytes. This study reveals that macrophytes are rich sources of minerals, n-6 and n-3 PUFAs.

Keywords: alpha-linolenic acid, linoleic acid, n-3 polyunsaturated fatty acids, sodium, iron, freshwater macrophytes

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INTRODUCTION

Freshwater macrophytes, the fastest growing aquatic plants, are abundant in tropical and subtropical countries. They grow profusely in nutrient-rich water. These macrophytes are broadly classified into four groups based on their occurrence in the water body: the surface floating (e. g. *Azolla* spp.), submerged (e. g. *Hydrilla* spp.), emergent (e. g. *Potamogeton* spp.), and marginal (e. g. *Ipomoea* spp.). The nutritional value of freshwater macrophytes has been recognized globally. The unchecked propagation of freshwater macrophytes creates problems in many water bodies. The judicial exploitation of these nutrient-rich plants may open a new avenue from a nutritional view point for humans and animals. The leaf protein extracted from freshwater macrophytes may be used for human or non-ruminant animals (1). Macrophytes are a rich source of protein, lipid, amino acids, fatty acids, and minerals (2). The amino acid and fatty acid profiles of duckweeds *Lemna minor* and *Spirodela polyrhiza* have been documented recently (3, 4). The mineral composition of macrophytes is different from the usual terrestrial vegetation. Calcium

(Ca), iron (Fe), and manganese (Mn) contents are higher in aquatic plants compared with the terrestrial ones (1). Minerals are important catalysts for various biochemical reactions. These are essential components for metabolism, growth, and development and help the animals to cope with the variable environmental conditions (5). There is an optimum dose for each mineral. Low/high concentrations may affect the physiology of the organisms. Toxic minerals like arsenic (As), mercury (Hg), antimony (Sb), cadmium (Cd) etc., are required by the body in little amounts, whereas excess levels of useful minerals like, sodium (Na), potassium (K), magnesium (Mg), Ca, Fe etc., may be harmful (5). Dietary inclusions of polyunsaturated fatty acids (PUFAs) have several health benefits for humans and other animals. The study of the profiles of fatty acids of feed ingredients ensures the quality of diets. Fish are unable to synthesize two essential fatty acids like n-6 (derived from linoleic acid, LA) and n-3 (derived from alpha-linolenic acid, ALA). So these fatty acids should be supplied to the diets of fishes (6).

The evaluation of minerals and fatty acids' compositions of aquatic macrophytes is essential for their selection as potential feed ingredients for fish and other animals. Some of the commonly occurring freshwater macrophytes are: *Azolla microphylla, A. pinnata*, *Enhydra fluctuans*, *Hydrilla verticillata*, *Ipomoea aquatica*, *Lemna minor*, *Marsilea quadrifolia*, *Pistia stratiotes*, *Salvinia molesta*, *S. natans*, *Spirodela polyrhiza,* and *Wolffia globosa*. These macrophytes are distributed throughout the temperate, sub-tropical, and tropical regions of the world. Some of these macrophytes like, *E. fluctuans*, *I. aquatica,* and *M. quadrifolia,* are consumed as vegetables by humans in India and Bangladesh (7), and *W. arrhiza* has been consumed in Thailand (8). Most of these macrophytes, except *H. verticillata* (submerged plant), *M. quadrifolia,* and *E. fluctuans* (marginal plants) are surface floating macrophytes. All these macrophytes propagate through vegetative reproduction. Mosquito fern *Azolla* spp. (Azollaceae) are heterosporous free-floating ferns. It lives symbiotically with nitrogen-fixing blue-green algae *Anabaena azollae*. Watercress *Enhydra fluctuans* (Asteraceae) is a hydrophytic plant and it grows in canals and marshy places. Waterthyme *Hydrilla verticillata* (Hydrocharitaceae) is a submerged, rooted aquatic plant. It can grow in water up to a depth of 6 m, and in transparent water it can survive up to a depth of 12 m. The water spinach *Ipomoea aquatica* (Convolvulaceae) with hollow roots floats in water easily. Three members of the family Lemnaceae, namely *Lemna* spp., *Spirodela* spp., and *Wolffia* spp. are known as duckweeds. The plant consists of a single leaf or frond with one or more roots. Water clover *Marsilea quadrifolia* (Marsileaceae) is a deciduous, aquatic fern. Each green and thin stalk rises from the rhizome to the water surface; it contains a single shamrock-like leaf with four leaflets. Water cabbage *Pistia stratiotes* (Araceae) is a perennial monocotyledon with thick, soft, and light green leaves that form a rosette. It floats on the surface of the water and roots are hanging beneath the leaves. The short stolon connects both the mother and daughter plants. Water fern *Salvinia* spp. (Salviniaceae) is a perennial free-floating macrophyte. During the period of high growth, leaf size decreases and both leaves and stems fold, doubling and layering to cover more of the water surface. The nutritional value of macrophytes in terms of proteins, lipids, ash etc. varies greatly (2). The culture medium influences the mineral contents of the macrophytes (8). The extracts of seven freshwater macrophytes show no cytotoxic and anti-proliferative effects on human cell lines (9). Therefore, macrophytes should be considered as useful feed ingredients. Production of macrophytes using a standard technique may help to maintain the nutritional value of the plant and also maximize the health benefits.

The aim of the present study is to evaluate the nutritional value, *viz.* proximate composition, minerals and fatty acids profiles of twelve cultured freshwater macrophytes. This study will help to evaluate the suitability of these macrophytes as feed ingredients for fish, poultry, and livestock.

MATERIALS AND METHODS

Culture of Macrophytes

Freshwater macrophytes were collected from water bodies of Delhi, Uttar Pradesh, and West Bengal and then identified. Macrophytes were cultured in outdoor cemented tanks (1.2 × 0.35 m) with clean dechlorinated tap water (3). A 10-cm layer of soil was used for the culture of *H. verticillata*, *M. quadrifolia,* and *E. fluctuans*. All other macrophytes were cultured without soil base. The depth of water was 30 cm in all culture tanks. A combination of organic manures *viz.* cattle manure, poultry droppings, and mustard oil cake was used (1:1:1) at the rate of 1.052 kg/m^3. All manures were decomposed for 5 days and then macrophytes were introduced individually in the outdoor cemented tanks. Three replicates were used for each macrophyte. For the steady supply of nutrients for the growth of macrophytes, the same combination of manures (at one-fourth dose of the initial one) was applied in the culture tanks. Manures were decomposed for 5 days in separate containers and then applied on day 6. This schedule was followed throughout the culture period. Culture tanks were monitored regularly and macrophytes were harvested when the whole surface of the tank was covered with plants. The freshly harvested macrophytes were washed twice with tap water and then with distilled water. After air drying, macrophytes were kept at 40°C for 3 h. Then the ground, sieved, and fine powders were kept in air-tight containers and stored in a refrigerator at 4°C for further assay.

Proximate Composition Analysis

The proximate composition of the macrophytes was analyzed (10). Three replicates were used for each assay. Moisture content was estimated after drying the sample at 105°C for 24 h. The dried samples were kept in a muffle furnace at 550°C for 8 h for the determination of ash contents. The crude protein contents were analyzed by measuring the nitrogen content (N x 6.25) with an automated micro-Kjeldhal apparatus (Pelican Instruments, Chennai, India). Crude lipid contents of the macrophytes were assayed gravimetrically (11). Carbohydrate contents were estimated by the subtraction method.

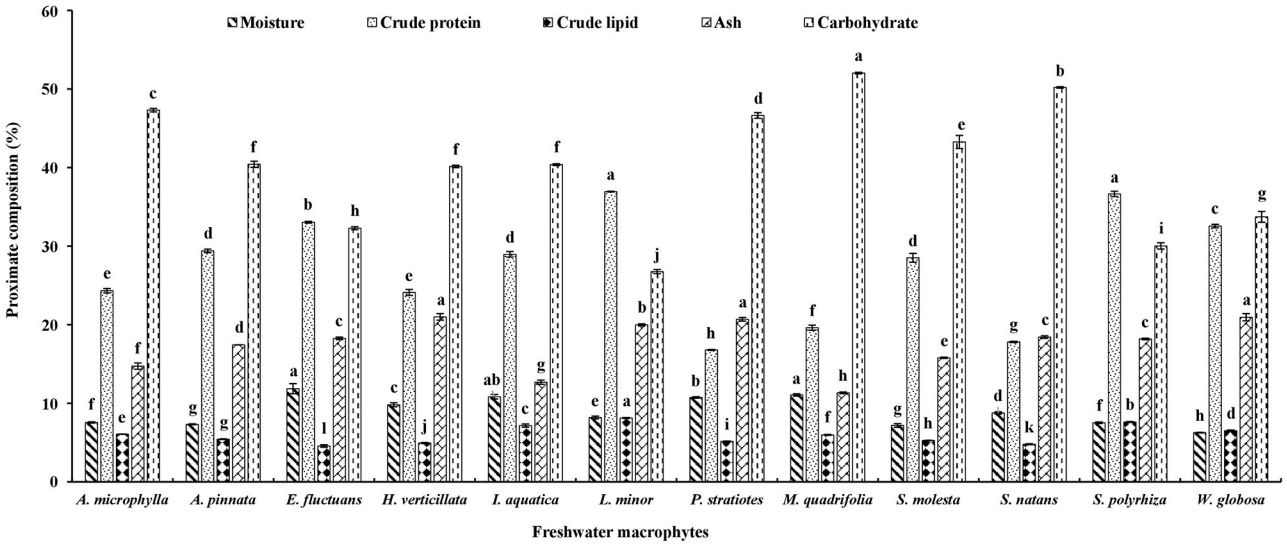

FIGURE 1 | Proximate composition of twelve freshwater macrophytes cultured with organic manures. Bars with different superscripts are significantly different ($n = 3$).

Mineral Assay

The mineral compositions of macrophytes were assayed using Inductively-Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7900, USA) following standard protocol at the Instrumentation Facility of Indian Institute of Technology, New Delhi. The powdered macrophyte sample (150 mg) was taken in a closed digestion vessel and 8 ml of suprapure 69% nitric acid (HNO_3, Merck, USA) was added to this. The sample was digested in Microwave digestion system (Multiwave PRO; Anton Paar, Austria). The digested sample was cooled at room temperature and transferred into a measuring cylinder; Milli-Q ultrapure water was added to make the volume 40 ml. Then the sample was filtered through a 0.2 μM syringe filter (Thermo Scientific, USA) and was collected in a glass vial. A 20 μL sample was injected through autosampler in the ICP-MS. The standard solution for each mineral was supplied with the equipment (Agilent Technologies, USA). It was diluted with Milli-Q ultrapure water containing 1% HNO_3 to make concentrations of 20, 40, 60, 80, 100, 250, 500, 1000 μg/l. The calibration (standard) curve was prepared. The blank was prepared with Milli-Q ultrapure water containing HNO_3 (1%). Minerals are divided into three major groups based on their concentrations in the mammal/human body *viz*. macro, trace, and ultra-trace minerals (5).

