



EVALUATION OF EXTRACTED *MENTHA ARVENSIS* LEAF MEAL IN THE DIET OF *CYPRINUS CARPIO* (LINN., 1758) REARED IN INLAND SALINE WATER

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

M.F.Sc. (Fish Physiology and Biochemistry)

By

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DEDICATED TO MY PARENTS,
PATNAIK UNCLE
&
FOR ALL THOSE WHO ENCOURAGED ME
TO FLY TOWARD MY DREAMS:
LET'S SOAR





केन्द्रीय मात्स्यिकी शिक्षा संस्थान

भारतीय कृषि अनुसंधान परिषद,

CENTRAL INSTITUTE OF FISHERIES EDUCATION

(A university Established Under Sec.3 of UGC Act 1956)

Indian Council of Agricultural Research,
Ministry of Agriculture Govt. of India



Dated: 30th June 2018

CERTIFICATE

Certified that the dissertation entitled "**Evaluation of extracted *Mentha arvensis* leaf meal in the diet of *Cyprinus carpio* (Linn., 1758) reared in inland saline water**" is a record of independent bonafide research work carried out by **Mr. Dharmaraj Patro (FPB-MA6-01)** during the period of study from August 2017 to June 2018 under our supervision and guidance for the degree of **Master of Fisheries Science (Fish Physiology and Biochemistry)** and that the Dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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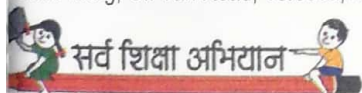
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DECLARATION

I hereby declare that the dissertation entitled “**EVALUATION OF EXTRACTED *MENTHA ARVENSIS* LEAF MEAL IN THE DIET OF *CYPRINUS CARPIO* (LINN., 1758) REARED IN INLAND SALINE WATER**” is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

अंरुस्थलीय खारा पानी (आईएसडब्लू) में साइप्रिनस कार्पीओ के विकास और तनाव प्रतिक्रिया का मूल्यांकन करने के लिए और मेन्था अर्वेन्सिस लीफ भोजन (ईएमएलएम) के साथ डी-तेल वाले चावल की चोटी (डीओआरबी) के प्रतिस्थापन के इष्टतम उपयोग प्रभाव का मूल्यांकन करने के लिए दो प्रयोग किए गए थे। साइप्रिनस कार्पीओ फिंगरलिंग में वृद्धि प्रदर्शन। पहला प्रयोग ताजा पानी और अंतर्देशीय नमकीन पानी (आईएसडब्लू) में साइप्रिनस कार्पीओ फिंगरलिंग के विकास और तनाव प्रतिक्रिया की तुलना करने के लिए नियंत्रण आहार के साथ चार प्रतिकृतियों में दो उपचारों के साथ 60 दिनों के लिए आयोजित किया गया था। परिणामों से संकेत मिलता है कि आईएसडब्लू में सामान्य कार्प के पालन में वृद्धि और फ्रीड रूपांतरण में कमी आई है। चयापचय और एंटीऑक्सीडेंट एंजाइमों की गतिविधि ने आईएसडब्लू में मछलियों में चयापचय में चयापचय और ऑक्सीडेटिव तनाव को भी इंगित किया है। आईएसडब्लू में दी गई मछलियों में प्रतिरक्षा को भी दबाया गया था, जैसा कि ग्लोबुलिन सामग्री में कमी के साथ बढ़े हुए A:G अनुपात से संकेत मिलता है। आहार में ईएमएलएम के उपयोग का मूल्यांकन करने के लिए दूसरा प्रयोग 60 दिनों के लिए आयोजित किया गया था। ईएमएलएम में 18.64% कच्ची प्रोटीन, 20.83% फाइबर और इन-विट्रो पाचन क्षमता 32.37% थी। ईएमएलएम में फाइटेट (4.43%), ऑक्सालेट (0.032%), कुल टैनिन (1.26%) और एल्कालोइड (0.68%) जैसे एंटीन्यूट्रिएंट मौजूद थे। उच्च स्तर पर फ्लैवोनोइड्स की उपस्थिति के कारण ईएमएलएम में एंटीऑक्सीडेंट संपत्ति थी। चार आईएसओ-नाइट्रोजेनस (30% कूड प्रोटीन) और आईएसओ-कैलोरीफेट आहार तैयार किए गए थे, डीओआरबी के प्रतिस्थापन 0% (सी, नियंत्रण आहार), 33% (टी 1 आहार), 66% (टी 2 आहार), और 100% (टी 3 आहार) ईएमएलएम द्वारा। इस अध्ययन के नतीजे से पता चला कि विकास प्रदर्शन और फ्रीड उपयोग में उल्लेखनीय कमी (पी < 0.05) थी। आरएनए और आरएनए / डीएनए अनुपात में वृद्धि हुई है (पी < 0.05) ईएमएलएम समावेशन के साथ, विकास प्रदर्शन के साथ संलग्न। ईएमएलएम फेड समूहों में पाचन एंजाइमों (एमाइलेज, प्रोटीयेज़, लाईपेज) की गतिविधि में कमी आई है। एएलटी, एएसटी, एलडीएच, एमडीएच जैसे चयापचय एंजाइम भी ईएमएलएम समावेशन से नकारात्मक रूप से प्रभावित थे, चयापचय तनाव का संकेत देते हैं। एंटीऑक्सीडेंट एंजाइमों की गतिविधि में आहार में ईएमएलएम के साथ 20% और 30% स्तर पर खिलाए गए प्रयोगात्मक समूहों में उल्लेखनीय कमी आई है। आहार में ईएमएलएम के साथ खिलाए गए समूहों में 20% और 30% के स्तर पर रक्त ग्लूकोज और कोर्टिसोल स्तर में भी काफी कमी आई है। ग्लोब्यूलिन प्रोटीन को सभी प्रयोग में वृद्धि ई.एम.एल.एम. के एक महत्वपूर्ण रोग प्रति रोधी क्षमता को दर्शाता है। कुल मिलाकर, परिणामों से संकेत मिलता है कि ईएमएलएम को मछलियों के भोजन में शामिल करने से तनाव में कमी, रोग, प्रतिरोधी क्षमता का विकास होता है परन्तु कुछ हदतक यह वृद्धि दार को कम करता है।

ABSTRACT

Two experiments were conducted to evaluate the growth and stress response of *Cyprinus carpio* in inland saline water (ISW) and to evaluate the optimum utilization effect of replacement of de-oiled rice bran (DORB) with extracted *Mentha arvensis* leaf meal (EMLM), on growth performance in *Cyprinus carpio* fingerlings. The first experiment was conducted for 60 days with two treatments in four replicates fed with control diet to compare the growth and stress response of *Cyprinus carpio* fingerlings reared in freshwater and ISW. The results indicated that rearing of common carp in ISW reduced growth and feed utilization. The activity of metabolic and antioxidant enzymes also increased indicating metabolic and oxidative stress in fishes reared in ISW. The immunity was also suppressed in the fishes reared in ISW, as indicated by the increased A:G ratio with decrease in globulin content. The second experiment was conducted for 60 days to evaluate the utilization of EMLM in the diet. The EMLM contained 18.64% crude protein, 20.83% fibre and the in-vitro digestibility was found to be 32.37%. The anti-nutrients such as phytate (4.43%), oxalate (0.032%), total tannin (1.26%) and alkaloids (0.68%) were present in EMLM. The EMLM had antioxidant property due to the presence of flavonoids at higher level. Four iso-nitrogenous (30% crude protein) and iso-calorific diets were formulated, with replacement of DORB at 0% (C, control diet), 33% (T1 diet), 66% (T2 diet), and 100% (T3 diet) by EMLM. The result of this study showed that there was significant decrease ($P<0.05$) in the growth performance and feed utilization. The RNA and RNA/DNA ratio decreased significantly ($P<0.05$) with EMLM inclusion, attuned with the growth performance. The activity of digestive enzymes (amylase, protease and lipase) were decreased in EMLM fed groups. The metabolic enzymes like ALT, AST, LDH, MDH, were also negatively affected by EMLM inclusion, indicating metabolic stress. The activity of antioxidant enzymes significantly decreased in the experimental groups fed with EMLM in the diet at 20% and 30% levels. The blood glucose and cortisol level also significantly reduced in the groups fed with EMLM in the diet at 20% and 30% levels. The globulin level increased in the serum across the experimental groups, revealing the immune-stimulant property of EMLM. Overall, the results indicated that the inclusion of extracted *Mentha arvensis* leaf meal (EMLM) has resulted in poor growth performance, whereas EMLM inclusion enhanced stress mitigation and immune responses in *Cyprinus carpio* reared in inland saline water.

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1. INTRODUCTION

Fish and fishery products are valuable sources of proteins and essential nutrients, and they provide human health benefits and facilitate environmental sustainability. Capture fisheries and aquaculture sector acts as a source of income and provides livelihood to millions of people around the world. Appetite for fish continues to expand around the globe, despite the stagnant levels of capture fish production. Aquaculture thus became the major supplier of fish and has the potential to be a major source of protein in the future. Aquaculture has the potential to produce substantial amounts of fish in the future to support increases in human population size. Also, aquaculture facilitate the tracking of safety and quality which is considered as advantageous over capture fisheries. According to an FAO report (FAO, 2016), more than 50% of food fish consumed globally will eventually be produced by aquaculture, and aquaculture is now the main source of increased fish supplies. The increasing demand for aquatic food products and the limited production from the capture fisheries had paved the way for rapid development of coastal and inland aquaculture. Indian fisheries has tremendous progress during the last five decades with the production levels increasing from 750,000 tonnes of fish in 1950-51 to 10.2 million tons in 2015-2016, of which the contribution from the inland sector is around 6.577 million tons (66.3 % of the total) compared to 3.7 million tons (33.3 %) from the marine sector. The contribution of fisheries to the gross domestic product (GDP) and agriculture GDP has been estimated to be 1.2 % and 4.2 %, respectively. India ranks second in the world in inland fish production, next to China. India contributes 6.62% of the world total fish production (SOFIA, 2014).

As the demand for food throughout the world increases, a great deal of effort has been expended finding ways to more efficiently produce food, both animal and vegetable, to satisfy the demand. Increasing demand for aquaculture has led to the development of new production systems. Inland saline aquaculture is such a new system which would help in utilizing the underutilized lands. Secondary salinization is a major environmental problem in many arid and semi-arid areas of the world, adversely affecting up to 380 million hectares of land, including 100 million hectares of arable land (Ghassemi *et al.*, 1995; Lambers, 2003). Inland saline aquaculture is land-based aquaculture using saline groundwater. Some species are cultured or are

being evaluated for their potential, including finfish such as tilapia, Asian sea bass, and trout, shrimp and oysters. Inland saline aquaculture has the potential for positive economic, social and environmental outcomes from groundwater in the salt-affected land.

In India, salinization of inland groundwater is prevalent mainly in the arid and semi-arid regions of Rajasthan, Haryana, Punjab, and Gujarat and to a lesser extent in Uttar Pradesh, Delhi, Madhya Pradesh Maharashtra, Karnataka, Bihar and Tamil Nadu. About 2 lakh sq.km area has been estimated to be affected by secondary salinization of land. The increased irrigation with the advent of agriculture increased salinity levels in soil water, surface water systems and/or aquifers (Van Weert *et al.*, 2009) Irrigation can also raise water tables, lead to waterlogging, and cause evaporation directly from the water table, increasing solute concentration in the soil (Van Weert *et al.*, 2009). The gradual rise of ground water levels with time has resulted in water logging and heavy evaporation in semi-arid regions lead to salinity problem in command areas. This saline water is not useful for irrigation or drinking purpose. As per recent assessment, about 2.46 m ha of the area under surface water irrigation projects is water logged or threatened by water logging. The water table in these areas is increasing due to the non-utilization of water. The ground water can only be decreased by pumping or by evapotranspiration. In order to utilize these vast water resources, aquaculture has been identified as one of the most suitable and potential options. This will help in reducing the underground saline water and prevent secondary salinization, which will help in development of agriculture land.

One of the major factor which determines the growth of aquaculture is the availability of feed ingredients. Rice bran is the most commonly incorporated ingredient in the aquaculture feed. Rice bran is a primary by-product of the traditional rice processing industry. One hundred kilograms of paddy on milling produces 10-12 kg of rice bran (Kahlon, 2009). Once oil is extracted from rice bran, the left over residue is called de-oiled rice bran (DORB) which has a crude protein content of 14-18%. In the recent past, the utilization of rice bran has been increased by multiple uses viz. as a functional food, in snacks food, bakery product, medical food, and poultry feed which resulted in increased price in the market. Furthermore, increasing fish production with respect to increaseing in human population requires additional amount of DORB which is falling short with the current production. It is therefore necessary to practically

search, explore, identify and utilize other plant protein source which could be cost effective, less competitive, not relatively in high demand and can replace the highly demanded de-oiled rice bran in fish feed. Due to changes in environmental conditions, unstable source and increase in the cost of raw materials, it is likely that the source and composition of aquaculture feeds will change considerably in the near future. Plant protein and carbohydrate have been considered as the important alternative ingredients for formulating fish feeds. Nowadays, most researchers focused on consumption of less expensive and locally available resources in fish feed, without sacrificing nutrition and quality of feed (Link *et al.*, 1999).