Fatty Acid Analysis

The fatty acid profiles of the macrophytes were analyzed using Gas Chromatograph (GC)–Flame Ionization Detector, Clarus 580 (Perkin Elmer, USA). The total lipid extracted from plants (11) was used to prepare fatty acid methyl esters (FAME) by transesterification using sulfuric acid in methanol at 50°C for 16 h (12). After extraction and purification of FAME (13), 1 ml sample was kept in a glass vial of autosampler of GC. The sample was separated and quantified in a GC column (60 m × 0.32 mm i.d. × 0.25 μm ZB-wax, Phenomenex, UK). The data were collected from pre-installed program software (TotalChrom Workstation Ver6.3, Perkin Elmer). The FAME was identified with the help of standards (Supelco FAME 37 mix, Sigma-Aldrich, USA).

Statistical Analysis

The compositions of twelve macrophytes are given as means ± standard error (SE). The differences in nutritional values of various macrophytes were tested using one-way analysis of variance (ANOVA) and Duncan's multiple range test (14). Statistical analyses were performed using the SPSS program (version 25.0). Statistical significance was accepted at $p < 0.05$.

RESULTS

Proximate Composition

The moisture content was highest (11.86%) and lowest (6.26%) in *E. fluctuans* and *W. globosa*, respectively (**Figure 1**). Significantly higher crude protein contents were found in two duckweeds, namely *L. minor* and *S. polyrhiza,* compared with others. The highest lipid content was also recorded in *L. minor*, followed by *S. polyrhiza*. The lipid content was minimum in *E. fluctuans*. Ash content was significantly higher in *H. verticillata*, *W. globose,* and *P. stratiotes* compared with other macrophytes. The ash content was minimum in *M. quadrifolia*. Carbohydrates levels were minimum and maximum in *L. minor* and *M. quadrifolia*, respectively.

Mineral Composition

Macrominerals

Among these twelve freshwater macrophytes, Na content was significantly higher in *P. stratiotes* compared with others (**Figure 2A**). This group was followed by *S. natans* and *E. fluctuans*. A significantly higher K level was found in *W. globosa* compared with others. This was followed by *L. minor*, *E.*

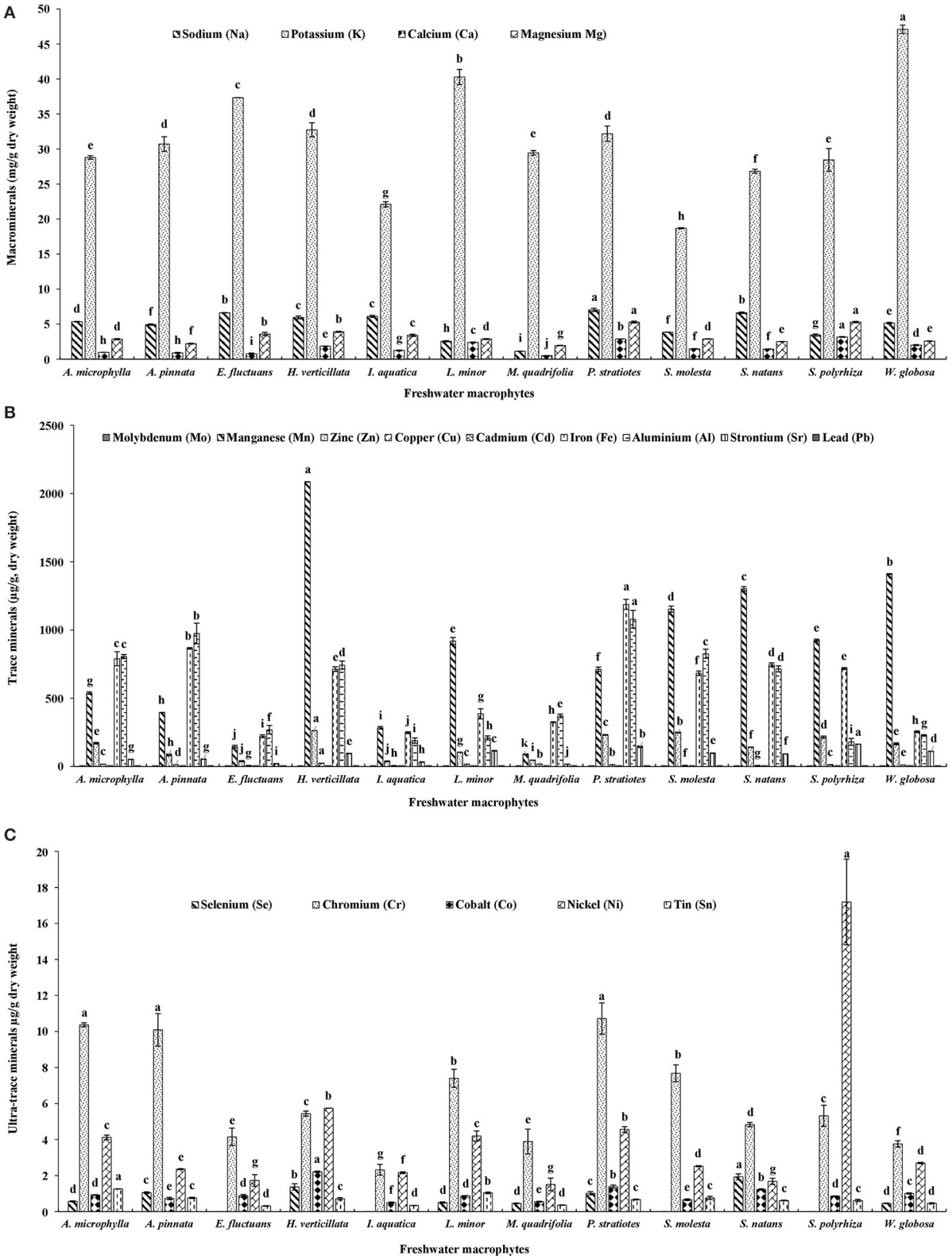

FIGURE 2 | (A) Macrominerals, **(B)** trace minerals, and **(C)** ultra-trace minerals contents of twelve freshwater macrophytes cultured with organic manures. Bars with different superscripts are significantly different ($n = 3$).

fluctuans, *H. verticillate,* and *P. stratiotes*. Ca content was highest in *S. polyrhiza*, followed by *P. stratiotes*. A significantly higher Mg level was found in *P. stratiotes* and *S. polyrhiza* compared with others. The Na, Ca, and Mg contents were minimum in *M. quadrifolia* compared with other macrophytes. It indicates the nutritional value of the macrophytes.

Trace Minerals

A total of nine trace minerals were found in these macrophytes (**Figure 2B**). Molybdenum (Mo) content was significantly higher in *A. microphylla, A. pinnata,* and *P. stratiotes* compared with others. Mn, zinc (Zn), copper (Cu), and Cd contents were significantly higher in *H. verticillata* compared with others. In *P. stratiotes*, significantly higher levels of Fe and aluminum (Al) were found compared with others. Among these macrophytes, *A. pinnata* ranked second for both Fe and Al. *A. microphylla* ranked third for Fe and fourth for Al contents. Maximum strontium (Sr) level was recorded in *S. polyrhiza* followed by *P. stratiotes*. In all these macrophytes lead (Pb) was found.

Ultra-Trace Minerals

Five ultra-trace minerals were found in these macrophytes (**Figure 2C**). A significantly higher level of selenium (Se) was found in *S. natans* compared with others. This plant was followed by *H. verticillata* and *P. stratiotes*. Se was absent in *S. molesta*, *E. fluctuans*, *I. aquatica,* and *S. polyrhiza*. Chromium (Cr) content was significantly higher in *P. stratiotes*, *A. microphylla,* and *A. pinnata* compared with others. Cobalt (Co) content was significantly higher in *H. verticillata* compared with others. This was followed by *P. stratiotes* and *S. natans*. Nickel (Ni) and tin (Sn) levels were significantly higher in *S. polyrhiza* and *A. microphylla*, respectively compared with others. Among these macrophytes, *P. stratiotes* ranked third for Ni content.

Fatty Acid Profile

The fatty acid profiles of twelve freshwater macrophytes were documented in the present study (**Tables 1–3**). The saturated fatty acids (SFAs) content was significantly higher in *W. globosa* compared with others. This was followed by *A. pinnata*, *L. minor,* and *I. aquatica*. SFA content was minimum in *P. stratiotes*. Among, SFA, palmitic acid (C16:0) was the dominant one in all these plants. Monounsaturated fatty acids (MUFA) content was significantly higher in *M. quadrifolia* compared with others. Among various MUFAs, oleic acid (C18:1n-9) was present in most of the plants and the amount was also higher compared with the others (**Supplementary Tables 1A–C**). MUFA content was also minimum in *P. stratiotes*. Though in small amounts two other monounsaturated fatty acids like, palmitoleic acid (C16:1n-9) and nervonic acid (C24:1), were present in all macrophytes, except *E. fluctuans* and *A. pinnata*. Another isomer of palmitoleic acid (C16:1n-7) was absent in two species of *Azolla* and *S. natans*.

The n-6 PUFA content was significantly higher in *W. globosa* compared with others. This was followed by *L. minor* and *A. pinnata*. The minimum level was found in *A. microphylla*. Among n-6 PUFA, LA (C18:2n-6) was the dominant one and was present in all macrophytes. Arachidonic acid (C20:4n-6) was the second dominant n-6 PUFA found in all macrophytes, except in *L. minor*. ALA (C18:3n-3) was the only member of n-3 PUFA present in all these macrophytes. ALA content was significantly higher in *I. aquatica* compared with others. This was followed by *L. minor* and *W. globosa*. The highest (4.04) n-3/n-6 was found in *I. aquatica* (**Supplementary Table 2**). The ratio ranged from 0.61 (*S. molesta*)−2.46 (*L. minor*) in other macrophytes.

DISCUSSION

A wide variation in the composition of freshwater macrophytes was recorded in the present study. The advantage of this study is that plants were cultured in the outdoor systems following a standard protocol (3). Therefore, almost the same quality of products is expected in a further study. There is scope for improvement in the nutritional value as the quality of the culture medium influences the composition of the plants.

In the present study, crude protein levels in three members of Lemnaceae family and *E. fluctuans* were above 30%, and protein contents of other macrophytes (except *P. stratiotes*, *S. natans,* and *M. quadrifolia*) were above 20%. The present study confirms the previous finding that macrophytes are rich sources of protein. The protein contents of *L. minor* and *S. polyrhiza* were 36.07 and 35.82%, respectively (3, 4). A previous study in Bangladesh reported that the protein contents of *E. fluctuans* and *I. aquatica* were 16.69 and 21.45%, respectively; macrophytes were collected from natural water bodies (15). In the present study, protein contents of *E. fluctuans* and *I. aquatica* were 16.35 and 7.51% higher compared with the same macrophytes studied in Bangladesh. Lipid contents of *I. aquatica*, *S. polyrhiza,* and *L. minor* ranged from 7.16 to 8.13% in the present study. The lipid contents of *E. fluctuans* and *I. aquatica* were 1.90 and 3.82% higher in the present study compared with the previous study (15). Ash contents of these two macrophytes were also higher in the present study compared with the previous one. Higher levels of ash contents of *H. verticillata*, *W. globosa,* and *P. stratiotes* compared with other macrophytes enhanced the nutritional value of these plants as feed ingredients for fish, poultry, and livestock. In the present study, lower levels of carbohydrates were observed in macrophytes compared with the plants harvested from the wild (15). Culture of macrophytes with organic manures enhanced the nutritional value of plants.