Nutritionists all over the world are constantly searching for the cost effective dietary energy sources for good production and growth within the shortest possible time. Leaf meals are one of the cheapest sources of proteins and energy that may be act as a potential alternative in future and will reduce the cost of fish feed. Several reviews have been made concerning the interest of foliage as feed, (Wanapat *et al.*, 2009). Many studies have been conducted using various sources of leaf meal proteins in diet of herbivores and carnivores fishes on cassava leaf meal (Ng *et al.*, 1989), on Alfalfa (Yousif *et al.*, 1994), (Reyes *et al.*, 2003) on *Carica papaya* and on *Leucaena leucocephala* (Bairagi *et al.*, 2004).

Mentha arvensis (corn mint, field mint or wild mint) is a species of flowering plant in the Mint family Lamiaceae. It is generally cultivated to extract the oil from its leaves. India is the largest producer and exporter of mentha oil in the world. At present the major producers of mint oil in the world are India, China, Brazil and US. *Mentha arvensis* is cultivated in India in the semi-temperate regions in the foothills of Himalayas in Punjab, Haryana, Himachal Pradesh, Uttar Pradesh and Bihar. The all-India area under *Mentha* in the country is estimated at 2.60 lakh ha (CIMAP, 2016). Extracted Corn Mint (*Mentha arvensis*) leaves are among the underutilized agricultural by-products. Corn mint leaves after the extraction of oil is a waste. The leaves after extraction can be utilized to make feed for fishes as it contains a good source of protein, 26.9% of dry weight (Yeoh and Wong, 1993). It is also a good source of amino acids, minerals, antioxidants and dietary fibre.

Nature has endowed with all plant to synthesize a wide variety of chemical substance called Anti-nutritional factors. Because of these anti-nutritional factors, they

are not frequently used as a feed ingredient. Anti-nutritional factors are those substances which either by themselves or through its metabolic products reduces the nutrient utilization, decreases digestibility, bind with divalent cations and make unavailable to fishes for their growth. ANFs are the secondary metabolites of plant they used it as a defense mechanism against the herbivores. After the consumption, it exerts deleterious effects when ingested by animals. (Soetan and Oyewole, 2009) found that the ANFs present in plants varies according to the occurrence and concentration vary according to the genetic origin, soil, fertilizer, pesticides and other environmental conditions, rainy season. The anti-nutrients present in corn mint includes flavonoids and tannins.

Common carp (*Cyprinus carpio*) is one among the cultured carp species which commands a higher price in the market. It is a stenohaline freshwater fish and can tolerate low levels of salinity. Common carp is a most preferred and delicious fish in south-east Asian countries and cultured throughout the world. It is the third most widely cultivated and important species in the world (after silver carp and grass carp) and contributing more than 4 million tonnes (FAO, 2014) to the world aquaculture production. High growth potential, compatibility with another species, wider feeding niche, etc. makes it preferable species for farming and aquaculture. India has already drawn up a strategic plan for doubling aquaculture production to cater the protein requirement of growing population. This will be only possible by the use of compounded feed, and this will increase the requirement of feed of ingredients like rice bran. However, increase in production of rice bran has very remote possibility due to adverse climatic condition for paddy production. Therefore there is explicit need to search for an alternative to rice bran. With this background, the present study has been undertaken with the following objectives:

Objectives:

1. To evaluate the growth of *Cyprinus carpio* in inland saline culture system fed with extracted *Mentha arvensis* leaf meal-based feed.
2. To evaluate stress responses in *Cyprinus carpio* fed with extracted *Mentha arvensis* leaf meal-based feed reared in inland ground saline water.

2. REVIEW OF LITERATURE

2.1. Inland saline water aquaculture

Global food production will need to increase by 38% by 2025 and by 57% by 2050 (Wild, 2003) if food supply to the growing world population is to be maintained at current levels. Most of the suitable land has been cultivated and expansion into new areas to increase food production is rarely possible or desirable. The aim, therefore was to achieve an increase in yield per unit of land rather than in the area cultivated. The efforts for increasing productivity lead to enhanced irrigation and salinization of ground water. It is estimated that about 15% of the total land area of the world has been degraded by soil erosion and physical and chemical degradation, including soil salinization (Wild, 2003). These salt affected areas pose a serious challenge for agriculture. Rising water tables and increasing salt content cause loss of fertility and lead to reduced productivity and alienation of valuable farm land. Secondary salinization of soil has become a case of global occurrence wherein around 20% of world's cultivated area and nearly half of the world's irrigated lands are seriously affected. These salt affected soil and water are unsuitable for agriculture purposes at most cases and hence the lands are left barren. On the other hand, these saline water reserves represent a potential opportunity for the development of inland aquaculture, which has been identified as the most suitable and potential measure for the utilization of these inland saline land and water resources. Inland saline aquaculture offers attractive prospects to the aquafarmers. Suitable sites for aquaculture in terms of land availability are often not easily and readily available as most of them are expensive and have been occupied by other users and purposes. Usage of Inland saline water for aquaculture purposes is an effective shift since most of such lands are located in remote areas where land is cheap and disease free, where there is better potential for aquaculture (Gong *et al.*, 2004).

2.1.1. Inland saline aquaculture scenario of the world

Salt affected areas and salinization of fertile agricultural lands and ground water is a common problem across the world. Salinization has affected over 380 million hectares of land in over 20 countries worldwide (Lambers, 2003). Inland saline water (ISW) have affected the agricultural outputs and environment in many countries of the

world like Australia, USA, China, Israel, and India. ISW have high levels of Ca^{2+} and Mg^{2+} resulting in higher water hardness with salinity ranging from 10-35 ppt (Jana *et al.*, 2004). Inland saline water aquaculture has potential to increase the source of income from unutilized resource (Trendall and Pitman, 1998 and Beresford *et al.*, 2001). Approximately, 103 million ha land in South-East Asia is under the grip of salinity (Massoud, 1974). Of this, 47 million ha is strongly saline, and 54 million ha is ranked as moderately saline soil. Thus, it provides the potential to turn an economic and environmental threat into an opportunity with both commercial and environmental benefits. The countries such as Australia, China, India, Israel and USA have a demonstrated utilization of their affected land and water resources for commercial mariculture (Zhu *et al.*, 2004). This water is suitable for culture of many euryhaline water species (Partridge *et al.*, 2002). Aquatic crustaceans and fin fishes have been reared in the inland saline water around the world (Saoud *et al.*, 2003). Inland saline areas of Australia constitutes 60% of the total of the world's saline soils and the salinity ranges of these water is from 5 ppt to 45 ppt.

2.1.2. Inland saline aquaculture scenario of India

Salinization in Inland ground water is prevalent mainly in the arid and semi-arid regions of Rajasthan, Haryana, Punjab and Gujarat and to a lesser extent in Uttar Pradesh, Delhi, Madhya Pradesh, Maharashtra, Karnataka, Bihar and Tamil Nadu. India has approximately 9.38 m ha area which is occupied by salt-affected soils in which 5.5 million ha are saline soils (including coastal) and 3.88 m ha alkali soils (IAB, 2000). Total area nearly 8.62 million hectares are salt-affected out of which 1.93 million square kilometers are under-laden with ground saline water (Lakra *et al.*, 2013). Almost 2.8 million hectares of salt affected soils are present within the Indo Gangetic alluvial plain occupying parts of Punjab, Haryana, Uttar Pradesh, Delhi, Bihar and Rajasthan states (Abrol *et al.*, 1971). In India, West Bengal has the highest area of salt affected soil followed by Uttar Pradesh and Haryana. The major reason for surge of Inland Saline Water (ISW) is due to high seepage of pond water and rising ground water tables which bring saline water to the surface, rising water salinity to a range of 10- 35 ‰ with elevated water hardness (Jana *et al.*, 2004). Studies on the utilization of salt affected inland saline soils and ground saline water for aquaculture have been initiated by Central Institute of Fisheries Education (CIFE) under ICAR sponsored Operational Research Project (ORP) during 1986 at Sultanapur, Gurgaon district,

Haryana. In 1996, the centre has been shifted from Sultanapur to Lahli Fish Farm, Rohtak, Haryana in order to strengthen research and field facilities. With the experience of Sultanpur, various programmes have been initiated for the culture of finfish and shellfish species.

The soil and ground saline water parameters were studied in various regions of Rajasthan (Jain *et al.*, 2006). Simultaneously, research programmes were undertaken to find out the suitability of ground saline water of Rajasthan for aquaculture and field trials on culture of milk fish *Chanos chanos* and laboratory trials on tiger shrimp, *Penaeus monodon* were undertaken. ICAR had sanctioned a niche area project, entitled 'Utilization of inland saline and sodic soils for Aquaculture'. CIFE conducted various studies on use of inland saline soils and ground saline water for aquaculture, pilot scale technologies were demonstrated for culture of various finfish and shellfish of freshwater, brackish water and marine origin viz., carps, Indian catfish, freshwater prawn, tiger shrimp, mullet and milkfish and commercially feasible technologies have been developed for few species (CIFE, 2002-03). As salinization of agricultural lands in inland and coastal states is one of the major problems, the Indian Council of Agricultural Research has identified as one of the thrust and niche area of research. In order to continue further research, the ICAR has sanctioned a consortium based NAIP sub-project entitled 'A value chain on fish production in fragile agricultural lands and unutilized aquatic resources in Maharashtra'.

2.2. Physiological changes in fishes reared in ISW

For physiological homeostasis, water homeostasis and osmoregulation are primary points of concern. Regulations of internal water balance and ion concentration are critical in fish since they either live in the hyperosmotic marine environment or in hypo-osmotic freshwater. Teleost fish are able to regulate the ionic concentration of their internal body fluids within narrow ranges (Potts, 1968).

Depending on the tolerance displayed to changes in external salt concentration, two types of freshwater fish can be distinguished, i.e., stenohaline and euryhaline fish, which display a different physiological response when exposed to saltwater (SW). Stenohaline freshwater fish hyper-regulate to allow a continuous influx of water necessary for the elimination of waste products via the kidneys. When exposed to saltwater, euryhaline freshwater teleosts are hypo-regulators that prevent net water

loss by drinking saltwater to keep the composition of the body fluids constant (Assem and Hankf, 1979).

Teleost exposed to hyperosmotic environments experience osmotic water loss coupled with diffusive ion gains. To compensate the loss of water, an increase in the drinking rate becomes necessary (Brocksen and Cole, 1972; Foskett *et al.*, 1981). In the intestine, water is absorbed osmotically, following active absorption of salts across the epithelium (Wilson *et al.*, 1996). To offset the increased ion load, and ultimately survive in saline waters; fish must actively excrete Na^+ and Cl^- . Gills are the primary organ involved in osmoregulation in teleost fishes. Ion-transporting chloride cells, located on the branchial filamental epithelium, are rich in Na^+ , K^+ -ATPase, which drives excretion mechanisms. Chloride cells are responsible for ion excretion in both juvenile and adult seawater-acclimated fish (Foskett *et al.*, 1981; Marshall and Bryson, 1998; Perry, 1997). The gills of teleost fish play an important role in ion regulation (Ay *et al.*, 1999; Weng *et al.*, 2002). The adaptation of Common carp to saline water involves some functional changes in gill epithelium chloride cells and Na^+ - K^+ ATPase activities. Chloride cells (mitochondria-rich cells (MRCs)) are found to be sparsely distributed on the filament, in inter-lamellar regions, and at the bases of lamellar (Greco *et al.* 1996; Perry 1997). These cells are the main location of the gill Na^+ - K^+ ATPase (McCormick 1993; Weng *et al.*, 2002).

Increased salinity results in the augmentation of Na^+ - K^+ ATPase activity as well as morphological changes in MRCs (Uchida *et al.*, 1996). The number, size, and location of branchial chloride cells are known to alter with changes in environmental salinity. The degree of change in cells can be influenced by the range of change in salinity as well as the species and age of fish (Hwang and Hirano 1985; Kelly and Woo 1999; Caberoy and Quinitio, 2000). Changes in morphology of chloride cells following transfer to saltwater and freshwater suggest that filament and lamellar chloride cells are essential in saltwater and freshwater osmoregulation, respectively. The Common carp (*Cyprinus carpio*) is a well-known example of a stenohaline freshwater fish (Gupta and Hanke 1982). The adaptive capacity of common carp to different salinities depends on the integrated osmoregulatory function of numerous organs, mainly the gills, digestive tract, and kidney (Cioni *et al.*, 1991).

McDonald and Grosell (2006) studied that secretion of HCO_3^- in the intestinal lumen will be more when fish exposed to high saline water to facilitate water absorption. Ca^{2+} is an impermeable cation and remains within the lumen, because of that the formation of CaCO_3 precipitates in the teleost gut is well known, and removal of this cation by precipitation thus contributes to a partial decrease of the luminal osmolality (Wilson *et al.*, 2002), and the concentration of Mg^{2+} and SO_4^{2-} increase osmolality. Salinity may influence physiological processes; it was shown to alter vital processes such as osmoregulation, rates of protein synthesis and oxygen uptake, the scope for growth, and extracellular acid–base balance (Guerin and Stickle 1997; Whiteley *et al.*, 2001).

2.3. Growth of fishes and shellfishes in ISW

Development and growth in teleost fishes take place following appropriate steps for each species and more or less directly under control of environmental factors. Fish are dependent on both internal (nervous, endocrinological and neuroendocrinological) and external (ecological) factors, which control or synchronize many activities or functions, including growth capacity.

In fish, growth is a specific physiological function, which differs from that observed in birds and mammals. Firstly, growth is continuous, so that fish become larger the longer they live, and they are much more dependent on external environmental conditions (Brett, 1979; Boeuf *et al.*, 1999). Among the ecological factors, salinity is specific to the aquatic environment.

The common carp (*Cyprinus carpio*) is a well-known example of a stenohaline freshwater fish (Gupta and Hanke, 1982). Although the animal displays some tolerance to increased salinity (Kulijev and Agayarova, 1984; Schildhauer *et al.*, 1992) and diluted sea water has been reported to enhance survival, growth and development of carp larvae (Lam and Sharma, 1985), exposure to higher levels of salinity appear to have adverse effects on carp. Growth decreases, glycogen stores are depleted, and fish become more susceptible to bacterial infections (De Boeck *et al.*, 1996). The food conversion ratio (FCR) and weight gain are significantly affected by fluctuating salinities in the short nose sturgeon, better growth was observed in the fish reared in

freshwater, and as the salinity increased, growth rate got decreased (Jarvis *et al.*, 2001).

Many physiological changes occur when the stenohaline freshwater fish are exposing to salt stress, those are depending on the salinity levels range, time of exposure to salinity changes and the effect of other ecological factors (Bailey *et al.*, 2002) which leads to increase energy demands in order to maintain the internal balance (Evans, 2002).

2.4. Effect of ISW rearing on immunity

Hemato-immunological parameters are indicators of physiological stress responses to endogenous or exogenous changes in fish (Santos and Pacheco, 1996). The blood parameters of fish may be affected by acute or chronic stress (Barton and Iwama, 1991).

Nile tilapia, exposed to saline water showed changes in blood parameters (Verdegem *et al.*, 1997), immune function parameters (Choi, 2004), and histopathology (Hassan *et al.*, 2013, de Azevedo *et al.*, 2015), which is suggested to make them more susceptible for infectious disease.

2.5. Effect of ISW rearing on stress response

Fish and other aquatic animals are subject to a broad variety of stressors because their homeostatic mechanisms are highly dependent on prevailing conditions in their immediate surroundings. In 1936 a scientist named Hans Selye, upon observing effects of noxious stimuli in laboratory animals coined the term “stress” and defined it as “the non-specific response of the body to any demand for change” (Selye 1936). Variations of water temperature, pH, and salinity outside the optimal species’ ranges can act as environmental stressors for aquatic organisms, affecting their survival and maintenance (Anger *et al.*, 1998; Heugens *et al.*, 2001).

Any stress factor may create an internal physiological imbalance in fish appears through disorder hormones and enzymes functions and changing in some blood picture characteristics, which need a systematic physiological response by fish against stressors returning to homeostasis (Eddy, 2006). Stress response in all vertebrates

including fish results in the activation of the neuroendocrine system, which brings about changes in metabolism, osmoregulation and blood parameters (Chatterjee *et al.*, 2006). The metabolism shifts from anabolism to catabolism to supply extra energy needed to combat stress (Chatterjee *et al.*, 2006). Much of the stress studies on fish have been focused on the primary response related to cortisol and catecholamine levels (Barton and Iwama, 1991). Among the secondary stress response parameters, blood glucose and lactate levels are most commonly tested (Chatterjee *et al.*, 2006).

Measurement of metabolic enzyme activity can also serve as a valuable stress indicator in organisms like marine invertebrates and fishes (Dahlhoff, 2004). The gluconeogenic pathway is active in fishes since they prefer to utilise the protein and lipid rather than carbohydrate for energy requirements and under stress, glucose is mainly derived by gluconeogenesis (Vijayan *et al.*, 1993). The stress responses in fish can be manifested through key gluconeogenic enzymes, enzymes of protein metabolism mainly transaminases, the terminal enzyme of glycolytic pathway i.e. lactate dehydrogenase and malate dehydrogenase.

The increment of Alanine transaminase (ALT) and Aspartate transaminase (AST) levels occur during nervous shock, hypoxia and stress where their standard plasma levels would be affected by several factors such as pollution, ammonia and nitrite toxicities and other environmental parameters like temperature and salinity (Das *et al.*, 2004).

Cortisol is one of the important fish hormones responsible in increasing the salinity tolerance during freshwater fish transferring to seawater through its effect on reducing body fluid concentration (hypo-osmotic) (Morgan and Iwama, 1991).

Cortisol regulates body fluid electrolytes of teleost through its direct effect on cellular membrane permeability, increases number and size of mitochondria and activating ATPase levels which control salt motion in gill chloride cells of teleost's (Uchida *et al.*, 1997).

Cortisol plays an important role in increasing ions uptake and it acts with prolactin hormones during acclimatisation to freshwater (Pelis and McCormick, 2001), whereas the hormone itself shares growth hormone in increasing fish capability to salinity stress (Hyde *et al.*, 2004).

An increase of cortisol hormone concentrations in blood plasma of freshwater fish when exposed to a gradual increase in salinity clarify fish adaptation towards the salted water. The hormone decreases the body fluids concentration of freshwater fish to osmoregulate the physiological responses which locate under hormonal control (McCormick, 1995).

2.6. Histological response

Evaluation of histopathological manifestations provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism (Menon *et al.*, 2005). Hypersalinity results in a qualitatively different type of negative response. Apoptosis of chloride cells (branchial cells that facilitate ion transport and have an integral role in acid-base regulation; Perry 1998) occurred in hybrid tilapia exposed experimentally to various concentrations of hypersaline water for a model of salinity tolerance (Sardella *et al.*, 2004).

2.7. Fish feed and ingredients availability

One of the problems faced by the aquaculture industry today is the high cost of fish feed. Nutritionist all over the world are continually searching for the dietary protein sources in which fish will maximise growth and increase production within the shortest possible time and at the lowest cost. Increasing raw material and farm operating costs, coupled with an often static and decreasing market value for many farmed species necessitates that the farmer reduces production costs to maintain profitability. Since feed usually represent the most significant single operating cost item, therefore, it should be focused at further reducing feed costs per unit of production through the development and use of non-conventional feed ingredients in fish feed.

De-oiled rice bran (DORB) is one of the major ingredient used in the fish feed. DORB is the by-product after extraction of oil from rice bran. At present about 7.0 MT of ingredients are being used for farm-made fish feed production (Ramakrishna *et al.*, 2013). The current aquaculture production of the country (4.3 MMT) which is projected to reach 12.7 MMT by the year 2030 as reported by World Bank report (Msangi *et al.*, 2013).

Considering the total aquafeed production of India which is about 1 MMT as reported by Alltech (2014) is set to reach around 3 MMT by the year 2030 but the feed demand will be around 23 MMT hence the significant part of feed will be the Mash feed that mainly contains DORB (about 80-85 %) (Ramkrishna *et al.*, 2013). The carps together contribute to more than 82% of total aquaculture production in India. About 97% of the carp feeds used by Indian farmers are farm-made (Ramkrishna *et al.*, 2013). However, looking into the DORB production of India which is presently about 6 MMT is not going to increase to support the demand that will be required for Indian Major Carp (IMC) and other cultured fish. Hence, It is essential to explore alternate fish feed ingredients of cheaper cost and high value which can replace the DORB and sustain the Indian aquaculture. Aquaculturists try to obtain the balance between growth and the quality and quantity of feed (Gokcek *et al.*, 2008). A well-balanced feed is the key to obtain adequate growth (Aderolu *et al.*, 2010). Protein is the most costly component of fish feeds. Therefore, efforts to reduce feed expenses have resulted in increased use of plant proteins as replacements for expensive animal ingredients, especially fish meal in diet formulations (Ping *et al.*, 2010).

2.8. Plant based feed ingredients

The major plant dietary nutrient sources, includes cereals, meals and oils, and protein concentrates.

- I. Cereals, including by-product meals and oils: includes milled/processed cereals (maize/corn, wheat, rice, barley, sorghum, oats, rye, millet, triticale, etc.); by-product meals (corn/maize gluten, wheat gluten, dried distillers grains with solubles, rice protein concentrate, rice bran, wheat bran); and extracted oils (corn/maize, rice).
- II. Oilseed meals and oils: includes full-fat (soybean) and solvent extracted oilseed meals (soybean, rapeseed, cotton, groundnut/peanut, sunflower, palm kernel, copra); by-product meals (soybean protein concentrates, rapeseed/canola protein concentrate); and extracted oils (palm, soybean, rapeseed, sunflower, linseed, cottonseed, olive).
- III. Pulses and protein concentrate meals: includes milled/processed pulses (peas, lupins) and by-product meals (pea protein concentrate, lupin protein concentrates).

Soybean meal has been the most frequently studied dietary ingredient as a fish meal replacer in diets for many fish species because of its high protein content, relatively well balanced amino acid profiles, reasonable price and steady supply (Storebakken et al., 2000). Soybean meal (SBM) is widely available protein source with nearly sufficient digestible protein, high energy content and good amino acid profile (Hertrampf and Piedadu-Pascual, 2000). It is used as a cost effective replacement feed ingredient to fish meal in aquafeeds (Yue and Zhou, 2008). Rapeseed meal is relatively less costly than soybean meal. The aminoacid content of rapeseed meal is comparable well with that of soya bean meal and richer in methionine (Clandinin, 1967). Glucosinolates are the main antinutrients present in rapeseed (Brassica spp.) meal and mustard oil cake, which are otherwise potentially attractive protein sources in fish feeds. Heat treatment by wet pressure cooking reduces the glucosinolate content of rapeseed meal from 40 to 26 $\mu\text{mol/g}$ of diet (Burel et al., 2000).

2.9. Anti-nutrients in plant feedstuffs

Nature has equipped many plants with the ability to synthesise a variety of chemical substances with the apparent function of protecting them from becoming food for microbes, insects and higher animals. Consequently, many of these compounds may exert harmful effects when ingested by humans and animals. Such substances are often called antinutritive factors (ANFs), although they may also have beneficial effects, such as being antioxidative, immunostimulatory or prebiotic, depending on the amount ingested. Possible harmful effects include reduced palatability, less efficient utilization of feed nutrients for growth, altered nutrient balances of the diets, inhibition of growth, intestinal dysfunction, altered gut microflora, immune modulation, goitrogenesis, pancreatic hypertrophy, hypoglycaemia or liver damage. The species of animal, its age, size, gender, state of health and plane of nutrition and any stress factors may modify these responses. Anti-nutritional factors are substances which either by themselves or through their metabolic products, interfere with feed utilization and affect the health and production of animal or which act to reduce nutrient intake, digestion, absorption and utilization and may produce other adverse effects (Akande et al., 2010). Anti-nutritional factors are compounds which reduce the nutrient utilization and/or food intake of plants or plant products used

as human foods or animal feeds and they play a vital role in determining the use of plants for humans and animals (Soetan and Oyewole , 2009b). There is a wide distribution of biologically-active constituents throughout the plant kingdom, particularly in plants used as animal feeding stuff and in human nutrition (Igile, 1995). Many plant components and seeds of legumes and other plant sources contain in their raw state wide varieties of anti-nutrients which are potentially toxic (D'Mello, 2000). The knowledge that the compounds elicit both toxic and advantageous biological responses has given rise to several investigations in recent times as to their possible physiological implications in various biological systems (Igile, 1995). Some of these chemicals are known as “secondary metabolites” and they have been shown to be highly biologically active and Most of these secondary metabolites elicit very harmful biological responses, while some are widely applied in nutrition and as pharmacologically active agents (Soetan, 2008).Anti-nutrients can be broadly divided into four groups (Agbo 2008):

I. Factors affecting protein utilization and digestion (e.g., protease (trypsin) inhibitors, tannins and lectins)

II. Factors affecting mineral utilization (e.g., phytates, gossypol pigments, oxalates and glucosinolates)

III. Antivitamins

IV. Miscellaneous (e.g., mycotoxins, mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytoestrogen). Antinutrients may also be classified according to their ability to withstand thermal processing, the most commonly employed treatment for destroying them. Heat labile factors include protease inhibitors, phytates, lectins, goitrogens and antivitamins, whereas heat stable factors are represented by saponins, non-starch polysaccharides, antigenic proteins, estrogens and some phenolic compounds (Van der Peol, 1989; Rumsey *et al.*, 1993). Oligosaccharides become somewhat more digestible after heat treatment, whereas the effect of thermal treatment on substances such as tannins remains uncertain. An overview on the effects of some antinutrients present in oilseeds and pulses on fish has been presented by Tacon (1997).

2.10. Rice bran as a feed ingredient

Rice (*Oryza sativa* L.) is an important cereal crop and a staple food for more than half of the world's population (Wani *et al.*, 2012). Rice is produced in more than 100 countries as a staple food, accounting for about 25% of all cereal grain production (Hernandez *et al.*, 2000). In India and other countries, approximately one million tons of bran are produced annually and used predominantly for animal feed (Pan *et al.*, 2006 & Sumantha *et al.*, 2006). Rice contains 80% carbohydrates, 7–8% proteins, 3% fat and 3% fiber (Saikia & Deka, 2011). Rice bran is a by-product of rice milling, is obtained from outer layer of the brown (husked) rice kernel during milling. Processing of rice produces several streams of materials such as husk, milled rice, and bran. Milling of paddy yields 70% of rice (endosperm) as the major product and byproducts consisting of 20% husk, 8% bran and 2% germ (Van Hoed *et al.*, 2006). Rice bran is an underutilized by-product of rough rice obtained during the production of white rice from brown rice. It is a good source of proteins, minerals, fatty acids and dietary fiber (Mccaskill & Zhang, 1999). Rice bran representing 5–8% of the total grain contains minerals including iron, phosphorus and magnesium, 11% and 13% crude protein, approximately 11.5% of fibers and good amount of oil because it may contain 20% of its weight in oil (Oliveira *et al.*, 2011).