Among these macrophytes, highest levels of macrominerals, Na and Mg, were found in *P. stratiotes*. K and Ca were highest in *W. globosa* and *S. polyrhiza*, respectively. In the present study, among various macrophytes, *P. stratiotes* ranked second and fifth for Ca and K, respectively. A previous study reported the highest Ca level in *Hydrilla* sp., followed by *P. stratiotes* and *E. crassipes*. There was no variation in Mg level among these three macrophytes (16). Macromineral profile of leaves and roots of *P. stratiotes* collected from a natural water body of Nigeria was documented (17). This study showed that Na, K, Ca, and Mg contents were 3.73, 32.83, 2.30, and 3.70 g/kg of leaves, respectively. In the present study, Na, Ca, and Mg contents were 47, 20, and 30%, respectively, higher in *P. stratiotes* compared with the plants studied in Nigeria. K content was almost the same in the plants grown in two different conditions. The Na,

TABLE 1 | Saturated fatty acids (SFA) profiles of freshwater macrophytes cultured with organic manures (mg/100 g, dry weight).

Macrophytes /Fatty acids	C14:0	C15:0	C16:0	C18:0	C20:0	C22:0	C24:0	∑SFA
Azolla microphylla	6.55 ± 0.06^{fg}	-	541.84 ± 18.38^{de}	1.76 ± 0.89^{h}	1.39 ± 0.23^{h}	-	5.65 ± 0.23^{ef}	**557 ± 19.79^{fg}**
Azolla pinnata	16.99 ± 0.69^{e}	17.76 ± 8.72^{a}	815.78 ± 3.39^{b}	27.06 ± 0.36^{cd}	$4.08 \pm 0.42e^{f}$	17.58 ± 1.45^{a}	16.23 ± 1.09^{a}	**915.51 ± 8.08^{b}**
Enhydra fluctuans	9.46 ± 0.29^{f}	6.90 ± 2.29^{bc}	377.47 ± 0.07^{f}	29.34 ± 0.06^{c}	$2.01 \pm 0.17g^{h}$	0.07 ± 0.03^{d}	5.63 ± 1.02^{ef}	**431.01 ± 0.83^{h}**
Hydrilla verticillata	20.03 ± 1.90^{de}	4.40 ± 1.49^{cde}	377.33 ± 16.67^{f}	21.53 ± 1.68^{ef}	17.91 ± 0.17^{a}	0.89 ± 0.12^{cd}	8.03 ± 0.57^{de}	**450.14 ± 5.075^{h}**
Ipomoea aquatica	14.97 ± 0.73^{ef}	3.64 ± 0.89^{def}	622.79 ± 0.87^{c}	68.69 ± 0.80^{a}	4.54 ± 0.33^{de}	0.07 ± 0.01^{d}	14.47 ± 0.41^{ab}	**729.22 ± 1.90^{d}**
Lemna minor	40.11 ± 0.05^{a}	8.25 ± 0.13^{b}	753.13 ± 0.05^{b}	$17.69 \pm 0.06f^{g}$	5.65 ± 0.11^{d}	5.04 ± 0.10^{b}	12.81 ± 0.15^{bc}	**842.69 ± 0.55^{c}**
Marsilea quadrifolia	14.50 ± 0.46^{e}	1.92 ± 0.36^{efg}	542.58 ± 6.39^{de}	$16.81 \pm 0.47f^{g}$	1.38 ± 0.10^{h}	0.84 ± 0.02^{cd}	10.37 ± 0.81^{cd}	**588.42 ± 5.72^{ef}**
Pistia stratiotes	5.29 ± 0.40^{g}	3.85 ± 0.19^{cde}	268.75 ± 0.06^{g}	$14.07 \pm 1.16g$	8.11 ± 0.10^{c}	0.57 ± 0.04^{cd}	10.10 ± 0.45^{cd}	**310.77 ± 0.70^{i}**
Salvinia molesta	34.04 ± 5.04^{b}	6.14 ± 0.85^{bcd}	574.15 ± 19.84^{cd}	23.35 ± 0.72^{de}	2.99 ± 1.18^{fg}	5.13 ± 0.02^{b}	10.28 ± 0.48^{cd}	**656.11 ± 16.58^{e}**
Salvinia natans	4.04 ± 0.37^{g}	-	486.68 ± 2.72^{e}	3.26 ± 0.07^{h}	2.39 ± 0.18^{gh}	-	5.08 ± 0.41^{f}	**501.45 ± 4.02^{gh}**
Spirodela polyrhiza	24.26 ± 2.07^{cd}	0.81 ± 0.04^{fg}	509.94 ± 16.48^{e}	24.84 ± 1.58^{cde}	5.51 ± 0.62^{d}	1.42 ± 0.03^{cd}	7.85 ± 0.89^{de}	**574.66 ± 22.02^{fg}**
Wolffia globosa	28.24 ± 0.60^{c}	3.08 ± 0.29^{def}	1084.31 ± 63.38^{a}	54.25 ± 4.57^{b}	11.31 ± 0.90^{b}	2.07 ± 0.01^{c}	10.87 ± 0.46^{c}	**1194.14 ± 71.13^{a}**

Values having the means (n = 3) in each row with different superscript are significantly ($p < 0.05$) different.

TABLE 2 | Monounsaturated fatty acids (MUFA) profiles of freshwater macrophytes cultured with organic manures (mg/100 g, dry weight).

Macrophytes /Fatty acids	C16:1*n*-9	C16:1*n*-7	C17:1	C18:1*n*-9	C20:1*n*-9	C22:1*n*-9	C24:1	∑MUFA
Azolla microphylla	9.85 ± 0.70^{cde}	-	12.49 ± 0.75^{c}	77.22 ± 1.20^{e}	-	-	2.83 ± 0.74^{b}	**102.39 ± 1.91^{e}**
Azolla pinnata	9.39 ± 0.52^{cde}	-	91.52 ± 0.17^{b}	106.75 ± 0.34^{c}	-	-	-	**207.67 ± 0.35^{b}**
Enhydra fluctuans	-	25.29 ± 0.52^{c}	-	44.99 ± 0.21^{g}	0.28 ± 0.13^{cd}	-	0.18 ± 0.13^{c}	**70.75 ± 0.73^{f}**
Hydrilla verticillata	0.13 ± 0.02^{g}	8.81 ± 0.42^{d}	-	48.97 ± 1.60^{g}	14.15 ± 0.14^{a}	-	0.21 ± 0.03^{c}	**72.28 ± 3.67^{f}**
Ipomoea aquatica	0.33 ± 0.02^{f}	27.25 ± 0.29^{c}	-	24.16 ± 1.20^{h}	-	-	3.45 ± 0.62^{b}	**55.20 ± 1.73^{g}**
Lemna minor	51.60 ± 0.08^{a}	86.47 ± 0.15^{a}	-	9.67 ± 0.10^{i}	2.39 ± 0.04^{b}	-	7.01 ± 0.11^{a}	**157.17 ± 0.12^{c}**
Marsilea quadrifolia	7.86 ± 0.26^{de}	24.61 ± 0.42^{c}	168.24 ± 2.69^{a}	127.93 ± 2.59^{b}	0.54 ± 0.40^{cd}	7.58 ± 0.76^{b}	1.14 ± 0.74^{c}	**337.94 ± 7.36^{a}**
Pistia stratiotes	0.65 ± 0.08^{f}	10.59 ± 0.63^{d}	-	30.66 ± 0.03^{h}	0.25 ± 0.08^{cd}	5.39 ± 0.25^{c}	0.82 ± 0.03^{c}	**48.38 ± 0.14^{g}**
Salvinia molesta	6.51 ± 0.89^{e}	10.41 ± 2.38^{d}	9.53 ± 0.49^{d}	94.04 ± 0.02^{d}	0.39 ± 0.05^{cd}	3.63 ± 0.25^{d}	0.51 ± 0.01^{c}	**125.06 ± 4.06^{d}**
Salvinia natans	16.32 ± 0.74^{b}	-	4.71 ± 0.34^{e}	68.48 ± 1.18^{f}	-	-	0.65 ± 0.01^{c}	**90.16 ± 0.28^{e}**
Spirodela polyrhiza	13.81 ± 1.55^{bc}	38.93 ± 2.70^{b}	-	49.23 ± 5.09^{g}	0.13 ± 0.02^{cd}	3.30 ± 0.59^{d}	0.06 ± 0.01^{d}	**105.49 ± 5.59^{e}**
Wolffia globosa	12.00 ± 0.69^{bcd}	40.67 ± 0.54^{b}	-	137.22 ± 0.61^{a}	0.64 ± 0.03^{c}	17.65 ± 0.92^{a}	0.20 ± 0.02^{c}	**208.40 ± 2.60^{b}**

Values having the means (n = 3) in each row with different superscript are significantly ($p < 0.05$) different.

TABLE 3 | Polyunsaturated fatty acids (PUFA) profiles of freshwater macrophytes cultured with organic manures (mg/100 g, dry weight).

Macrophytes /Fatty acids	C18:2 *n*-6	C18:3 *n*-6	C20:2 *n*-6	C20:3 *n*-6	C20:4 *n*-6	∑*n*-6 PUFA	C18:3 *n*-3	∑ *n*-3 PUFA	*n*-3/n-6
Azolla microphylla	96.72 ± 4.71^{j}	-	-	-	16.71 ± 0.14^{d}	**113.43 ± 4.85^{i}**	149.05 ± 4.98^{i}	**149.05 ± 4.98^{i}**	1.31 ± 0.01^{f}
Azolla pinnata	443.29 ± 1.13^{c}	10.57 ± 0.31^{b}	-	3.17 ± 0.62^{de}	81.32 ± 0.42^{a}	**538.36 ± 2.49^{c}**	591.33 ± 0.66^{e}	**591.33 ± 0.66^{e}**	1.09 ± 0.01^{h}
Enhydra fluctuans	329.01 ± 3.35^{e}	-	1.11 ± 0.10^{bc}	-	5.24 ± 0.24^{e}	**335.38 ± 3.01^{e}**	608.37 ± 5.12^{e}	**608.37 ± 5.12^{e}**	1.81 ± 0.01^{d}
Hydrilla verticillata	168.35 ± 2.01^{g}	-	0.97 ± 0.08^{bc}	-	1.20 ± 0.17^{g}	**170.53 ± 1.89^{g}**	405.91 ± 5.50^{g}	**405.91 ± 5.50^{g}**	2.38 ± 0.06^{b}
Ipomoea aquatica	384.86 ± 1.85^{d}	-	0.95 ± 0.63^{bc}	-	2.87 ± 0.09^{f}	**388.69 ± 1.31^{d}**	1572.23 ± 2.87^{a}	**1572.23 ± 2.87^{a}**	4.04 ± 0.01^{a}
Lemna minor	601.47 ± 0.10^{b}	-	3.35 ± 0.10^{a}	5.33 ± 0.07^{c}	-	**610.16 ± 0.07^{b}**	1505.63 ± 10.10^{b}	**1505.63 ± 10.10^{b}**	2.46 ± 0.02^{b}
Marsilea quadrifolia	255.25 ± 1.33^{f}	-	0.91 ± 0.49^{bc}	55.44 ± 0.55^{a}	0.06 ± 0.01^{i}	**311.68 ± 2.31^{f}**	473.56 ± 1.79^{f}	**473.56 ± 1.79^{f}**	1.51 ± 0.01^{e}
Pistia stratiotes	132.20 ± 0.67^{h}	-	0.71 ± 0.04^{c}	1.03 ± 0.03^{f}	0.27 ± 0.01^{h}	**134.22 ± 2.11^{h}**	322.97 ± 0.32^{h}	**322.97 ± 0.32^{h}**	2.40 ± 0.03^{b}
Salvinia molesta	131.23 ± 1.29^{h}	-	2.39 ± 0.54^{ab}	23.88 ± 1.30^{b}	0.21 ± 0.01^{h}	**157.72 ± 0.56^{g}**	97.09 ± 1.18^{j}	**97.09 ± 1.18^{j}**	0.61 ± 0.01^{j}
Salvinia natans	118.57 ± 0.78^{i}	-	-	-	19.21 ± 0.22^{c}	**137.78 ± 1.00^{h}**	116.51 ± 0.89^{j}	**116.51 ± 0.89^{j}**	0.85 ± 0.01^{i}
Spirodela polyrhiza	368.28 ± 7.45^{d}	-	1.00 ± 0.01^{bc}	3.80 ± 0.34^{cd}	0.93 ± 0.02^{g}	**374.03 ± 6.06^{d}**	724.41 ± 12.66^{d}	**724.41 ± 12.66^{d}**	1.93 ± 0.02^{c}
Wolffia globosa	728.27 ± 17.19^{a}	16.54 ± 1.18^{a}	2.70 ± 0.05^{a}	1.65 ± 0.01^{ef}	22.44 ± 1.57^{b}	**771.63 ± 17.37^{a}**	909.28 ± 16.17^{c}	**909.28 ± 16.17^{c}**	1.17 ± 0.01^{g}

Values having the means (n = 3) in each row with different superscript are significantly ($p < 0.05$) different.