Rice bran contains about 15% protein with a desirable amino acid profile and high digestibility (Wang *et al.*, 1999) and it is a good source of dietary fiber (Abdul-Hamid and Luan, 2000). Its nutritional value is much higher than rice endosperm protein or protein from any cereals or legumes (Tang *et al.*, 2003). Rice bran is also considered as a protein source for hypoallergenic ingredients (Gnanasambandam and HelTiarachchy, 1995). Rice bran is rich in antioxidant compounds like polyphenols, vitamin E, tocotrienols, oryzanol and carotenoids that help prevent the oxidative damage to DNA and other body tissues (Jariwalla, 2001; Godber and Wells, 1994). Full fat rice bran contains 18–22% oil including an array of bio-active phytochemicals such as oryzanols, phytosterols, tocotrienols, squalene, polycosanols, phytic acid, ferulic acid, and inositol hexaphosphate to mention a few (Ardiansyah *et al.*, 2006).

The composition of rice bran varies with the rice type, climatic conditions and rice processing methods (Grist, 1985). Chemical profiles of rice bran also differ concerning rice variety. Rice bran, a valuable byproduct contains a large number of essential nutrients such as minerals, vitamins, fibre, amino acids and antioxidants

(Younas *et al.*, 2011). Additionally, various antinutritional factors present in rice bran limit its use as a food ingredient. The factors include trypsin inhibitors, haemagglutinin, lectin and phytates (Younas *et al.*, 2011). Rice bran is the major by product generated during milling, which is further extracted for oil. The defatted residues of bran contain 15.4% protein (Hamada, 2000). Proteins extracted from heat stabilized defatted rice bran could be used as a nutraceutical food ingredient (Tang *et al.*, 2003).

De-oiled rice bran is a fat free rice bran or rice police and is a good replacer of other aqua feed ingredients such as maize and other cereals (Limbu *et al.*, 2016). Extraction of oil leads to concentration of nutrient such as protein minerals and greater concentration of NDF (neutral detergent fiber), ADF (acid detergent fiber) and lignin and phytate phosphorus (Warren and Farrel, 1990). DORB contains 15–17% protein along with 12–15% crude fiber (Sereewatthanawut *et al.*, 2008). It is reported that, the cost of aqua-feed can be reduced substantially by using DORB at higher levels (Ramakrishna *et al.*, 2013). Each year 90% of the rice bran produced in the world is utilized cheaply as a feed stock for cattle and poultry and the remainder is used for extraction of rice bran oil (Schramm *et al.*, 2009). Rice protein is hypoallergenic and contains good quantity of lysine. Thus it may act as a suitable ingredient for infant food formulations while adding variety to the restricted diets of children with food allergies (Helm and Burks, 1994). Hence, rice bran has a lot of potential uses as food ingredients in a range of food applications.

2.11. Leaf meal as a feed ingredient

Considering the importance of nutritionally balanced and cost-effective artificial diets for fish, there is an increasing research effort to evaluate the nutritive value of different non-conventional feed resources, including terrestrial and aquatic macrophytes (Edwards *et al.*, 1985; Wee and Wang, 1987; Patra and Ray, 1988; Ray and Das, 1992, 1993, 1995; Mondal and Ray, 1999). Plant leaf meals are cheap ingredients available with the potential to replace rice bran. Aquatic and terrestrial macrophytes have been used as supplementary feeds in fish farming since the early times of freshwater fish culture (Bardach *et al.*, 1972) and still play an important role as fish feed in extensive culture systems (Edwards, 1987). The efficacy of various plant sources for partial or complete replacement of fish meal in aquafeeds has been tested (Atack and Matty, 1979; Viola *et al.*, 1982; Tacon, 1993; Ray and Das, 1995;

Mondal and Ray, 1999). Cruz and Laudencia (1978) used mulberry leaf meal for Nile tilapia diet with 60% inclusion in combination with 40% rice bran and reported highest weight gain and food conversion ratio in fish. Various leaf meals have been tested in shrimp and fish diets for replacing fishmeal partially and to decrease diet cost (Penaflorida, 1995; Eusebio, P.S. and Coloso, 1998). CY *et al.* (2007) isolated two bacterial strains, *Bacillus subtilis* CY5 and *Bacillus circulans* TP3, from the gastrointestinal tracts of common carp, *Cyprinus carpio* and *Oreochromis mossambicus* respectively and were identified as potent cellulase producers. They studied the effect of different production parameters, such as fermentation condition, moisture, pH, temperature, inoculum size, and nitrogen sources on cellulase production by the isolated bacterial strains. Mohapatra and Patra (2014) evaluated the nutritional quality and acceptability of *Pistia stratiotes* meal for *Cyprinus carpio* fry under laboratory culture condition. An inclusion of *Pistia* up to 45% level could be used as a replacement for fish meal in the diet of common carp fry.

2.12. Extracted *Mentha arvensis* leaf meal

Mentha arvensis (the corn mint, field mint, or wild mint) is a species of flowering plant in the mint family Lamiaceae. It has a circumboreal distribution, being native to the temperate regions of Europe and western and central Asia, east to the Himalaya and eastern Siberia, and North America. India is the largest producer of corn mint oil and its products include menthol crystals, dementholised oil, mint terpenes, etc (Alvi *et al.*, 2001). The cultivation of corn mint or menthol mint (*M. arvensis* f. *piperascens*) is largely confined to northern and northwest (Aflatuni, 2003). *Mentha arvensis* leaf is a rich source of crude protein and fat, and they constitute 26.9 and 16.8 respectively on a dry weight basis. *Mentha* is an excellent source of amino acids, minerals, antioxidants and dietary fibre and has a high calorific value. (Yeoh and Wong, 1993). The *Mentha arvensis* (menthol mint) is an important essential oil-bearing plant and the l-menthol crystallized from the essential oil, de-mentholated oil and specific terpene fractions thereof are widely used in food, flavour, pharmaceutical and cosmetic industries (Singh *et al.*, 2007). *Mentha* is cultivated in a large area in the Indo-Gangetic Plains in the states of Punjab, Haryana, Uttarakhand, Uttar Pradesh and Bihar, with maximum area in Uttar Pradesh (Khanuja *et al.*, 2005). Singh and Khanuja (2007) reported that the area under mint cultivation in India has increased from 0.8 lakh

hectare in 1995 to 1.6 lakh hectare in the year 2009. The cultivation area under mint is increasing due to the introduction of improved varieties of mint and utilisation of early mint technology (EMT). The estimated area under mint cultivation in the year 2016 is 2.6 lakh hectare (CIMAP, 2017). Thus, the oil extracted residue of mentha is plentifully available, as a prospective feed ingredient.

2.13. Biological and nutraceutical properties of *Mentha arvensis*

The essential oils of *Mentha* plants have been reported to have anti-inflammatory, antimicrobial, antioxidant, anticarcinogenic, insecticide and analgesic properties (Shaikh *et al.*, 2014) and also have widely used in the perfumery, pharmaceutical and food industries (Thawkar *et al.*, 2016). Out of a huge number of essential oil-bearing plants belonging to *Mentha* species, (*Mentha arvensis*) locally known as pudina (Khan and Khatoon, 2008) constitutes the most important source of therapeutic agents and is used in the alternative systems of medicine (Naeem *et al.*, 2011). The mint plant has been reported to contain many active ingredients such as menthol (40-50 %) (Malik *et al.*, 2012), carvone (67.3 %), Limonene (13.5%), 1, 8-cineole (5.4%), Linalool (2.8 %), menthone (2.9 %), and someone (1.2 %) (Younis *et al.*, 2004) which provide positive effects on health and productivity (Ghazaghi *et al.*, 2014). The ancient Egyptians, Greeks and Romans knew peppermint as flavouring agent for food and as medicine while the essential oils of mint were used as perfumes, food flavours, deodorants and pharmaceuticals (Baris *et al.*, 2006). During the middle ages, powdered mint leaves were used to whiten teeth (Hajlaoui *et al.*, 2008). Mint is mentioned in the Icelandic Pharmacopoeias of the thirteenth century (Dai, 1981).

3. MATERIALS AND METHODS

3.1. Site of the experiment

The feeding trial was conducted in the wet laboratory of ICAR-CIFE, Rohtak Centre, Haryana, India. The periodical water quality analysis was carried out at soil and water analysis laboratory at CIFE Centre Rohtak whereas physiological, biochemical and hematological parameters were analyzed at Fish Nutrition, Biochemistry, and Physiology laboratory in CIFE, Mumbai.

3.2. Experimental animal

Experiments were performed on *Cyprinus carpio* fingerlings with an average weight of 5-6. The fishes for both Experiments I & II were procured from a private farm, located at Baniyani village, Rohtak, Haryana and transported in aerated tanks to the wet lab of CIFE Rohtak centre. The fishes were then transferred to aerated FRP tanks of 500-liter capacity and left undisturbed for overnight. The fishes were treated with 0.2% salt water for disinfection and Vitamin C to reduce the stress caused due to transportation and handling on the next morning. Then, the fishes were acclimatized to the saline condition by slowly increasing the salinity of the water. During the acclimatization period, fishes were fed with control feed containing 30% crude protein.

3.3. Chemicals and glasswares

The following equipment's and lab wares were used in the present study:

- Glassware, Borosil, India.
- Flame Photometer
- Osmometer (OSMOMAT 030 Gonotec, Germany)
- Micropipettes from Eppendorf (Germany), Gene Mate (USA) and Tarsons, India
- Plasticware, Tarson Pvt. Ltd., India.
- Digital pH meter (control dynamics)
- Electronic balance (Shimadzu)
- Hot air oven (Newtronic, India)

3.4. Cleaning and siphoning

The tanks were cleaned manually and siphoning was done on every three days to remove the excess feed and faecal matter. An equal volume of water of the same temperature was filled after the siphoning to maintain the water level in the tank. The water of required salinity was prepared and kept in aerated condition to fill the experimental tanks when required. This was carried out throughout the experimental period for all the experimental groups. The body weight was measured at intervals of 15 days to assess the growth and calculate the feeding ration for the next 15 days. The fishes were starved overnight before taking the body weight.

3.5. Physicochemical parameters of water

Water quality parameters viz., temperature, pH, dissolved oxygen, total hardness, ammonia, free carbon dioxide, nitrite, and nitrate were recorded during the experimental period of all the experiments.

3.5.1. Temperature

The water temperature of all the experimental tubs was recorded using dissolved oxygen meter (MERCK, Germany). That also measures the temperature.

3.5.2. pH

The pH was measured by using digital pH meter for all the experimental tubs.

3.5.3. Dissolved oxygen

The dissolved oxygen was measured by membrane electrode method using dissolved oxygen meter (MERCK, Germany) for all the experimental tubs.

3.5.4. Free carbon dioxide

The dissolved free Carbon dioxide was measured by the titrimetric method (APHA, 1998) and calculated using the following formula

$$\text{CO}_2 \text{ (mg/L)} = A \times N \times 44 \times 1000$$

Where, A = Volume of titrant (NaOH)

N = Normality of titrant (N/44)

3.5.5. Carbonates hardness

Carbonate hardness was estimated by carbonate hardness test kit (Carbonate hardness test, MERCK, Germany).

3.5.6. Ammonia

Un-ionized ammonia concentration was estimated spectrophotometrically at 635 nm wavelength by phenate method (APHA, 1998) and compared with the standard graph. The concentration was expressed as mgL⁻¹.

3.5.7. Nitrite-N

Nitrite concentration was estimated spectrophotometrically at 543 nm wavelength (APHA, 1998) and compared with the standard graph. The concentration was expressed as mg/l.

3.5.8. Nitrate-N

Nitrate concentration was estimated spectrophotometrically at 543 nm wave length (APHA, 1998) and compared with the standard graph. The concentration was expressed as mg/l.

3.6. Experimental diets

Four iso-nitrogenous and iso-caloric practical diets were prepared with graded levels of extracted *Mentha arvensis* leaf meal (EMLM) replacing the DORB in the diet. A control (C) with 0% EMLM and 30% DORB inclusion in the feed. Three treatment feeds (T1, T2 & T3) were prepared with 10%, 20% and 30% EMLM inclusion respectively replacing 33%, 66% and 100% of DORB in the diet. The composition of the experimental diets for the Experiment I & II is given in Table 1.

3.7. Feeding

Feeding was done at the rate of 2% of body weight initially, and the feeding rate was adjusted according to the feed intake. The daily ration was divided into two equal parts and was fed at 9:00 am in the morning and 5:00 p.m.

Table 1: Composition of experimental diets for Experiment I & II

<i>Ingredients</i>	<i>C/FW/ISW</i>	<i>T1</i>	<i>T2</i>	<i>T3</i>
DSBM	30	30	30	30
GNOC	20	20	20	20
Wheat Flour	12.18	12.18	12.18	12.18
DORB	30	20	10	0
EMLM	0	10	20	30
Oil	5	5	5	5
Vit-min mix	1.5	1.5	1.5	1.5
Choline Chloride	0.3	0.3	0.3	0.3
CMC	1	1	1	1
BHT	0.02	0.02	0.02	0.02
Total	100	100	100	100

Abbreviation:- T-Treatment; C-Control; GNOC-groundnut oil cake; EMLM- Extracted *Mentha arvensis* leaf meal; DORB-deoiled rice bran; DSBM defatted soybean meal; CMC-carboxymethyl cellulose; BHT-Butylated Hydroxytoluene; Vit-min mix-vitamin mix and mineral mixture

Composition of vitamin mineral mix (PREEMIX PLUS) (quantity/kg): Vitamin A, 55,00,000 IU; Vitamin D3, 11,00,000 IU; Vitamin B2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6 mcg; Calcium Pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 L- lysine, 10 g; DL- Methionine, 10 g; Selenium

3.8. Experimental design and setup

3.8.1. Experiment 1

The experiment aimed to compare growth and evaluate stress response of *Cyprinus carpio* fingerlings reared in two different waters viz. freshwater (FW) and inland saline water (ISW) fed with control diet. Ninety fingerlings (avg wt 5 ± 0.5 g) were randomly distributed into two experimental groups in triplicates following a completely randomized design. The two treatments were FW (fishes reared in fresh water) and ISW (reared in inland saline water of salinity 5ppt). The fishes were fed with control diet.

Table 2: Design of experiment 1

Experimental Group	Condition
FW	Fishes reared in fresh water and fed with normal diet
ISW	Fishes reared in inland saline water (ISW) of 5ppt salinity and fed with normal diets

3.8.2. Experiment 2

The experiment aimed to evaluate growth and stress response of *Cyprinus carpio* fingerlings fed with graded levels of EMLM in the diet and reared in inland saline water (ISW) of 5ppt salinity. The four experimental groups were C, T1, T2 and T3. In the control (C) group, no EMLM was included in the feed and contained 30% DORB in the formulation. In the feeds of T1, T2 and T3 groups, the EMLM was included at 10%, 20%, 30% level which replaced 33%, 66% and 100% of DORB respectively in the diet. The four experimental groups were taken in triplicates. Fish of average weight 5 ± 0.5 g were used for the experiment.

Table 3: Design of experiment 2

Experimental Group	Condition
C (control)	Basal diet without leafmeal, i.e., EMLM (30% DORB in the Diet)
T1	10% EMLM, replacing 33% of DORB in the Diet
T2	20% EMLM, replacing 66% of DORB in the Diet
T3	30% EMLM, replacing 100% of DORB in the Diet

3.9. Preparation of extracted *Mentha arvensis* leaf meal (EMLM)

Extracted *Mentha arvensis* leaf was obtained from CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Regional centre, Pantnagar. The leaves were separated from the twigs and sun-dried. The leaves were powdered in a grinder to fine particle size and were sieved using a mesh. The powdered leaf meal was used in the diet preparation.



3.10. Proximate analysis of leaf meal, diet and fish carcass

The fishes were collected from the experimental tubs, and the biomass of the respective tanks was noted down. The samples after weighing are then used for proximate analysis. Proximate analysis of the diets was made by standard methods (AOAC, 1995) at the Fish Nutrition Laboratory, CIFE, Mumbai. Similarly, the proximate composition of leaf meal and experimental diets were done.

3.10.1. Moisture

The sample left after complete evaporation of moisture is called dry matter. All nutrients of feed are expressed on dry matter basis. Dry matter is, therefore a common denominator for the comparison of feeds as well as fish regarding nutritive value. The moisture content of the experimental diets was determined by taking a known weight of the sample in the petri dish and drying it in a hot air oven at 100 - 105°C till a constant weight was achieved. The difference in weight of the sample gave the moisture content, which was calculated by using the following formula

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

3.10.2. Crude protein (CP)

The nitrogen content of the dried samples was estimated quantitatively by Kjeltex semi-automated method (2200 Kjeltex Auto Distillation, Foss Tecator, Sweden) using titration as the means for determining the nitrogen percentage. The sample is digested to release the nitrogen content from the component proteins by strong H₂SO₄ in the presence of digestion mixture (CuSO₄ & K₂SO₄). The released nitrogen gets collected in the Boric acid (4%). This collected nitrogen gives green color in the presence of Toshio's indicator. This N₂ is titrated with N/50 H₂SO₄ and quantity of H₂SO₄ consumed is measured which gives the nitrogen content. The crude protein percentage was obtained by multiplying the nitrogen percentage by a factor of 6.25.

$$\text{Crude protein}(\%) = \text{N}_2(\%) \times 6.25$$

3.10.3. Ether extract (EE)

Ether extract of the moisture-free samples was estimated by Soxhlet apparatus using diethyl ether (Boiling point $65 \pm 5^\circ \text{C}$) as the solvent. Crude fat can be extracted from the dried samples by using organic solvents like diethyl ether, petroleum ether, etc. the solvent is evaporated, and fat is estimated gravimetrically. The calculation was made as follows.

$$EE(\%) = \frac{\text{Initial weight of sample} - \text{final Weight of sample}}{\text{Initial weight of sample}} \times 100$$

3.10.4. Ash

The ash content of the experimental diets was estimated by taking a known weight of dried samples in a silica crucible and placing it in a muffle furnace at 600°C for 5-6 hours. The calculation was done as follows:

$$Ash(\%) = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

3.10.5. Fibre

Fibre estimation was done in fibre tech (Tulin equipment, India) apparatus and further ashing was done using a muffle furnace at 550°C for 5 hours.

3.10.6. Total carbohydrate and nitrogen free extract

The total carbohydrate (TC) of the fish carcass was calculated by subtracting the percentage of other nutrients from 100.

$$TC(\%) = 100 - [\text{Crude protein}(\%) + \text{Ether extract}(\%) + \text{Ash}(\%)]$$

In case of feed and leaf meal, the NFE was calculated as follows

$$NFE(\%) = 100 - [\text{Crude protein}(\%) + \text{Ether extract}(\%) + \text{Ash}(\%) + \text{Crude Fibre}(\%)]$$

3.10.7. Digestible energy value (DE)

The digestible energy value of leaf meal and experimental diets was calculated by standard physiological values (Halver, 1976) as per the following formula.

$$\text{DE (kcal/100g)} = \{\text{Protein (\%)} \times 4 + \text{Lipid (\%)} \times 9 + \text{Carbohydrate (\%)} \times 4\}$$

3.11. Determination of anti-nutritional factors (ANFs) in EMLM

3.11.1. Phytic acid

For phytic acid estimation, 0.5 g of dried sample was weighed and transferred to a 125-mL Erlenmeyer flask and extracted with 10 mL of 3.5% HCl. The samples were constantly shaken in an orbital shaker (ORBITEK®; Scigenics, India) at 200rpm for 1 hrs, followed by centrifugation at 5000 rpm for 10 min, and the supernatant was collected for analysis (Makkar *et al.*, 2007). The phytic acid in the extract was determined according to the colorimetric assay described by Gao *et al.* (2007) with slight modification. Exactly 0.1mL of sample extract was diluted to 3 mL with distilled water and then thoroughly mixed with 1mL of wade Reagent (0.03% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ + 0.3% sulphosalicylic acid). The mixture was centrifuged at 5000 rpm for 10 min after keeping for 30 min, and the supernatant was taken for absorbance at 500nm against distilled water. The phytic acid content was calculated from the calibration curve of phytic acid standard and expressed as milligrams of phytic acid (PA) per gram of sample (mg^{-1}). All the extractions were carried out in triplicates.

3.11.2. Total flavonoids

Total flavonoid content of the leaf meal was determined following a method by Park *et al.*, 2008. Aqueous extract of the sample was done taking 0.5g of the sample in 10ml of distilled water in a beaker and kept for stirring for overnight. For estimation 0.3 ml of aqueous extract was transferred to a test tube then 3.4ml 30% methanol and 0.15ml of NaNO_2 (1M) was added. The solution was mixed well using a vortex. Absorbance was measured against the reagent blank (without sample) at 506nm. Total flavonoid content in the sample was expressed as milligrams of Rutin equivalents per gram of dried fraction.

3.11.3. Alkaloid content

The alkaloid content was determined by gravimetric method (Haborne, 1973). 5g of each sample was weighed and dispersed into 50 ml of 10% acetic acid solution in ethanol. The mixture was well shaken and then allowed to stand for about four hours before it was filtered. The filtrate was then evaporated to one-quarter of its original volume on a hot plate. Concentrated ammonium hydroxide was added dropwise to precipitate the alkaloids. The pre-weighed filter paper was used to filter off the precipitate, and it was then washed with 1% ammonium hydroxide solution. The filter paper containing the precipitate was dried on an oven at 60°C for 30 min, transferred into desiccators to cool and then reweighed until a constant weight was obtained. The constant weight was recorded. The weight of the alkaloid was determined by weight difference of the filter paper and expressed as a percentage of the sample weight analyzed. The sample was taken in triplicate.

3.11.4. Oxalate content

The procedure for oxalate determination followed was described by Nwosu (2011). In this process one gram sample was weighted in 100 ml beaker, 20ml of 0.03N HCl was added, warmed to 40-50°C using a magnetic hot plate and stirred for one hour. The extract was diluted to 100 in a volumetric flask. The oxalate was estimated by pipetting 5 ml of extract into a conical flask and made alkaline with 1 ml of 5N ammonium hydroxide. A little indicator paper was placed in a conical flask to enable to know the alkaline regions. It was also made acidic by adding three drop phenolphthalein followed by dropwise addition of glacial acetic acid. 1ml of 5% CaCl₂ was then added and allowed to stand for three hours and centrifuged to 3000rpm for 15 minutes. The supernatant was discarded, and 2 ml of 3N H₂SO₄ was added to each tube and precipitate was dissolved by warming in a water bath (70-80°C). The content of all the tube was carefully poured into a clean conical flask and titrated with freshly prepared 0.01N KMNO₄ at room temperature until the first pink color appeared throughout the solution. It was allowed to stand until the solution became colorless. The solution was then warmed to 70-80°C and titrated until a permanent pink color persisted for at least 30 sec was attend.

$$\% \text{ Oxalate} = \frac{100}{\text{weight of sample}} \times 0.00225 \times \text{total titre volume}$$

3.11.5. Total tannin

Total tannin was estimated using the spectrophotometric method as described by Makkar *et al.* (2007). The reagent used was Folin-Ciocalteu reagent, and the results were expressed as tannic acid equivalents. The total tannin content was calculated from the calibration curve of tannic acid standard and expressed as milligrams/100g of tannin. All the extractions were carried out in triplicates

3.12. *In Vitro* Digestibility

3.12.1. *In vitro* Relative protein digestibility of EMLM

In vitro protein digestibility study was carried out using the method of Ali *et al.* (2009). The fresh tissue of alimentary canal of the fish was homogenized under the cold condition and diluted with distilled water (1:10 w/v). The homogenized tissue was centrifuged at 12000 rpm for 15 min at 4 °C for enzyme extraction. An equivalent amount of finely powdered EMLM that provided 160mg of crude protein was weighed and mixed with 20 ml of distilled water and 2mL of the enzyme to obtain 8mg crude protein per milliliter, and the pH was adjusted to 8 (Eutop pH tutor, Thermo Fisher Scientific, Singapore). The pH drop was recorded at every minute interval for 10 min, and casein was used as the reference protein. Relative Protein Digestibility was calculated using the formula:

Relative Protein Digestibility (RPD %) = $(-\Delta\text{pH of ingredients} / -\Delta\text{pH of casein}) \times 100$.

3.13. Growth parameters

Sampling was done at intervals of 15 days to assess the body weight of the fishes. Fishes were starved overnight before taking the weight. The weight was recorded using an electric balance.