Ca, Zn, and Cu contents were higher in *I. aquatica* grown in Bangladesh compared with the macrophytes assayed in the present study (15). Although, Mg, K, and Fe contents were higher in the *I. aquatica* assayed in the present study compared with the plants studied in Bangladesh, Na, Mg, and K contents were higher in *E. fluctuans* evaluated in the present study compared with the previous study in Bangladesh. The Na, K, Mg, and Ca contents were higher in *A. filiculoides* and *S. molesta* grown in swine lagoons compared with the present study (18). In *A. filiculoides*, Na, K, Mg, and Ca contents were 2.77, 22.5, 5.04, and 9.3 g/kg (dry matter), respectively. In *S. molesta* Na, K, Mg, and Ca contents were 4.44, 34.7, 5.18 and 10.6 g/kg (dry matter), respectively.

In the present study, Na to K ratio ranged from 0.038 (*M. quadrifolia*) −0.276 (*I. aquatica*). The ratio was 0.063, 0.109, 0.121, 0.160, 0.177, 0.182, 0.185, 0.205, 0.218, and 0.238 in *L. minor*, *W. globosa*, *S. polyrhiza*, *A. pinnata*, *E. fluctuans*, *H. verticillata*, *A. microphylla, S. molesta*, *P. stratiotes,* and *S. natans*, respectively. In all these macrophytes, the ratio of Na to K is less than the WHO/FAO-recommended ratio for an adult human, i. e., <0.49 (19). Various studies showed the effect of culture medium on the mineral profile of macrophytes (8, 20, 21). In different species of duckweeds Na: K varied from 0.027–1.49 (K: Na = 0.67–37). In *Wolffia*, the ratio was 0.025 (K: Na = 40) and in another species, *W. microscopica* it was 0.003 (K: Na = 276).

In the present study, the Mg: Ca varied from 1.20 (*L. minor*)−4.65 in (*E. fluctuans*). Ca has been serving as the main structural mineral and helps in metabolism. It serves as a signal for vital physiological processes. Mg, the fourth most abundant cation in the body, is a co-factor for 350 cellular enzymes, most of which are involved in energy metabolism (22), hence, the ratio of Mg: Ca should be maintained. The Mg: Ca ratio was 0.4 in duckweed (21) and 0.5 in other species, *W. microscopica* (8). In the present study, the ratio was 1.28 for *W. globosa*.

The trace minerals analysis showed that among these macrophytes, *P. stratiotes* was a rich source for Mo, Fe, and Al. This macrophyte also has considerable amounts of Mn, Zn, and Sr. *A. microphylla* and *A. pinnata* were also rich sources of Fe and Mo. In a different strain of *W. arrhiza*, Fe contents ranged from 0.16–0.29 μg/g freeze-dried sample (23). The Fe content of *W. globosa* was 254.12 μg/g in the present study. Higher levels of Zn and Cu were found in *I. aquatica* grown in Bangladesh compared with the present study; Fe content was higher in the present study compared with the previous one (15). Fe content of *E. fluctuans* grown in two different environments was the same. Zn and Cu contents were lower in the plants assayed in the present study compared with the plants studied in Bangladesh. The Cu content of *S. molesta* grown in swine lagoons was 13 g/kg, dry weight (18). In the present study, Cu content of *S. molesta* was less compared with the previous study.

In the present study, the highest level of ultra-trace mineral Se was found in *S. natans*. This important mineral was also present in *H. verticillata* and *P. stratiotes*. It was interesting to record that Se was absent in *S. molesta*, *S. polyrhiza*, *E. fluctuans,* and *I. aquatica*. The Se content of freeze-dried *W. arrhiza* was <0.03 μg/g (23). In the present study, Se content of *W. globosa* was higher compared with the previous study. Significantly higher Cr levels were found in *P. stratiotes*, *A. microphylla,* and *A. pinnata* compared with other macrophytes. A significantly higher Co level was found in *H. verticillata* compared with the others. This macrophyte was followed by *P. stratiotes and S. natans*. Co content in all these macrophytes was >0.50 μg/g (dry weight). Among these macrophytes, the highest Ni content was found in *S. polyrhiza,* and this macrophyte was followed by *H. verticillata* and *P. stratiotes*. In the present study, the contents of heavy metals *viz*. Cd, Cu, Pb, and Sn of macrophytes were within the permissible limits (Cd: 0.2, Cu: 73.3, Pb: 0.3, Sn: 250 Zn: 99.40; mg/kg of wet weight) of WHO/FAO (24). In the present study, the mineral composition was evaluated in the dry sample. Therefore, the moisture (minimum 90%) contents of the samples should be considered at the time of comparison with the permissible limit of WHO/FAO for humans (where fresh plants were considered). In seaweeds, there is no regulation on the maximum heavy metals contents (25).

Various studies showed the dietary requirements of different macro, trace, and ultra-trace minerals for different animals (**Supplementary Tables 3A,B**). Na requirements of grass carp (*Ctenopharyngodon idella*), poultry, cattle, and humans are 2, 0.012–0.200, 0.96 g/kg diet, and 2.4 g/day, respectively. Among various fishes and prawns (*Pinneaus indicus*), Mg requirements vary from 0.4 to 0.946 g/kg of diets. K requirements recorded for common carp (*Cyprinus carpio*), grass carp, and Nile tilapia (*Orechromis niloticus*) are as follows: 0.9–12.4, 4.6, and 2.1–3.3 g/kg diets. K requirements for poultry, cattle, and humans are 0.3 and 2.4 g/kg diet and 3.5 g/day, respectively. Among different groups of fishes, rohu (*Labeo rohita*), common carp, grass carp, catla (*Catla catla*), and Nile tilapia require 1.9, 0.1, 2, 1.9 and 7 g Ca/kg diet, respectively. Ca requirements for poultry, cattle, and humans are 8 and 5.12 g/kg diet and 1.0 g/day, respectively. Among various fishes, Mn, Fe, Zn, and Co requirements vary from 12–25, 30–200, 15–79, and 0.01–0.5 mg/kg diet, respectively. Nile tilapia requires Se and Cr at the rate of 0.4 and 139.6 mg/kg diet, respectively. Fe, Zn, Cu, Se, Cr, and Co requirements are also evaluated for poultry, cattle, and humans. In channel catfish *Ictaluraus punctatus*, Fe, Cu, Mn, Zn, Se, and Co requirements were 30, 5, 25, 200, 0.1, and 0.05 mg/kg feed, respectively (26). In fish, Fe deficiency causes hypochromic microcytic anemia, Co and Mn deficiencies result in poor growth; Zn deficiency causes growth depression, cataract, and caudal fin and skin erosion; Se deficiency results in muscular dystrophy. In fish nutrition, Co plays a significant role. In common carp, the addition of cobalt chloride/cobalt nitrate enhanced the growth and hemoglobin formation (27). Therefore, supplementation of freshwater macrophytes may help to overcome the mineral deficiency in fish and other animals without showing any negative impact (9).

The fatty acid compositions of the two duckweeds *L. minor* and *S. polyrhiza* showed similarity with the previous study (3, 4). In the present study, palmitic acid and oleic acid were the dominant SFA and MUFA, respectively. Similar results were

also found in four duckweeds, *Landoltia*, *Lemna*, *Wolffiella,* and *Wolffia* (8). The fatty acid compositions of four aquatic plants *S. cuculata*, *Trapa natans*, *L. minor,* and *I. reptans* showed that cis-15 tetracosenoic acid and 9-hexadecenoic acid were the dominant fatty acids, and highly unsaturated fatty acids contents were higher compared with the saturated fatty acids (28). In the present study, LA was the major contributor for n-6 PUFA in all plants, and except in *L. minor*, arachidonic acid was also found in all macrophytes. ALA was the only member of n-3 PUFA present in these macrophytes. The presence of LA and ALA were recorded in duckweeds (8). The freshwater teleosts are capable of converting ALA to long-chain polyunsaturated fatty acids (LC-PUFA) like eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6n-3) (29–31). Therefore, the feeding of fish with freshwater macrophytes-based diets helps to fulfill the LC-PUFA requirements of cultured fish (32, 33). The n-6/n-3 PUFA was always <1; it ranged from 0.48–0.94 in different *Wolffia* species (23). A similar result was also found in the present study, except in two species of *Salvania*, where the ratio was >1.0.

CONCLUSION

Among these macrophytes, Na, Mg, Cr, and Fe contents were maximum in *P. stratiotes*; this macrophyte ranked second for Co, Sr, and Ca. *H. verticillata* was the richest source for Cu, Mn, Co, and Zn, and it ranked second for Se. Ca, Mg, Sr, and Ni contents were higher in *S. polyrhiza* compared with the others. *S. natans* and *W. globosa* were rich sources for Se and K, respectively. All these macrophytes were rich sources of n-6 and n-3 fatty acids. This study shows that macrophytes have an immense potential to be used as rich sources of minerals, as well as n-6 and n-3 PUFA for fish, poultry, and livestock.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JS, RC, and DRT designed the study. GK, RG, AS, NK, JS, and RC grown the macrophytes and analyzed samples. RC, DRT, GK, and JS wrote the manuscript. GK, AS, RG, NK, RC, DRT, and JS prepared tables. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.869425/full#supplementary-material

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The study of effect of vitamin C and *Achyranthes aspera* seeds enriched diets on the growth, biochemical composition, digestive enzyme activities and expressions of genes involved in the biosynthesis of fatty acids in Snow trout *Schizothorax richardsonii* (Gray, 1832)

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ABSTRACT

Snow trout, *Schizothorax richardsonii* (Gray, 1832), is an economically important fish in the Himalayan region. Snow trout fry (118.53 ± 5.42 mg) were cultured in flow-through systems for 60 days. Fry were fed with four different diets containing: 0.08% vitamin C (D1), 0.5% *Achyranthes aspera* seeds (D2), both vitamin C and seeds (D3), and a control (D4). Average weight (271.82 ± 4.90 g) and specific growth rate (1.39 ± 0.03) were significantly higher in fish fed D3 compared to the others. Digestive enzyme activities were significantly higher in D3 compared to the others. EPA content was significantly higher in D2 (110.33 ± 0.94 mg 100 g^{-1}) and D3 (109.87 ± 1.87 mg 100 g^{-1}); DHA (342.70 ± 0.77 mg 100 g^{-1}) and *n*-3 PUFA (496.17 ± 1.82 mg 100 g^{-1}) contents were significantly higher in D3 compared to the others. The expression of *fads2d6* was significantly higher in D3 (6.70-fold) and *elovl5* in D2 and D3 (1.51–1.45-fold) compared to the others. Enriched diets improved fish flesh composition.

KEYWORDS

Schizothorax richardsonii; Vitamin C; *Achyranthes aspera*; DHA and EPA; *fads2d6* and *elovl5*

Introduction

Snow trout, *Schizothorax richardsonii* (Gray, 1832), belongs to the family Cyprinidae and is a commercially important fish of the Himalayan region. It contributes the maximum yield in the capture fisheries of hills and is a potential species for aquaculture (Kamalam et al. 2019; Petr 2002). Although artificial breeding of snow trout is successful (Joshi 2004), the proper culture technique has not been developed (Sarma et al. 2013). Moreover, the growth rate of snow trout is very slow, and the fish are susceptible to diseases

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(Moses, Nilssen, and Chakrabarti 2017). Nutrition plays a significant role in the health status of the cultivable species (Lall and Olivier 1993). Supplementation of growth promoters and immunostimulants in the diet enhances the growth and improves the immunity of the fish (Kord et al. 2021). An earlier study shows that the enrichment of feeds with vitamin C and *Achyranthes aspera* seeds increases the growth rate of snow trout larvae. The enhanced levels of digestive enzymes, myeloperoxidase, nitric oxide synthase, etc., are found in fish fed enriched diets (Moses, Nilssen, and Chakrabarti 2017). The enzyme L-gulonolactone oxidase, which helps in the synthesis of ascorbic acid from L-gulonolacetone, is lacking in teleosts (Sato, Nishikimi, and Udenfriend 1976). Many studies show that supplementation of vitamin C in diets of fishes improves health conditions, e.g., in rohu *Labeo rohita* (Misra et al. 2007), Jian carp *Cyprinus carpio* (Liu et al. 2011), tilapia *Oreochromis karongae* (Nsonga et al. 2009), GIFT tilapia *O. niloticus* (Huang et al. 2016), and discus fish *Symphysodon haraldi* (Liu et al. 2018). Various factors like species, development stage, and environment etc., influence the requirement of vitamin C. The supplementation is needed more at the earlier stages compared to adults (Imanpoor, Imanpoor, and Roohi 2017; Kolkovski et al. 2000).

The herb *Achyranthes aspera* L. belongs to the family Amaranthaceae. It grows in tropical climatic conditions and has pharmaceutical applications. Many studies show that the seed of *A. aspera* enhances the immunity of different carps, and it protects the fishes against pathogens (Chakrabarti and Srivastava 2012; Chakrabarti et al. 2014; Kumar et al. 2019; Rao and Chakrabarti 2005; Rao et al. 2004; Sharma et al. 2019). The feeding of a seed-enriched diet protects the carps from harmful UV-B radiation (Singh, Sharma, and Chakrabarti 2013a, 2013b). The seed-supplemented diet also enhances the survival and growth rate of fishes. Chakrabarti et al. (2012) have observed the presence of saturated fatty acids (SFA), palmitic acid (23%), stearic acid (2%), and two long-chain polyunsaturated fatty acids (LC-PUFA)—oleic acid (33%) and linolenic acid (42%)—in the seeds of *A. aspera*.

Earlier studies show that freshwater teleosts are capable of converting linoleic acid (LA; 18:2 *n*-6) and α-linolenic acid (ALA; 18:3 *n*-3) to LC-PUFA—arachidonic acid (AA; 20: 4 *n*-6), eicosapentaenoic acid (EPA; 20:5 *n* − 3), and docosahexaenoic acid (DHA; 22:6 *n*-3) (Buzzi, Henderson, and Sargent 1997; Cook 1996; Glencross 2009). Therefore, the essential fatty acid requirements of freshwater teleosts can usually fulfill dietary LA and ALA (Tocher et al. 2001; Tocher, Dabrowski, and Hardy 2010). The conversion of LA and ALA to LC-PUFA requires a series of fatty acyl desaturase (*fad*) and elongation of very long-chain fatty acids (*elovl*) enzymes such as *elovl5* and *elovl2* (Torstensen and Tocher 2010). Fatty acid synthase (*fas*) is a key enzyme that regulates the de novo biosynthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH (Dong et al. 2014). So far,

no study has been conducted to evaluate the effect of seeds of *A. aspera* on the fatty acid composition and the expressions of genes involved in the biosynthesis of fatty acids in snow trout.

The present study aims to evaluate the effect of vitamin C and seeds of *Achyranthes aspera* supplemented diets on the growth performance, digestive enzyme activities, biochemical composition, and expressions of specific genes involved in the biosynthesis of fatty acids—*fads2d6, fas, elovl5*, and *elovl2*—in the fry of snow trout (*Schizothorax richardsonii*).

Materials and methods

Culture of fry

Snow trout were bred in the wet laboratory of DCFR, Champawat, Uttrakhand, India, in September 2017, and larvae were grown up to fry stage (118.53 ± 5.43 mg). The experiment was started October 8 and was continued up to December 6, 2017. Fry were cultured under four different feeding regimes and maintained in four separate flow-through systems. Each flow-through system consisted of four culture units (10 L each); these four units were considered replicates. The flow rate of the water was 1.5 L min^{-1}. The stocking density was 10 fry per $unit^{-1}$ (10 × 4 = 40 fry per treatment).

Formulation of diets and feeding

Four diets were formulated using fish meal as protein source (Table 1). The three experimental diets were: diet 1 (D1) was supplemented with vitamin C (0.08%), diet 2 (D2) was supplemented with *A. aspera* (0.5%) seeds, and diet 3 (D3) was enriched with vitamin C (0.08%) and seeds (0.5%); the control diet 4 (D4) had no vitamin C or seeds. The concentrations of vitamin C and seeds in the diets were selected based on a previous experiment by Moses, Nilssen, and Chakrabarti (2017). The amino acids and fatty acids compositions of the

Table 1. Formulation of diets and their composition.

Ingredient (g kg^{-1})	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal	450	450	450	450
Fish oil	10	10	10	10
Wheat flour	535.2	531	530.2	536
Vitamins & minerals	4	4	4	4
Vitamin C	0.8	–	0.8	–
A. aspera	–	5	5	–
Proximate composition of diets (% dry weight)				
Lipid	7.43 ± 0.03	6.86 ± 0.03	7.00 ± 0.05	7.09 ± 0.21
Protein	35.84 ± 1.12	35.92 ± 0.26	36.00 ± 0.23	36.15 ± 1.13
Moisture	7.37 ± 0.08	7.39 ± 0.12	7.00 ± 0.08	7.38 ± 0.03
Ash	9.36 ± 0.11	9.20 ± 0.03	8.84 ± 0.11	9.04 ± 0.05

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

formulated diets were evaluated (Tables S1 and S2 in the supplementary materials). Snow trout fry were fed at the rate of 5% of body weight every day. The whole amount of feed was divided into two parts and was given at 9:00 a.m. and 4:00 p.m. Satiation limit was assessed by feeding the fish with different amount of feeds; it was observed that feeding of fish at the rate of 2.5% of body weight was suitable. After 1 h of feeding, the uneaten feed was removed. The experiment was continued for 60 days.

Survival rate, growth, and feed conversion ratio

Survival rate (%) of snow trout was recorded. The weight of individual fish was recorded (Libror AEG-120, Shimadzu, Japan) after 60 days of culture. Specific growth rate (SGR) and feed conversion ratio (FCR) were calculated as follows:

$$Specific\ growth\ rate\ (SGR\ \%) = [ln\ (final\ weight) - ln\ (initial\ weight)] \times 100/study\ period$$

$$Feed\ conversion\ ratio\ (FCR) = feed\ intake/body\ weight\ gain$$

Digestive enzyme assays

The digestive tract was collected from individual snow trout. The digestive tracts of six fish were pooled to make one replicate, and four replicates were used per treatment. The sample was prepared in the following sequence: homogenization of pooled sample in ice-cold distilled water, following centrifugation (10,000 g for 30 min at 4°C) and collection of supernatant. The supernatant was used for the enzyme assay. All enzymes were assayed using a fluorometer (Biotek Synergy H1, USA). The EnzChek ® Ultra Amylase Assay Kit (E33651, Invitrogen, Oregon, USA) was used for the estimation of amylase activity. The excitation and emission were recorded at 485 nm and 520 nm respectively. The total protease activity was estimated based on a green fluorescence technique. The EnzChek® Protease Assay Kit (E6638, Molecular ProbesTM, Invitrogen) was used for the study. The change in fluorescence was estimated at excitation 485 nm and emission 530 nm. The trypsin activity was assayed using Na-benzoyl-L-arginin-methyl-coumarinylamide (Sigma, USA) as a substrate (Ueberschar 1988). The change in fluorescence was recorded at 380 nm (excitation) and 440 nm (emission). The chymotrypsin activity was assayed using succinyl-Leu-Val-Tyr-4-methyl-coumaryl -7-aminde (Sigma) as substrate (Cao et al. 2000). The excitation and emission were recorded at 380 nm and 450 nm respectively. Lipase was estimated using 4-methylumbelliferyl butyrate (4-MUB, Sigma) as substrate (Roberts 1985).

The excitation and emission were studied at 365 nm and 450 nm respectively. Total protein was estimated using bovine serum albumin as a standard (Bradford 1976).

Biochemical composition

The proximate composition—crude protein, crude lipid, moisture, and ash contents were assayed following standard methods (AOAC 2000). The crude protein was estimated following the Kjeldahl technique. In this assay, total nitrogen (%) was measured and was multiplied with 6.25 (N × 6.25) to obtain the protein (%) content. The crude lipid content was assayed gravimetrically (Folch, Lees, and Sloane-Stanley 1957). Moisture content was estimated by keeping the sample at 105°C for 3 h; for the estimation of ash, the sample was kept at 550°C for 8 h.

Fatty acids

The acid transesterification reaction was used for FAME (fatty acid methyl esters) estimation using a methylation reagent (1%). Crude lipid (1 mg) isolated from each sample was added with 2 mL of methylation reagent, 1 mL of toluene, and 1 mL of (C17:0) internal standard. After vortexing and proper mixing, the lipid sample was flushed with nitrogen. The sample was incubated at 50°C for 16 h (Christie 2003). The FAME (1 mg mL^{-1}) was isolated, purified, separated, and quantified using a Perkin Elmer Gas Chromatograph (Clarus 580 Autosampler, USA). Zebronics wax column 60 m (length) × 0.32 mm (internal diameter) × 0.25 μm (film thickness) was used for fatty acid analysis. FAMEs were compared with the known standards and the published data (Tocher and Harvie 1988). The fatty acid content was expressed as mg 100 g^{-1} of wet sample.