3.13.1. Percentage weight gain

The percentage weight gain was calculated using the following formula

$$\text{Weight gain(\%)} = \frac{\text{Final weight} - \text{Initial Weight}}{\text{Initial weight}} \times 100$$

3.13.2. Specific growth rate (SGR)

The Specific Growth rate was calculated by the following formula

$$SGR(\%) = \frac{\log_e Finalweight - \log_e Initialweight}{Numberofdays} \times 100$$

3.13.3. Feed conversion ratio (FCR)

The Feed conversion ratio was calculated by the following formula:

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

3.13.4. Protein efficiency ratio (PER)

Protein efficiency ratio was calculated by the following formula:

$$PER = \frac{\text{Net weight gain (g)}}{\text{Protein Fed (g)}}$$

3.14. Body indices

3.14.1. Hepatosomatic index (HSI)

The liver weights of fishes of different treatment groups were recorded, and the hepatosomatic index was calculated as follows:

$$HSI = \frac{LiverWeight(g)}{Weightoffish(g)} \times 100$$

3.14.2. Gastro somatic index (GaSI)

The weights of the gastrointestinal tract of fishes from different treatment groups were recorded, and the gastro somatic index was calculated as follows:

$$GaSI = \frac{Weight\ of\ intestinal\ tract}{Weight\ of\ fish} \times 100$$

3.15. Quantification of DNA, RNA, and ratio between RNA and DNA

Quantitative determination of nucleic acids in tissue was performed by pentose analysis following the method of Schneider (1957) and was calculated as follows: The DNA content of the nucleic acid extract is presented by the following equation:

$$\mu\text{g DNA/ mL}^{-1} = \frac{1}{4} \text{ OD at 600nm} \times 0.019$$

The RNA content of the nucleic acid extract is presented by the following equation:

$$\mu\text{g RNA/ml}^{-1} = [(\text{OD at 600nm} + 0.0081) - (\mu\text{gDNA/ml}^{-1} \times 0.013)] / 0.116$$

3.16. Enzyme assays

3.16.1. Sample preparation for enzyme assays

At the end of the experiment, ten fishes were collected from each tank and anesthetized with clove oil (50 μ L⁻¹). Fishes were then dissected and the tissues viz., liver, intestine, and muscle, were immediately removed. A 5% tissue homogenate was prepared in chilled 0.25 M sucrose solution using Teflon coated mechanical homogenizer (REMI Equipment's, Mumbai, India). The whole procedure was followed in cold condition. Homogenised samples were centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was collected in glass vials and stored in the deep freezer (-20°C) for enzyme assay. Suitable dilution of the samples was prepared as and when required.

3.16.2. Digestive enzymes activity

3.16.2.1. Amylase activity

Amylase activity was estimated as the reducing sugars produced due to the action of glucoamylase and α -amylase on carbohydrate using di-nitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Specific amylase activity was expressed as a mole of maltose released from starch per min at 37 °C temperature.

3.16.2.2. Protease activity

Protease activity was determined by the casein digestion method (Drapeau, 1974). One unit of enzyme activity was defined as the amount of enzyme needed to release acid-soluble fragments equivalent to $\Delta 0.001$ at 280 nm per minute at 37 °C and pH 7.8.

3.16.2.3. Lipase activity

The lipase activity was determined by the titrimetric method of Cherry and Crandell (1932) which is based on the measurement of fatty acids released by the enzymatic hydrolysis of triglycerides present in a stabilized emulsion of olive oil. The amount of a standard sodium hydroxide solution used to titrate the fatty acids released, taken as an index of lipase activity of the crude enzyme extract. The assay system consists of 1.5 ml of stabilized lipase substrate and 1.5 ml of 0.1 M Tris-HCl buffer at PH 8.0, to which 1.0ml of the crude enzyme extract was added. The assay mixture was incubated for 24 hr. At 4° C, after which the reaction is stopped by addition of 3 ml of 95% ethyl alcohol. The mixture is then titrated with 0.01 N NaOH using 0.9% (w/v) phenolphthalein as indicator.

3.17. Activities of metabolic enzymes

3.17.1. Aspartate aminotransferase (AST)

AST activity from different tissues of *Cyprinus carpio* was assayed in different tissue homogenates as described by Wooton (1964). The substrate comprised of 0.2M DL-aspartic acid and 2mM α -ketoglutarate in 0.05M phosphate buffer (pH 7.4). In the treatment and control tubes, 0.5ml of the substrate was added. The reaction was started by adding 0.1ml of tissue homogenate in the treatment tube. The assay mixture was incubated at 37°C for 60 minutes. The reaction was terminated by adding 0.5ml of 1mM 2, four dinitrophenyl hydrazine (DNPH). In the control tubes, the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 minutes with occasional shaking. Then 5ml of 0.4M NaOH solution was added, and the contents were thoroughly mixed. After 10 minutes, the OD was recorded at 540nm against blank. The AST activity was expressed as Nanomoles oxaloacetate formed/mg protein/minute at 37°C.

3.17.2. Alanine aminotransferase (ALT)

The procedure adopted for the estimation of ALT activity was same as that for AST activity estimation except that the substrate comprised of 0.2 M D, L-alanine instead of aspartic acid. The ALT activity was expressed as nanomoles pyruvate formed / mg protein/minute at 37°C.

3.17.3. Lactate dehydrogenase (LDH)

The lactate dehydrogenase (LDH) activity was assayed by the method of Wrobleiuski and Laude (1955). The total 3ml of the reaction mixture comprised of 2.7ml of 0.1M phosphate buffer (pH 7.5), 0.1ml of NADH solution (2mg NADH dissolved in 1ml of phosphate buffer solution), 0.1ml of tissue homogenate and 0.1ml of sodium pyruvate. The reaction was started after addition of substrate sodium pyruvate. The OD was recorded at 340nm at 30s interval. The specific enzymatic activity was expressed as unit mg protein-1 min-1 at 25 °C where 1 unit was equal to $\Delta 0.01$ OD min-1.

3.17.4. Malate dehydrogenase (MDH)

The Malate dehydrogenase (MDH) activity was assayed in different tissues by the method of Ochoa (1955). The total 3ml of the reaction mixture comprised of 2.7ml of 0.1M phosphate buffer (pH 7.5), 0.1ml of NADH solution (2mg NADH dissolved in 1ml of phosphate buffer solution), 0.1ml of tissue homogenate and 0.1ml of freshly prepared oxaloacetate (2 mg oxaloacetate dissolved in 2ml of chilled distilled water). The reaction was started after addition of oxaloacetate solution as substrate. The OD was recorded at 340nm at 30s interval. The specific enzymatic activity was expressed as unit mg protein-1 min-1 at 25 °C where 1 unit was equal to $\Delta 0.01$ OD min-1.

3.17.5. Glucose-6-phosphatase

The G-6-Pase activity in the tissues (liver) was assayed by the method of Marjorie (1964). The assay mixture consisted of 0.3 ml of malate buffer (pH6.5), 0.1 ml of 0.1M glucose-6-phosphate solution and 0.1 ml of tissue homogenate and was incubated for 15min at 37°C. The reaction was terminated by addition of 1ml of 10% TCA solution. One millilitre of the aliquot of the supernatant was used for phosphate

(Pi) estimation by method of Fiske and Subbarow (1925). One unit of enzyme activity was defined as the amount that released 1 μ g of phosphorous per minute.

3.18. ATPase

Total adenosine triphosphates (ATPase) (Adenosine triphosphate phosphohydrolase, E.C.3.6.1.3) was assayed in a reaction mixture of 0.1 M Tris-HCl buffer (pH 7.8), 100mM NaCl, 20mM KCl, 3mM MgCl₂ and 5mM ATP. The mixture was incubated for 15 min, and then the reaction was terminated using 10% trichloroacetic acid (Post and Sen, 1967). Phosphate liberated was estimated at 660nm (Fiske and Subbarow, 1925).

3.19. Oxidative stress enzymes

3.19.1. Superoxide dismutase (SOD)

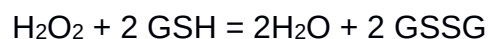
Superoxide dismutase was assayed according to the method described by Mishra and Fridovich, (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme. Fifty microlitres of the sample was taken in the cuvette, and the 1.5 ml 0.1M carbonate-bicarbonate buffer is containing 57mg/dl EDTA (pH 10.2), and 0.5 ml epinephrine (3mM) was added and mixed well. Change in optical density at 480nm was read immediately for 3 min in a spectrophotometer (Shimadzu–UV). One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto-oxidation. SOD expressed as unit activity (amount of protein required to give 50% inhibition of epinephrine auto-oxidation).

3.19.2. Catalase (CAT)

Catalase was assayed according to the method described by Takahara *et al.* (1960). To a reaction mixture of 2.45ml phosphate buffer (50mM, pH 7.0), enzyme source was added, and the reaction was started by the addition of 1.0ml of H₂O₂ solution. The decrease in absorbance was measured at 240nm at 15 sec intervals for 3 min. The enzyme blank was run simultaneously with 1.0ml distilled water instead of the H₂O₂ solution. Enzyme activity was expressed as nanomoles H₂O₂ decomposed /min /mg protein.

3.19.3. Glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was determined according to the method of Hafemann *et al.* (1974). GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H₂O₂ and NaN₃.



Tissue homogenate (approximately 0.5 mg protein) was incubated with 0.1 ml of 5mM GSH, 0.1 ml of 1.25 mM H₂O₂, 0.1ml of 25 mM NaN₃ and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37°C for 10 min. The reaction was stopped by adding 2 ml of 1.65 % HPO₃, and the reaction mixture was centrifuged at 1500 rpm for 10 min. 2 ml of the supernatant was mixed with 2 ml 0.4 M Na₂HPO₄ and 1ml of 1mM DTNB. The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 min at 37°C against distilled water. A sample without the tissue homogenate processed in the same way was kept as a non-enzymatic reaction.

3.19.4. Glutathione-s-transferase (GST) assay

GST activity was determined by the method of Habig *et al.* (1974), following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2, 4- dinitrobenzene (CDNB) using as substrate at the presence of reduced glutathione (GSH). The reaction mixture was prepared by mixing 1.5 ml sodium phosphate buffer 0.1 M pH 6.5, 0.2 ml GSH 9.2 mM, 0.02 ml CDNB 0.1 M and 0.1 ml of the sample. The absorbance was measured at 340 nm and the temperature +25°C spectrophotometrically using Specol-211 (Germany). The increase in absorbance was recorded for three mins. The reaction solution without the fish tissue homogenates and erythrocytes lysates were used as a blank.

3.20. Non enzymatic antioxidant parameter

3.20.1. Estimation of reduced glutathione (GSH)

The amount of reduced glutathione in the samples was estimated by the method of Boyne and Ellman, 1972. 1ml of the sample extract was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67g of glacial metaphosphoric acid,

0.2g EDTA, and 30g NaCl dissolved in 100ml water). After centrifugation, 2.0ml of the protein-free supernatant was mixed with 0.2ml of 0.4 M Na₂HPO₄ and 1.0ml of DTNB reagent (40mg DTNB in 100ml of aqueous 1% trisodium citrate). Absorbance was read at 412nm within 2min. GSH concentration was expressed as nmol/mg protein.

3.21. Protein estimation

Quantification of the protein of the different tissues was carried out using Lowry's method (Lowry, 1951). Tissue homogenate (0.1ml) was taken and precipitated using 1ml of 10% TCA. The protein residue was collected by discarding the supernatant produced by centrifugation at 500rpm for 20min. The residue was dissolved in 0.5 ml of 0.1N NaOH. An amount of 0.1ml of the dissolved protein residue was used for further analysis. Alkaline copper sulfate (5ml) was added and left for 10min. To this 1N Folin's reagent was added and incubated for 30min in the dark. Reading was taken at 660nm against the blank. Bovine serum albumin was used as a standard. Protein content was expressed in mg g⁻¹ wet tissue.

3.22. Immuno-haematological parameters

3.22.1. Collection of blood

Each fish was anesthetized with clove oil (50 µl of clove oil per liter of water) before taking blood from fish. Blood was taken from a caudal vein using a medical syringe (N-24) of 1 ml which was previously rinsed with 2.7% EDTA solution. Blood collected was then transferred immediately to test tube containing a thin layer of EDTA (as an anticoagulant) and shake well slowly to prevent hemolysis of blood.

3.22.2. Collection of serum

Each fish was anesthetized with clove oil (50µl of clove oil per liter of water) before taking blood from fish. Blood was taken from a caudal vein using a medical syringe without using anticoagulant (EDTA) solution. Blood collected was then transferred immediately to a dried Eppendorf tube. The tubes were allowed to stand in tilted position at room temperature for 1hrs which allows the blood to clot. After clotting the blood, centrifuge at 6000 rpm for 10 min, the yellow straw color serum was carefully collected and transferred to another Eppendorf tube and immediately stored at -200°C for further use.