Amino acids

The amino acid composition of the sample was assayed using a Hitachi L-8900 Amino acid analyzer (Japan). All amino acids (except methionine, cysteine, and tryptophan) were hydrolyzed using HCl (6 N). In a digestion tube, a 30 mg sample was taken; 10 mL HCl was added to the sample, then flushed with nitrogen and sealed. The tube was kept for 22 h at 110°C for hydrolysis (Chakrabarti et al. 2018). Then the sample was kept in a nitrogen evaporator (PCi Analytics Pvt. Ltd., Maharastra, India) to evaporate acid. The dried samples were dissolved in HCl (0.02 N), and the final concentration of protein was 0.5 mg mL^{-1}. The sample (20 μL) was injected in the autosampler. The performic acid was first used to oxidize methionine and cysteine, and then hydrobromic acid (48%) was added in

the sample. The methanesulfonic acid (4 N) and 3-(2-aminoethyl) indole were used for the measurement of tryptophan. The amino acid concentrations were measured using standard solutions (Wako Pure Chemical Industries, Limited). The glutamine and tryptophan were estimated using freshly prepared standard (Sigma-Aldrich, USA). The amino acid content was expressed as g 100 g^{-1} of sample (wet weight).

Gene expression

Four genes—*fads2d6* (delta-6 desaturase), *fas* (fatty acid synthase), *elovl5* (elongase of very long chain fatty acids 5), and *elovl2* (elongase of very long chain fatty acids 2) were selected for the study; β-actin was used as reference gene for normalization. Primers were designed as per the sequences of genes available on the NCBI (Table 2). RNA was isolated using Trizol reagent following the instructions provided with the chemical. The purity of isolated RNA was estimated. The RNA was treated with DNase 1 to avoid DNA contamination (DNase kit, Sigma Aldrich). Purified RNA was converted to cDNA with an ABi kit (USA). The PCR amplification with β-actin confirmed cDNA, and bands were separated on agarose gel. SYBR green was used for the study of relative mRNA expressions (RT-PCR, Quant Studio 6 Flex, ABi, USA). The amplification efficiency of primers was estimated with their melt curves. Total volume of reaction mixture per well was 10 µL that was composed of 5 µL of SYBR green, 0.5 µL of forward primer, 0.5 µL of reverse primer, 3 µL of nucleic acid-free water, and cDNA 1 µL. The amplification cycle in the RT-PCR was as follows: 10 min for preliminary denaturation, number of cycles was 40 (like 15 s at 95°C and 1 min at 60°C). The plate was sealed with a 96-well optical adhesive cover. The optical plate was placed in RT-PCR and a program was run using QuantStudio 6 Flex software v1.1 on the computer. On the basis of CT value, the fold change of a particular mRNA was estimated (Livak and Schmittgen 2001).

Table 2. Primers used for gene expression analysis.

Gene	Accession No.	Direction	Primer Sequence
β-actin	KU715835	Forward	5′-GCCGTGACCTGACTGACTAC-3′
		Reverse	5′-CTGCTCGAAGTCAAGAGCCA-3′
fads2d6	KJ576791	Forward	5′-CACAATGCCTCGGCACAAT-3′
		Reverse	5′-CCAGCCAGAGTTCTCCAGATTT-3′
fas	MF289408	Forward	5′-CAATGCCCAGCAGCATAAGG-3′
		Reverse	5′-TGGGCTTGTTGAACCTCGG-3′
elovl5	KF924199	Forward	5′-GGGCTGGCTGTATTTCCAGAT-3′
		Reverse	5′-GATGCCACCCCATTAGTGTGA-3′
elovl2	KR706498	Forward	5′-ATCAGTTTGGTCTGCCGGTT-3′
		Reverse	5′-CAGCACAATGAAGATGGTGTCC-3′

Water quality

Water quality parameters were monitored regularly. The Hach multimeter (HQ40D, USA) was used for the estimation of temperature, pH, conductivity, dissolved oxygen, and ammonia levels of water. The nitrite and nitrate contents were measured following the APHA (2017) method. Temperature, pH, and conductivity ranged from 9.0 to 19.2°C, 7.15 to 7.88, and 241.3 to 251.6 μS cm^{-1} respectively in different culture systems throughout the study period. The experiment was started October 8 and completed December 6, 2017. At the start of the experiment the water temperature was 19.2°C; in November the water temperature gradually reduced. The minimum 9.0°C was recorded in the first week of December, and the experiment was completed. Snow trout is a coldwater fish and survives well in temperatures ranging from 5 to 20°C (Barat et al. 2019). Dissolved oxygen, ammonia, nitrite, and nitrate levels of fish culture units ranged from 7.36 to 9.38, 0.06 to 0.8, 0.10 to 0.18, and 0.21 to 0.28 mg L^{-1} respectively throughout the experiment.

Statistical analysis

The growth parameters, digestive enzyme activities, and biochemical composition of snow trout were expressed as means ± SEM. Data were analyzed using ANOVA, one-way analysis of variance, and Duncan's multiple range test (DMR) (Montgomery 1984). Statistical significance was accepted at the $P < 0.05$ level.

Results

Survival and growth

In snow trout fed the control diet (D4), the survival rate was 93%; all fish survived (100%) in the other treatments. A significantly higher final average weight was recorded in snow trout fed the D3 diet (271.82 ± 4.90 mg) compared to fish fed the other diets. The weight gain was 1.45–2.76-fold higher in fish fed the experimental diets compared to the control. Significantly higher SGR (1.39 ± 0.03%) and lower FCR (1.55 ± 0.07) were observed in snow trout fed the D3 diet compared to the fish cultured in the other three different feeding regimes (Table 3).

Table 3. Survival, average weight, specific growth rate (SGR), feed conversion ratio (FCR), and digestive enzyme activities found in *S. richardsonii* fed with four different diets. Means (n = 4) sharing different letters in the same row are significantly ($P < 0.05$) different.

Parameter	Diet 1	Diet 2	Diet 3	Diet 4
Survival (%)	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a	93 ± 2.50b
Average weight (mg)	198.72 ± 2.55b	201.22 ± 1.71b	271.82 ± 4.90a	173.74 ± 2.67 c
SGR (%)	0.86 ± 0.02b	0.87 ± 0.02b	1.39 ± 0.03a	0.64 ± 0.01 c
FCR	2.48 ± 0.03b	2.61 ± 0.16b	1.55 ± 0.07 c	3.26 ± 0.05a
Amylase (mU $\mu l^{-1}mg^{-1}$protein min^{-1})	19.93 ± 0.51b	21.46 ± 0.58b	33.28 ± 0.20a	12.09 ± 0.23 c
Total Protease (Fluorescence change $unit^{-1}$)	108.55 ± 4.07b	109.71 ± 1.83b	136.69 ± 3.0a	99.96 ± 2.54 c
Trypsin (μmol AMC mg^{-1}protein min^{-1})	141.66 ± 5.19b	150.87 ± 7.78b	254.63 ± 5.45a	72.86 ± 2.31 c
Chymotrypsin (μmol AMC mg_{-1}protein min^{-1})	74.80 ± 1.44b	80.34 ± 1.77b	162.35 ± 1.72a	59.83 ± 0.85 c
Lipase (μmol 4MU mg^{-1}protein min^{-1})	74.28 ± 2.06b	69.70 ± 1.66 c	157.68 ± 6.23a	78.68 ± 1.58b

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

Digestive enzyme activities

Significantly higher amylase, total protease, trypsin, chymotrypsin, and lipase activities were observed in snow trout fed the D3 diet compared to fish cultured in the other three different feeding regimes (Table 3). There was no significant difference in amylase, protease, trypsin, and chymotrypsin activities between snow trout fed the D1 and D2 diets. These enzyme activities were minimum in fish fed the control diet. Lipase activity was minimum in snow trout fed the D2 diet.

Proximate composition of muscle

The moisture contents ranged from 76.25% to 76.65% in different diets fed snow trout. The protein content was significantly higher in snow trout fed the D1 and D4 diets compared to the fish fed diets D2 and D3 (Table 4).

Table 4. Biochemical composition of muscle (wet weight) of *S. richardsonii* fed with four different diets. Means (n = 4) sharing different letters in the same row are significantly ($P < 0.05$) different.

Parameter (%)	Diet 1	Diet 2	Diet 3	Diet 4
Moisture	76.25 ± 0.36a	76.65 ± 0.54a	76.55 ± 0.17a	76.45 ± 0.35a
Protein	16.85 ± 0.10a	16.22 ± 0.27b	16.17 ± 0.16b	16.76 ± 0.20a
Lipid	3.20 ± 0.03b	3.32 ± 0.04b	3.54 ± 0.02a	3.11 ± 0.02 c
Ash	2.61 ± 0.05a	2.39 ± 0.02b	2.41 ± 0.01b	2.37 ± 0.02b

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

Table 5 (A) Amino acid profile (g 100 g^{-1} wet weight) of muscle of *S. richardsonii* fed with four different diets. Means ($n = 4$) sharing different letters in the same row are significantly ($P < 0.05$) different. Essential amino acids.

Amino acid	Diet 1	Diet 2	Diet 3	Diet 4
Histidine (His)	0.395 ± 0.001b	0.392 ± 0.001b	0.405 ± 0.003a	0.390 ± 0.001b
Isoleucine (Ile)	0.630 ± 0.002a	0.616 ± 0.002b	0.632 ± 0.001a	0.620 ± 0.001b
Leucine (Lue)	1.126 ± 0.004a	1.072 ± 0.005 c	1.101 ± 0.004b	1.109 ± 0.005b
Lysine (Lys)	1.247 ± 0.006a	1.210 ± 0.005 c	1.247 ± 0.002a	1.230 ± 0.002b
Methionine (Met)	0.396 ± 0.001a	0.289 ± 0.001 c	0.298 ± 0.003 c	0.388 ± 0.006b
Phenylalanine (Phe)	0.641 ± 0.010a	0.607 ± 0.003 c	0.624 ± 0.002b	0.628 ± 0.016b
Threonine (Thr)	0.725 ± 0.004a	0.686 ± 0.006 c	0.701 ± 0.001b	0.725 ± 0.012a
Tryptophan (Trp)	0.219 ± 0.002d	0.222 ± 0.002 c	0.228 ± 0.004a	0.226 ± 0.010b
Valine (Val)	0.731 ± 0.002a	0.715 ± 0.004 c	0.733 ± 0.006a	0.721 ± 0.004b
∑ Essential amino acids	6.109 ± 0.022a	5.808 ± 0.029d	5.970 ± 0.011 c	6.037 ± 0.046b

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

Table 5 (B) Nonessential and free amino acids.