3.22.3. Serum glucose

Serum glucose estimation was carried out by the method of Trinders (1972) using Erba-Manheim Glucose Kit (catalogue no. BLT00027). Glucose in the sample is oxidized to yield gluconic acid and hydrogen peroxide in the presence of glucose oxidase. The enzyme peroxidase catalyzes the oxidative coupling of 4-aminoantipyrine with phenol to yield a colored quinonimine complex, with absorbance proportional to the concentration of glucose in the sample. Three test tubes labelled as Blank (B), Standard (S) and Test (T) were taken. In all the tubes add 1 ml of Reagent 1. Add 10 µl of sample in the tube marked as test (T), then add 10 µl of standard in the tube marked as standard (S). Add 10 µl of distilled water in reagent blank (B). Mix well and incubate for 5-10 min and take the OD at 490-550nm wavelength. Then calculate the glucose concentration using the formula:

$$\text{Glucose } \left(\frac{\text{mg}}{\text{dl}} \right) = \frac{\text{absorbance of Test}}{\text{absorbance of standard}} \times \text{conc. of standard}$$

3.22.4. Serum proteins

3.22.4.1. Serum total protein

Serum protein was estimated by biuret method (Reinhold, 1953) using the kit (ERBA). Proteins present in the plasma binds with copper ions in an alkaline medium of the biuret reagent and produce a purple colored complex, whose absorbance is proportional to the protein concentration. Three test tubes labelled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1ml of biuret reagent and 2ml of distilled water were added. 0.05 ml of protein standard was taken in the test tube labelled as standard and 0.05ml of plasma was added into the test tube labelled as a test. It was then mixed well and incubated at 37° C for 10 min. The absorbance of Standard (S) and Test (T) was measured against the blank (B) in a spectrophotometer at 630 nm. The calculation was done as follows:

$$\text{Serum Protein } \left(\frac{\text{mg}}{\text{dl}} \right) = \frac{\text{absorbance of Test}}{\text{absorbance of Standard}} \times \text{conc. of standard}$$

3.22.4.2. Albumin

Albumin was estimated by bomocresol green binding method (Doumas *et al.*, 1972). Albumin in a buffered medium binds with bromocresol green (BCG) and produces a green color whose absorbance is proportional to the albumin concentration. Three test tubes labeled as blank (B), standard (S) and test (T) were taken. Into all the tubes, 1ml of buffered dye reagent and 2ml of distilled water were added. 0.01ml of albumin standard was taken in the test tube labeled as standard and 0.01ml of plasma was added into the test tube labeled as a test. It was then mixed well and incubated at 37°C for 10 min. The absorbance of Standard (S) and test (T) were measured immediately against a blank (B) in a spectrophotometer at 630nm. The calculation was done as follows

$$\text{Serum Albumin } \left(\frac{\text{mg}}{\text{dl}} \right) = \frac{\text{absorbance of Test}}{\text{absorbance of Standard}} \times \text{conc. of standard}$$

3.22.4.3. Globulin

Globulin was calculated by subtracting albumin values from total plasma protein.

$$\text{Globulins in (g/dl)} = \text{Total protein in (g/dl)} - \text{Albumin in (g/dl)}$$

3.22.4.4. Albumin- globulin ratio

A/G ratio was calculated by dividing albumin values by globulin values

$$\frac{A}{G} \text{ ratio} = \frac{\text{Albumin in (g\%)}}{\text{Globulin in (g\%)}}$$

3.22.5. Serum lipid profile

3.22.5.1. Cholesterol

The cholesterol level in plasma was determined by using the Erba-Manheim Cholesterol kit. Test tubes labelled as Blank (B), Standard (S) and Test (T) were taken, and in all tubes, 500 µl of reagent R was added. Five µl of cholesterol standard was taken in the test tube labelled as standard, and five µl of plasma was added in the test tube labelled as a test and five µl of distilled water in the test tube

marked as blank. It was mixed well and incubated at 37°C for 10 minutes. The absorbance of standard (S) and test (T) was measured against a blank (B) in a spectrophotometer at 505 nm. The calculation was done as follows:

$$\text{Total cholesterol } \left(\frac{\text{mg}}{\text{dl}} \right) = \frac{\text{Absorbance of Test (T)}}{\text{Absorbance of Standard (S)}} \times \text{conc. of standard}$$

3.22.5.2. Triglycerides

The triglyceride level in plasma was determined by using the Erba-Manheim triglycerides kit. Test tubes labelled as Blank (B), Standard (S) and Test (T) were taken, and in all tubes, 500 µl of reagent R was added. Five µl of triglyceride standard was taken in the test tube labelled as standard, and five µl of plasma was added in the test tube labelled as a test and five µl of distilled water in the test tube marked as blank. It was mixed well and incubated at 37°C for 10 minutes. The absorbance of standard (S) and test (T) was measured against a blank (B) in a spectrophotometer at 505nm. The calculation was done as follows:

$$\text{Triglycerides } \left(\frac{\text{mg}}{\text{dl}} \right) = \frac{\text{Absorbance of Test (T)}}{\text{Absorbance of Standard (S)}} \times \text{conc. of standard}$$

3.22.6. Serum creatinine

The creatinine level in serum was determined by using the Erba-Manheim Creatinine Kit. Test tubes labelled as Standard (S) and Test (T) were taken and in all tubes, 500 µl of reagent R was added. 50 µl of creatinine standard was taken in the test tube labelled as standard (S), mixed well and read initial absorbance (A1) 20 seconds after mixing and final absorbance (A2) 80 seconds after mixing. The reading was taken at 505nm. Similarly, the test (T) readings were taken with 500 µl of reagent R, and 50 µl plasma sample was added. The calculation was done as follows:

$$\Delta A = A_2 - A_1$$

$$\text{Creatinine } \left(\frac{\text{mg}}{\text{dl}} \right) = \frac{\Delta A \text{ of Test}}{\Delta A \text{ of Standard}} \times \text{Conc. of Standard}$$

3.22.7. Cortisol

Cortisol level was quantified in the serum samples of all the treatment groups through ELISA. The quantification was done using commercially available Cortisol Assay kit (Catalogue no. MBS480481) procured from MyBioSource, USA. The Assay was performed according to the protocol provided with the kit.

3.22.8. Hemoglobin content

The hemoglobin level of blood was analyzed following the Cyanmethemoglobin method using Drabkins Fluid (Qualigens). Blood (20 µl) was mixed with 5 ml of Drabkin's working solution. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens Diagnostics). The hemoglobin concentration was then calculated by using the following formula:

$$\text{Hemoglobin content (g\%)} = \frac{OD(T)}{OD(S)} \times \frac{251}{1000} \times 60$$

Where, OD (T) = Absorbance of the test

OD (S) = Absorbance of standard

3.22.9. Total erythrocyte count (TEC)

Blood (20 µl) was mixed with 3980 µl of RBC diluting fluid in a clean test tube. The mixture was shaken well to suspend the cells uniformly on the solution. A small drop of this mixture was charged to Neubauer's counting chamber of a hemocytometer. Care was taken that there were no air bubbles trapped. The red blood cells were counted in five groups of squares. All the cells lying inside the five small squares are counted under high power (40X) of the light microscope. The following formula is used to calculate the number of RBC per mm³ of the blood sample:

$$\text{Number of RBC/mm}^3 = \frac{NX\text{dilution}}{\text{Area counted} \times \text{depth of fluid}}$$

$$\frac{NX200}{0.2X0.1}$$

$$= N \times 10000$$

Where N is the total number of red blood cells counted in 5 squares of the hemocytometer.

3.22.10. Total leucocyte count (TLC)

Twenty microlitres of blood were mixed with 3980µl of WBC diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly on the solution. Care was taken that there were no air bubbles trapped. The numbers of cells were counted in four big squares under high power (40x) magnification of a light microscope. The number of WBC per mm³ of the blood sample is calculated using the following formula:

$$\text{Number of WBC/mm}^3 = \frac{NX\text{dilution}}{\text{Area counted} \times \text{depth of fluid}}$$

$$\frac{NX200}{4X0.1}$$

$$= N \times 500$$

Where N denotes the total number of white blood cells counted in 4 squares of the hemocytometer.

3.21. Statistical analysis

Statistical significance of different enzyme activities was analyzed using one-way analysis of variance (ANOVA) using SPSS 22.0 for Windows. Duncan's multiple range test was used for post hoc comparison of mean (P<0.05) between different acclimation temperatures. All data presented in the text, figures, and tables are means ± standard error, and statistical significance for all statistical tests was set at (P< 0.05).

4. RESULTS

4.1. Physico-chemical parameters of water

Physico-chemical parameters of water such as dissolved oxygen (mg L^{-1}), temperature ($^{\circ}\text{C}$), pH, free carbon dioxide (mg L^{-1}), total hardness (mg L^{-1}), ammonia (mg L^{-1}), nitrite - N (mg L^{-1}) and nitrate - N (mg L^{-1}) were estimated and average values of all the treatments are presented in Table 4.

4.1.1. Dissolved oxygen

The dissolved oxygen concentration of all the experimental tanks was recorded within the range of 6.1 to 8 mgL^{-1} during the experimental period.

4.1.2. Temperature

The water temperature of the different experimental treatment ranged from 25°C to 28.5°C during the experimental period of 60 days.

4.1.3. pH

The pH values were recorded within the range of 7.4 to 8.7.

4.1.4. Carbonate hardness

The carbonate hardness was found to be 1310 - 1380 mgL^{-1} during the experimental period.

4.1.5. Free carbon dioxide

The free carbon dioxide in water was too low to get detected during the experimental period.

4.1.6. Ammonia

The total ammonia content of all the experimental tubs was recorded before water exchange. It was found to be in the range of 0.13 to 0.27 mg L^{-1} .

4.1.7. Nitrate

The nitrate content was found to be in the range of 0.01 to 0.07 mg L⁻¹, throughout the experimental period.

4.1.8. Nitrite

The nitrite - N content was found to be in the range of 0.001 to 0.005 mgL⁻¹.

Table 4: Physico-chemical parameters of water during the experimental period for different experimental groups in Experiment I & II

Parameters	FW ¹	ISW ² & Control	T1	T2	T3
Temperature (°C)	25.6-28.4	25.3-28.5	25.1-28.3	25.7-28.3	25.4-28.1
DO (mgL ⁻¹)	6.7-7.8	6.3-7.7	6.2-7.5	6.1-7.6	6.3-8
pH	7.6-8.4	7.5-8.1	7.4-8.7	7.6-8.2	7.4-8.4
Hardness (mgL ⁻¹)	210-240	1335-1360	1320-1367	1340-1375	1315-1360
Free CO ₂ (mgL ⁻¹)	ND	ND	ND	ND	ND
Ammonia (mgL ⁻¹)	0.21-0.24	0.13-0.20	0.22-0.26	0.16-0.19	0.18-0.27
Nitrite (mgL ⁻¹)	0.001-0.002	0.001-0.002	0.001-0.002	0.003-0.005	0.002-0.003
Nitrate (mgL ⁻¹)	0.01-0.03	0.03-0.04	0.02-0.04	0.04-0.06	0.05-0.07
Salinity (gL ⁻¹)	0.5	5.1	5.4	5.8	5.2

¹ Fresh water, ² Inland saline water, ND: Not Detected

4.2. Proximate composition of the EMLM

The results of proximate composition of corn mint (*Mentha arvensis*) leaves are represented in Table 5. The values for moisture, crude protein, ether extract, nitrogen free extract, crude fibre, and total ash were 6.22, 18.64, 2.35, 36.52, 20.83, 15.42% dry matter respectively. The digestible energy was found to be 241.81Kcal/100 g for EMLM.

Table 5: Proximate composition of extracted *Mentha arvensis* leaf meal (EMLM)

Parameters	EMLM	DORB
Moisture (%)	6.22±1.23	7.93±0.85
Crude Protein (%)	18.64±0.84	14.97±1.10
Ether Extract (%)	2.35±0.35	0.54±0.23
Nitrogen Free Extract (%)	36.52±1.56	50.16±1.82
Crude Fibre (%)	20.83±1.24	14.10±1.16
Total Ash (%)	15.42±0.73	12.30±0.76
Digestible Energy (Kcal/100g)	241.81±2.61	265.38±2.36

Data expressed as mean ± SE, n=3

Calculated Digestible energy (Kcal/100g) = (%CP×4) + (%EE×9) + (%NFE×4) (Halver, 1976)

4.3.1. Anti-nutritional factors (ANFs) of extracted *Mentha arvensis* leaf meal (EMLM)

The results of analysis of antinutritional factors (Table 6) revealed the presence of tannins (1.26%), oxalate (0.03%), phytic acid (4.42%), alkaloids (0.68%) and flavonoids (25.20 mg/g rutin equivalents).

Table 6: Anti-nutritional factors (ANFs) in EMLM

Antinutritional factors	Quantity
Total tannins (%)	1.26
Total oxalate (%)	0.032
Phytate (%)	4.43
Alkaloids (%)	0.68
Flavanoids (mg rutin equivalent/g)	25.20

4.3.2. In vitro relative protein digestibility of EMLM

The crude protein content of EMLM was 18.50% and relative in-vitro protein digestibility was found to be 32.37% in comparison with casein (SRL, CP: 95%). Relative pH change is given in Table 7 and Fig 1.