Amino acid	Diet-1	Diet-2	Diet-3	Diet-4
Nonessential amino acids				
Alanine (Ala)	0.878 ± 0.002a	0.845 ± 0.005c	0.871 ± 0.002a	0.865 ± 0.003b
Arginine (Arg)	1.504 ± 0.009b	1.352 ± 0.211c	1.575 ± 0.046a	1.574 ± 0.002a
Asparatate (Asp)	1.500 ± 0.003a	1.435 ± 0.009d	1.465 ± 0.002c	1.485 ± 0.010b
Cysteine (Cys)	0.171 ± 0.001a	0.154 ± 0.003b	0.157 ± 0.001b	0.170 ± 0.001a
Glutamic Acid (Glu)	2.387 ± 0.009a	2.293 ± 0.006a	2.345 ± 0.002a	2.375 ± 0.016a
Glycine (Gly)	0.939 ± 0.004a	0.901 ± 0.008a	0.927 ± 0.001a	0.926 ± 0.003a
Proline (Pro)	0.526 ± 0.003b	0.527 ± 0.004b	0.541 ± 0.004a	0.518 ± 0.002 c
Serine (Ser)	0.697 ± 0.001a	0.658 ± 0.004c	0.671 ± 0.002b	0.695 ± 0.009a
Tyrosine (Tyr)	0.512 ± 0.002a	0.485 ± 0.001c	0.502 ± 0.002b	0.501 ± 0.004b
∑ Non-essential amino acids	9.113 ± 0.033a	8.650 ± 0.251b	9.054 ± 0.062a	9.110 ± 0.051a
Free amino acids				
Phosphoserine	0.034 ± 0.002a	0.023 ± 0.001b	0.025 ± 0.001b	0.022 ± 0.002b
Taurine	0.170 ± 0.001b	0.209 ± 0.001a	0.214 ± 0.004a	0.164 ± 0.001 c
Cystathionine	0.072 ± 0.002a	0.065 ± 0.001c	0.069 ± 0.001b	0.073 ± 0.001a
β-Amino isobutyric acid	0.266 ± 0.040a	0.204 ± 0.009b	0.207 ± 0.004b	0.259 ± 0.066a
γ-Amino-n-butyric acid	0.041 ± 0.016a	0.032 ± 0.002b	0.029 ± 0.001b	0.043 ± 0.002a
Ethanol amine	0.141 ± 0.007a	nd	nd	0.135 ± 0.013a
Hydroxylysine	0.144 ± 0.005a	0.142 ± 0.005a	0.147 ± 0.001a	0.154 ± 0.020a
Ornithine	0.034 ± 0.003a	0.035 ± 0.001a	0.039 ± 0.001a	0.036 ± 0.005a
3 Methylhistidine	0.013 ± 0.000a	0.016 ± 0.001a	0.017 ± 0.001a	0.012 ± 0.000a
Hydroxy proline	0.140 ± 0.006b	0.104 ± 0.007c	0.092 ± 0.005c	0.156 ± 0.004a
∑ Free amino acids	1.054 ± 0.154a	0.832 ± 0.030b	0.840 ± 0.015b	1.057 ± 0.213a

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

A significantly higher lipid level was observed in snow trout fed diet D3 compared to the other treatments. The ash content was significantly higher in snow trout fed the D1 diet compared to the others.

Amino acid profile of muscle

Significantly higher histidine and tryptophan contents were observed in snow trout fed the D3 diet compared to fish fed the other diets (Table 5). Significantly higher isoleucine, lysine, and valine contents were found in snow trout fed diets D1 and D3 compared to the others. Significantly higher leucine, methionine, and phenylalanine levels were observed in snow trout fed the D1 diet compared to fish fed the other diets. Significantly higher threonine levels were recorded in snow trout fed the D1 and D4 diets compared to the fish fed diets D2 and D3. The total essential amino acids content was significantly higher in snow trout fed the vitamin C-supplemented diet (D1) compared to the fish cultured in the other three feeding regimes.

Nonessential amino acid alanine content was significantly higher in snow trout fed the D1 and D3 diets, and arginine content was significantly higher in snow trout fed the D3 and D4 diets compared to the others. Aspartate and tyrosine levels were significantly higher in snow trout fed the D1 diet compared to the fish cultured in the other three different feeding regimes. Cysteine and serine contents were significantly higher in snow trout fed the D1 and D4 diets compared to the D2 and D3 treatments. In snow trout fed different diets, glutamic acid and glycine contents were not significantly different. Proline content was significantly higher in snow trout fed the D3 diet compared to fish fed the other diets. The total nonessential amino acids content was significantly lower in snow trout fed the D2 diet compared to the others.

The free amino acids phosphoserine level was significantly higher in snow trout fed the D1 diet and taurine content in the D2 and D3 diets compared to the others. Cystathionine, β-amino isobutyric acid, and γ-amino-n-butyric acid contents were significantly higher in snow trout fed the D1 and D4 diets compared to the others. The hydroxyproline content was significantly higher in snow trout fed the D4 diet compared to the others. Significantly higher total free amino acids were recorded in snow trout fed the D1 and D4 diets compared to the D2 and D3 diets.

Fatty acid profile of muscle

In the muscle of snow trout fed the different diets, various saturated (SFA), monounsaturated (MUFA), *n*-6 polyunsaturated (*n*-6 PUFA), and *n*-3 polyunsaturated (*n*-3 PUFA) fatty acids were observed. Significantly higher myristic acid and pentadecanoic acid was observed in snow trout fed the D2 and D3 diets compared to the other two treatments (Table 6). Palmitic acid was significantly higher in snow trout fed the D2 diet compared to fish fed the

Table 6. Fatty acid profile (mg 100 g^{-1} wet weight) of muscle of *S. richardsonii* fed with four different diets. Means (n = 4) sharing different letters in the same row are significantly (P < .05) different.

Fatty acids	Diet-1			Diet-2			Diet-3			Diet-4		
Saturated fatty acids												
14:0	269.39	±	1.00b	271.31	±	1.34a	274.02	±	1.67a	264.34	±	2.09c
15:0	33.06	±	4.94b	38.63	±	0.84a	38.44	±	1.88a	33.48	±	3.56b
16:0	714.18	±	9.04b	728.72	±	1.14a	719.19	±	14.72b	705.27	±	10.99b
18:0	88.35	±	3.70ab	92.17	±	0.68a	85.03	±	4.16b	91.49	±	2.11a
22:0	5.37	±	0.31a	5.53	±	0.33a	4.41	±	0.55b	5.23	±	0.15a
24:0	9.78	±	0.82a	7.97	±	0.19b	7.96	±	0.41b	9.97	±	0.57a
∑SFA	1120.14	±	18.80a	1144.34	±	2.38a	1129.04	±	18.82a	1109.78	±	14.45a
Monounsaturated fatty acids												
16:1 *n*-9	669.40	±	1.49b	674.17	±	2.76a	679.20	±	7.07a	657.01	±	5.98c
18:1 *n*-9	829.03	±	3.05a	838.09	±	2.16a	838.70	±	7.41a	816.74	±	7.96b
20:1 *n*-9	100.17	±	0.64a	101.27	±	0.43a	100.55	±	0.43a	98.76	±	1.06a
22:1 *n*-9	37.70	±	0.31a	38.06	±	0.35a	37.00	±	0.91a	37.41	±	1.49a
24:1	62.98	±	0.83a	62.22	±	0.70a	63.81	±	0.93a	59.40	±	0.97b
∑ MUFA	1699.27	±	4.04b	1713.82	±	5.45a	1719.25	±	14.15a	1669.31	±	17.14c
n-6 Polyunsaturated fatty acids												
18:2 *n*-6	347.89	±	1.50b	351.11	±	0.54a	352.29	±	3.08a	343.82	±	2.90b
18:3 *n*-6	14.59	±	0.82b	15.91	±	0.20a	14.27	±	0.23b	14.59	±	0.36b
20:2 *n*-6	17.55	±	0.44a	17.32	±	0.49a	17.81	±	0.46a	18.55	±	0.65a
20:3 *n*-6	112.49	±	0.32a	113.18	±	0.35a	112.84	±	0.89a	111.48	±	0.70a
20:4 *n*-6	8.10	±	0.25a	7.94	±	0.31a	8.01	±	0.24a	7.63	±	0.41a
∑ *n*-6 PUFA	500.63	±	1.94a	505.46	±	0.64a	505.22	±	3.76a	496.07	±	4.23a
n-3 Polyunsaturated fatty acids												
18:3 *n*-3	33.89	±	0.15a	34.33	±	0.39a	34.44	±	0.25a	33.57	±	0.64a
18:4 *n*-3	8.79	±	0.26a	8.76	±	0.09a	9.16	±	0.19a	8.76	±	0.19a
20:5 *n*-3	107.81	±	0.78b	110.33	±	0.94a	109.87	±	1.88a	105.72	±	1.67b
22:6 *n*-3	339.73	±	2.67b	338.94	±	0.68b	342.70	±	0.77a	335.39	±	2.37c
∑ *n*-3 PUFA	490.22	±	2.37b	492.36	±	1.28b	496.17	±	1.82a	483.44	±	3.02c
n-3/*n*-6	0.98	±	0.00a	0.97	±	0.00a	0.98	±	0.00a	0.97	±	0.01a

Diet-1, vitamin C 0.08%; Diet-2, *A. aspera* seeds 0.5%; Diet-3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet-4, control.

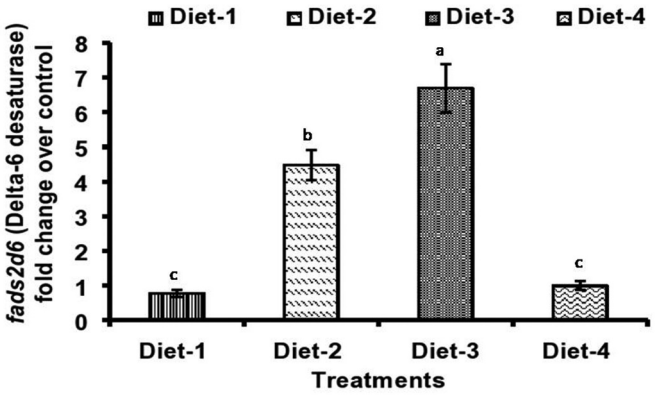

Figure 1. Relative mRNA expression of *fads2d6* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly (P < 0.05) different (*n* = 4).

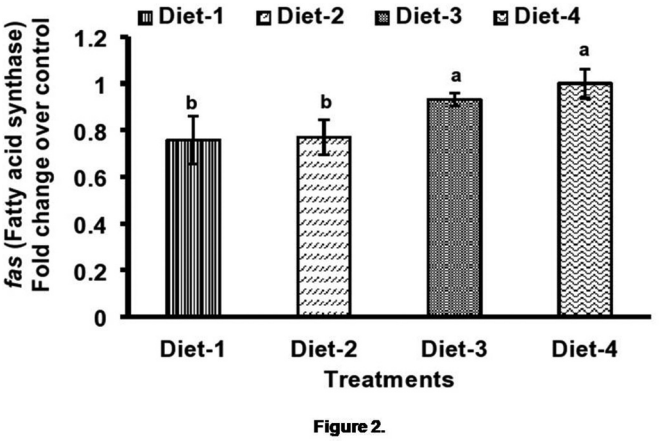

Figure 2. Relative mRNA expression of *fas* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly (P < 0.05) different (*n* = 4).

other diets. In snow trout fed the D3 diet, significantly lower stearic acid and behenic acid were found compared to others. SFA content was minimum in snow trout fed the control diet (D4).

Among MUFAs, palmitoleic and oleic acids contents were significantly higher in snow trout fed the D2 and D3 diets compared to the others. The gondoic acid and erusic acid levels were not significantly different among fish fed different diets. The nervonic acid content was significantly lower in snow trout fed the D4 diet compared to the others. The total monounsaturated fatty

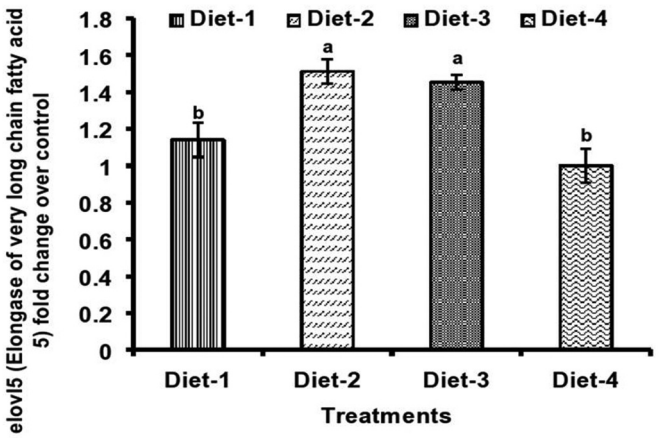

Figure 3. Relative mRNA expression of *elovl5* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly ($P < 0.05$) different ($n = 4$).

acid content was significantly higher in snow trout fed diets D2 and D3 compared to the others. In snow trout fed the D4 diet, the lowest MUFA content was observed.

Among *n*-6 PUFAs, the linoleic acid level was significantly higher in snow trout fed diets D2 and D3 and gamma linolenic acid in fish fed the D2 diet compared to the other diets. In snow trout fed the different diets, eicosadienoic, eicosatrienoic, and arachidonic acids contents were not significantly different.

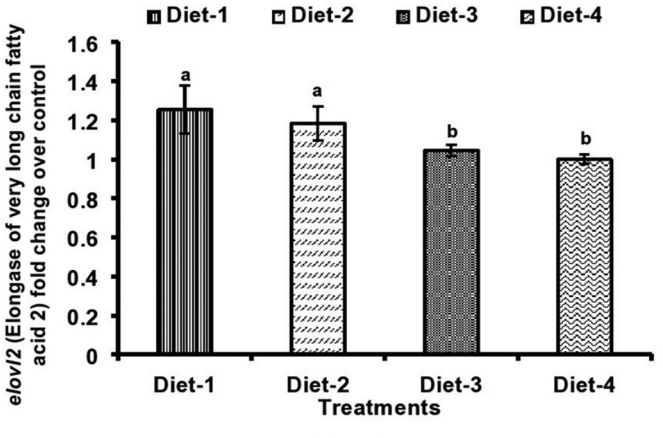

Figure 4. Relative mRNA expression of *elovl2* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly ($P < 0.05$) different ($n = 4$).

In snow trout fed the different diets, α-linolenic acid and stearidonic acid contents were not significantly different. A significantly higher eicosapentaenoic (EPA) acid level was observed in snow trout fed the D2 and D3 diets compared to the others. The docosahexaenoic acid (DHA) content was significantly higher in snow trout fed the D3 diet compared to other diets. Total *n*-3 PUFA content was significantly higher in snow trout fed the D3 diet compared to fish cultured in the other feeding regimes. A minimum *n*-3 PUFA content was found in snow trout fed the control diet (D4). The *n*-3/*n*-6 ranged from 0.97 to 0.98 in fish fed different diets.

Gene expression analysis

There was upregulation of delta-6 desaturase (*fads2d6*) in snow trout fed the D3 diet, and this feeding regime was followed by fish fed the D2 diet (Figure 1). Fatty acid synthase (*fas*) mRNA expression was downregulated in snow trout fed the enriched diets compared to the control (Figure 2). There were upregulations of *elovl5* in snow trout fed all enriched diets (Figure 3) and *elovl2* in snow trout fed the D1 and D2 diets compared to the others (Figure 4).

Discussion

The diets of snow trout fry were enriched with vitamin C, seeds of the plant *A. aspera*, and a combination of vitamin C and seeds. The positive effect of enriched diets was reflected on the survival rate of snow trout. The survival rate of snow trout was 93% in the control; in other three feeding regimes all fish survived (100%). The enhanced average weight was observed in snow trout fed the enriched diets compared to fish fed the control diet. Previous studies showed that the addition of vitamin C in diets enhanced the survival and growth and reduced the FCR of cultivable fishes such as rohu (Misra et al. 2007) and snow trout (Moses, Nilssen, and Chakrabarti 2017). In discus fish, supplementation of vitamin C and vitamin E resulted in higher SGR and lower FCR compared to the fish fed the control diet (Liu et al. 2018). Zou et al. (2019) recommended 71.46–150.26 mg kg^{-1} vitamin C for optimum growth of Chu's croaker *Nibea coibor*. Significantly higher average weight and SGR were found in snow trout fed both vitamin C and seeds enriched diet, D3 compared to the fish fed diets enriched with vitamin C/ seeds/control. In rohu, incorporation of 0.5% seeds of *A. aspera* increased the average weight and SGR (Sharma et al. 2019). The presence of ecdysterone in seeds increased the growth of fish (Chakrabarti et al. 2012). Ecdysterone increased protein synthesis in skeletal muscle (Goerlich-Feldmann et al. 2008) and thereby enhanced the growth of fish. The enrichment of diet with both vitamin C and seeds gave better results compared to the diet enriched with individual ingredients (vitamin C or seeds).

The digestibility of the consumed feed depends on the digestive enzyme activities of fish. All digestive enzymes activities assayed were significantly higher in snow trout fed the D3 diet. This resulted in efficient digestion of consumed diet and thereby the lowest FCR compared to fish fed other diets. All digestive enzyme activities (except lipase) were higher in fish fed diets with vitamin C and seeds compared to fish fed the control diet. Therefore, the highest FCR was observed in snow trout fed the control diet. In rohu fed seed-enriched diets, a lower FCR was recorded (Sharma et al. 2019; Singh et al. 2019).

Snow trout fed the D1 and D4 diets showed significantly higher protein contents compared to fish fed the D2 and D3 diets. There was no variation in the amino acid composition in snow trout cultured in four different feeding regimes; the amount of a specific amino acid varied. There were higher values of seven essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine) in snow trout fed the D1 diet and five amino acids (histidine, isoleucine, lysine, tryptophan, and valine) in fish fed the D3 diet compared to the fish fed the control diet, D4. Among nonessential amino acids, a higher value of alanine was found in snow trout fed both D1 and D3 diets compared to the fish fed diet D4; aspartate and tyrosine contents were maximum in fish fed the D1 diet and proline content was higher in D3 diets compared to snow trout fed the D4 diet. The free amino acid taurine content was significantly higher in fish fed the D2 and D3 diets. The enrichment of the diet with vitamin C and seeds increased the specific amino acid level in the muscles of fish. Several amino acids were found in the seeds of *A. aspera* (Goyal, Goyal, and Mehta 2007). Thus, feeding snow trout a seed-supplemented diet improved the amino acids contents of muscle. The enrichment of vitamin C in the diet of snow trout enhanced the protein synthesis and produced a higher amino acid profile. Vitamin C is a hydrogen donor and is associated with many hydroxylation reactions—in collagen maturation, catecholamine synthesis, and carnitine synthesis. Vitamin C helps in the synthesis of phenylalanine and tyrosine (Guillaume et al. 2001).

A significantly higher lipid content was found in fish fed the D3 diet. Enrichment of diets with seeds or vitamin C and seeds improved the fatty acid contents of the muscle of snow trout. Vitamin C showed no influence on the lipid and fatty acid contents of muscle of pacu (*Piaractus mesopotamicus*) or rainbow trout (*Oncorhynchus mykiss*) (Trattner et al. 2007; Trenzado, Morales, and de la Higuera 2008). Supplementation of vitamin C in the diets of grass carp (*Ctenopharyngodon idella*) improved the fatty acids (palmitoleic, oleic, linoleic, and linolenic acids and *n*-3 PUFA) contents in muscle compared to fish fed the control diet (Han et al. 2019). In snow trout fed the seed-enriched diet (D2), seven SFAs (myristic, pentadecanoic, palmitic, stearic, behenic acids, etc.) were found in highest proportion

compared to fish fed the other diets. In the muscle of snow trout fed the D1, D3, and D4 diets, 2 (behenic and lignoceric acids), 2 (myristic and pentadecanoic), and 3 (stearic, behenic, and lignoceric acids) SFAs were maximum respectively. Three MUFAs (palmitoleic, oleic, and nervonic acids) contents were maximum in snow trout fed the D2 and D3 diets. Among *n*-6 PUFA, the highest amount of linoleic acid was found in snow trout fed the D2 and D3 diets; γ-linolenic acid was maximum in fish fed the D2 diet. Among *n*-3 PUFAs, EPA content was significantly higher in fish fed the D2 and D3 diets; DHA content was highest in fish fed the D3 diet. Minimum EPA and DHA contents were observed in snow trout fed the D4 diet.

The present study showed that the supplementation of seeds in diets enhanced the fatty acids, especially DHA and EPA levels in the snow trout muscle. In common carp, linseed oil and sunflower oil induced substantial LC-PUFA production (Nguyen et al. 2019). Enhanced levels of EPA and DHA were found in snow trout fed the D2 and D3 diets in the present study. The presence of palmitic, oleic, and linolenic acids in seeds influenced the fatty acid compositions in snow trout fed the seed-supplemented diets. Moreover, the higher levels of EPA and DHA in snow trout fed the vitamin C and *A. aspera* seed-supplemented diet showed that the fish has the capacity to synthesize long-chain polyunsaturated fatty acids endogenously.

A significantly higher expression of *fads2d6* was found in fish fed the vitamin C and *A. aspera* seed-supplemented diet (D3). The expression was also higher in fish fed the *A. aspera* seed-supplemented diet (D2) compared to the fish fed the vitamin C-supplemented (D1) and control diets (D4). This showed that the presence of fatty acids (Chakrabarti et al. 2012) in the seed influenced the *fads2d6* expression. A higher expression of *delta 6 desaturase* was found in rainbow trout (*Onchorynchus mykiss*) fed a plant-based diet (Ve´ron et al. 2016). The upregulation of *fads-6a* was found in common carp fed sunflower oil (Nguyen et al. 2019). There was downregulation of *fas* in fish fed all enriched diets compared to the control. The downregulation of *fas* was found in grass carp fed a vitamin C-supplemented diet (Han et al. 2019). The mRNA expression of *elovl5* was higher in fish fed the D2 and D3 diets. The *elovl2* was upregulated in snow trout fed the D1 and D2 diets. There was no effect of plant-based oil in the expression of *elovl5* in common carp (Nguyen et al. 2019).

Conclusions

The enrichment of diets with vitamin C and *A. aspera* seeds enhanced the survival rate and growth of *S. richardsonii*. The enrichment also improved the flesh composition. Vitamin C improved the amino acids composition; seeds

improved the fatty acid composition of fish. Seeds enhanced the EPA and DHA contents in fish.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contribution

RC, JGS, GK, and SC designed the experiment; GK and SC conducted the experiment; GK, RKG, NK, and AKS analyzed the samples; RC, JGS, and GK prepared the manuscript; GK and NK prepared the tables and figures.

Data availability statement

The data that support the finding of this study are available within the article.

Ethical clearance

The ethical clearance for conducting this experiment was approved by the animal ethics committee of the University of Delhi. Protocol No - DU/ZOOL/IAEC-R/02/2019.

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