Table 7: pH change of Casein and EMLM using *C. carpio* intestinal extract

Time (min)	Casein (-ΔpH/min)	EMLM (-ΔpH/min)
0	8.0±0.01	8.0±0.01
1	7.69±0.01	7.91±0.01
2	7.47±0.01	7.79±0.01
3	7.36±0.01	7.77±0.01
4	7.25±0.01	7.75±0.01
5	7.19±0.01	7.70±0.01
6	7.11±0.01	7.68±0.01
7	6.80±0.01	7.62±0.01
8	6.74±0.01	7.59±0.01

9	6.61±0.01	7.55±0.01
-ΔpH/10 min	1.39±0.01	0.45±0.01

All values are expressed as Mean ± SE (n=3)

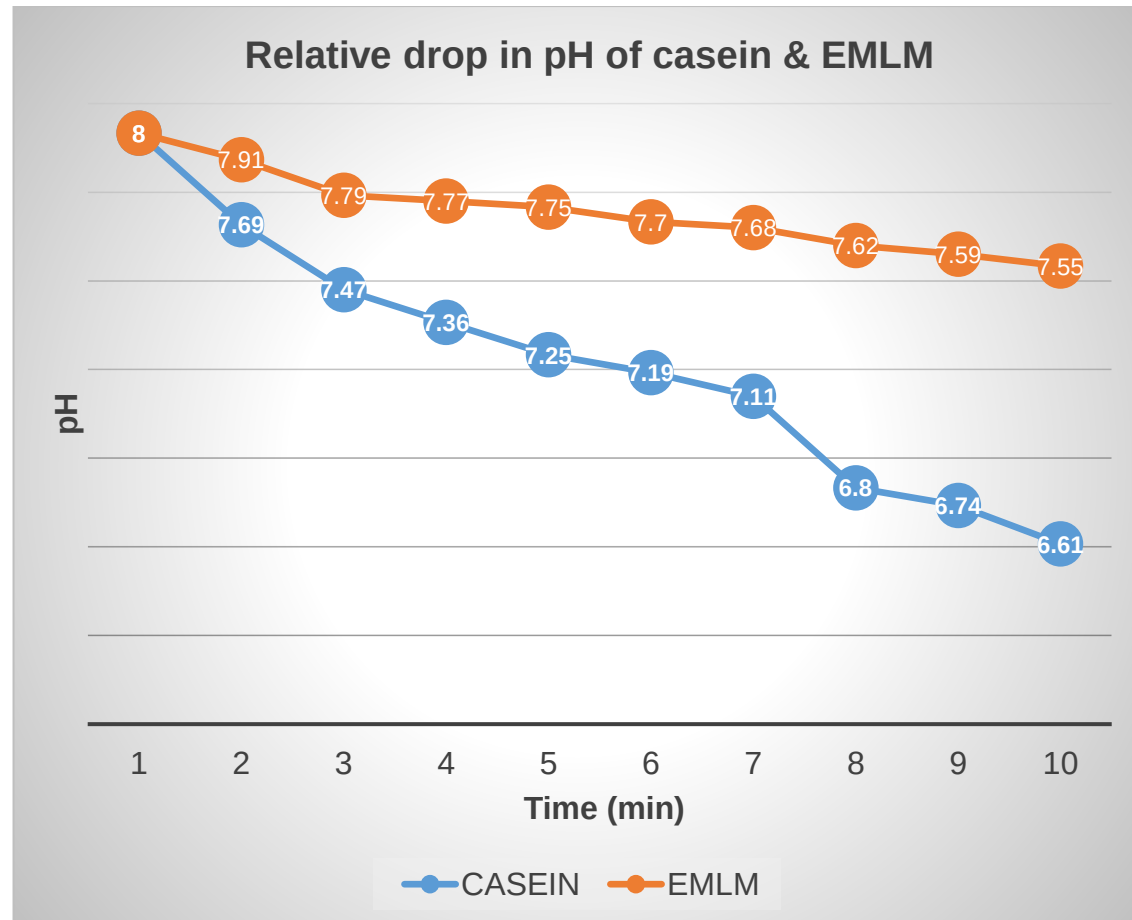


Fig 1: pH change of casein and EMLM using *C. carpio* intestinal extract

4.4. Experiment I

4.4.1. Proximate composition of diets

The proximate composition of the diets is shown in Table 8. The experimental diets were prepared containing 30% crude protein and 5% lipid for both the experimental groups. After proximate analysis, it was found that the moisture, crude protein, ether extract, nitrogen free extract, crude fibre and ash was found to be 10.11, 30.81, 5.31, 39.36, 7.16, and 7.25% respectively. The calculated digestible energy of the feed was 328.47 Kcal/100g.

4.4.2. Proximate composition of fish tissues

The proximate composition of the fish tissues is given in table 9. There was no significant difference ($P < 0.05$) observed between the experimental groups, however the values was different. The moisture content of the experimental groups varied from 76.10 to 74.82%. The dry matter content varied from 23.89-25.18%. The crude protein content on wet weight basis varied from 14.91 to 15.26%. The ether extract of all the fish was estimated within 3.94 to 3.45%. The total carbohydrate varied from 2.35 to 3.07% and the total ash content varied from 2.83 to 3.29%.

Table 8: Proximate composition of the experimental diets

Treatments	Moisture (%)	Crude Protein (%)	Ether Extract (%)	Nitrogen Free Extract (%)	Crude Fiber (%)	Ash (%)	Energy(DE Kcal/100g)
FW/ISW	10.11±0.21	30.81±0.26	5.31±0.09	39.36±0.45	7.16±0.18	7.25±0.05	328.47±0.73

Data expressed as mean ± SE, n=3. Calculated Digestible energy (Kcal/100g) = (%CP×4) + (%EE×9) + (%NFE×4) (Halver, 1976)

Table 9: Proximate composition of whole body of *Cyprinus carpio* fingerlings of different experimental groups (%wet weight basis)

Treatments	Moisture	Dry matter	Crude protein	Lipid	Carbohydrate	Ash
FW	76.10±0.49	23.89±0.48	15.26±0.06	3.45±0.12	2.35±0.29	2.83±0.14
ISW	74.82±0.23	25.18±0.23	14.91±0.27	3.94±0.09	3.07±0.15	3.29±0.16

All values are expressed as Mean±SE (n=3)

4.4.3. Growth performance and nutrient utilization

4.4.3.1. Percentage weight gain (PWG)

The body weight gain expressed in percentage, so as to avoid the initial variation in body weight and is given in Table 10 and Fig 2. The weight gain percentage was found to be significantly different ($P<0.05$) among the FW and ISW treatment groups. The fresh water reared fishes has shown significantly higher ($P<0.05$) growth than the fishes reared in inland saline water (ISW).

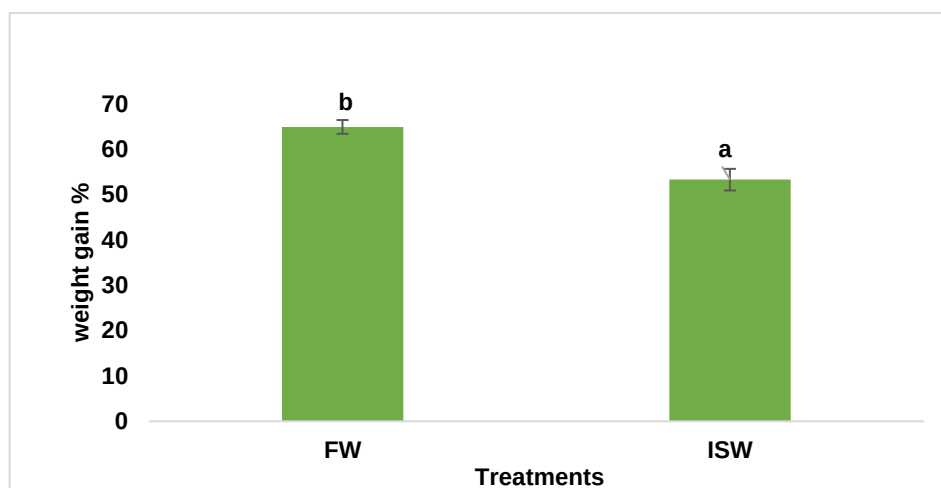


Fig 2. Percentage weight gain (PWG) of *C. carpio* reared in ISW

4.4.3.2. Specific growth rate (SGR)

The SGR of the different experimental groups is shown in the Table 8 and Fig 3. The SGR was significantly different ($P<0.05$) between FW and ISW groups. The significantly higher ($P<0.05$) SGR values were recorded in the FW (fresh water reared) group compared to that of ISW

4.4.3.3. Feed conversion ratio (FCR)

The FCR of the feed in different experimental groups is shown in the Table 13 and Fig 3. The FCR varied significantly ($P<0.05$) between the groups, with lower values observed in the FW group.

Table 10: Growth parameters of *Cyprinus carpio* reared in ISW

Treatments	PWG ¹	SGR ²	FCR ³	PER ⁴
FW	64.89 ^b ±1.52	0.82 ^b ±0.02	2.319 ^a ±0.06	1.44 ^b ±0.04
ISW	53.27 ^a ±2.38	0.71 ^a ±0.02	2.712 ^b ±0.07	1.22 ^a ±0.03
P value	0.015	0.015	0.012	0.012

Different superscripts in the same column signify statistical differences (P<0.05). Values are expressed as mean ± SE (n=12). ¹Percentage Weight Gain, ²Specific Growth Rate, ³Feed Conversion Ratio, ⁴Protein Efficiency Ratio

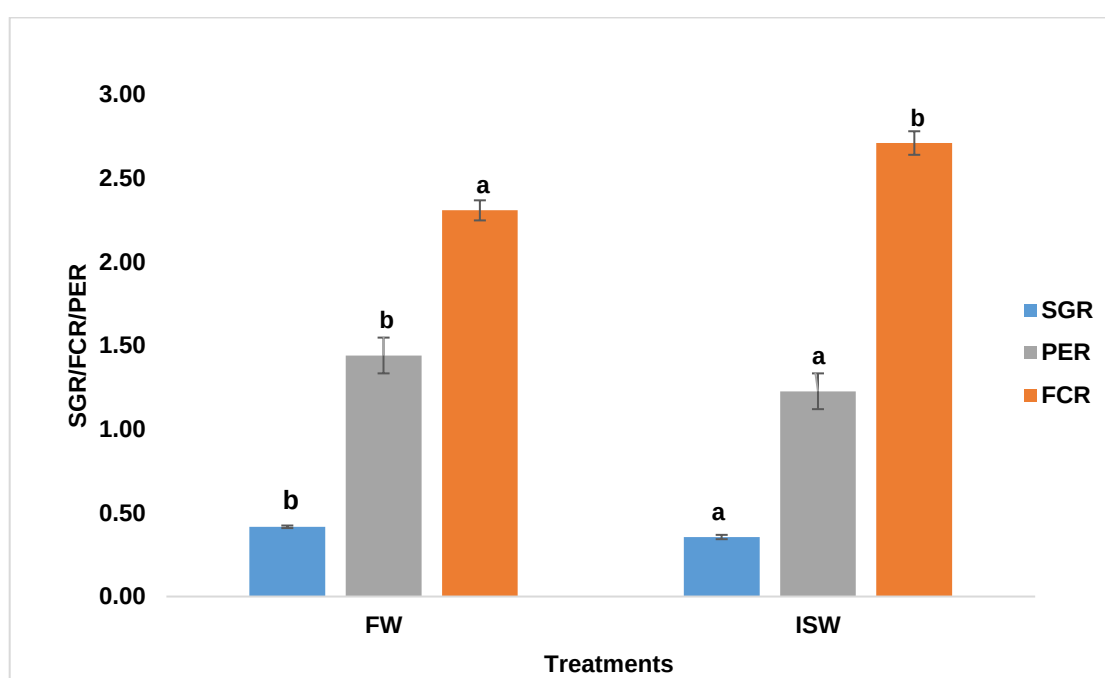


Fig 3. Growth parameters (SGR, PER, FCR) of *Cyprinus carpio* reared in ISW

4.4.4. DNA, RNA and RNA/DNA ratio

DNA, RNA and RNA/DNA ratio in the muscle of *C. carpio* fingerlings reared in FW and ISW is shown in Table 11. There was no significant difference in DNA and RNA levels in muscle (P>0.05) of the fishes reared in different waters. There was no

significant difference ($P>0.05$) between the two experimental groups in the RNA/DNA ratio as well.

Table 11: DNA, RNA and RNA/DNA ratio of *Cyprinus carpio* reared in ISW

Treatments	DNA ($\mu\text{g/g}$)	RNA ($\mu\text{g/g}$)	RNA/DNA
FW	2.72 $\pm$ 0.02	0.76 $\pm$ 0.04	0.28 $\pm$ 0.01
ISW	2.70 $\pm$ 0.06	0.65 $\pm$ 0.03	0.24 $\pm$ 0.01
P value	0.82	0.172	0.149

Values are expressed as Mean $\pm$ SE (n=12)

4.4.5. Digestive enzyme activities

The digestive enzymes (Amylase, Protease and Lipase) activity of *Cyprinus carpio* is given the Table 12. The digestive enzyme activity varied significantly ($P<0.05$) between the experimental groups. Amylase, protease and lipase activity was significantly higher ($P<0.05$) in the ISW group than that of the FW group.

Table 12: Different digestive enzyme activity of *Cyprinus carpio* reared in ISW

Treatments	Amylase	Protease	Lipase
FW	0.24 ^a $\pm$ 0.02	0.33 ^a $\pm$ 0.06	0.22 ^a $\pm$ 0.04
ISW	0.27 ^b $\pm$ 0.01	0.48 ^b $\pm$ 0.05	0.38 ^b $\pm$ 0.05
P value	0.004	0.034	0.048

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean $\pm$ SE (n=12)

4.4.6. Metabolic enzymes activity

4.4.6.1. Amino-transferases

The amino-transferase enzymes (AST and ALT) activity is given the Table 13, Fig 4 and Fig 5. Aspartate amino-transferase (AST/GOT) activity was significantly different

($P < 0.05$) in the muscle and liver between the experimental groups. The AST activity in liver and muscle of FW reared fishes was significantly ($P < 0.05$) lower than ISW reared fishes. Alanine amino-transferase (ALT/GPT) activity varied significantly between the treatments ($P < 0.05$) in both the tissues, with significantly highest ($P < 0.05$) activity in the ISW group.

Table 13: Amino-transferase activity of *Cyprinus carpio* reared in ISW

Treatments	AST (Muscle)	AST (Liver)	ALT (Muscle)	ALT (Liver)
FW	15.35 ^a ±0.69	16.86 ^a ±1.87	15.79 ^a ±2.39	21.49 ^a ±1.02
ISW	23.76 ^b ±1.02	24.96 ^b ±0.51	24.39 ^b ±1.66	26.13 ^b ±0.56
P value	0.002	0.014	0.042	0.017

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean $\pm$ SE (n=12)

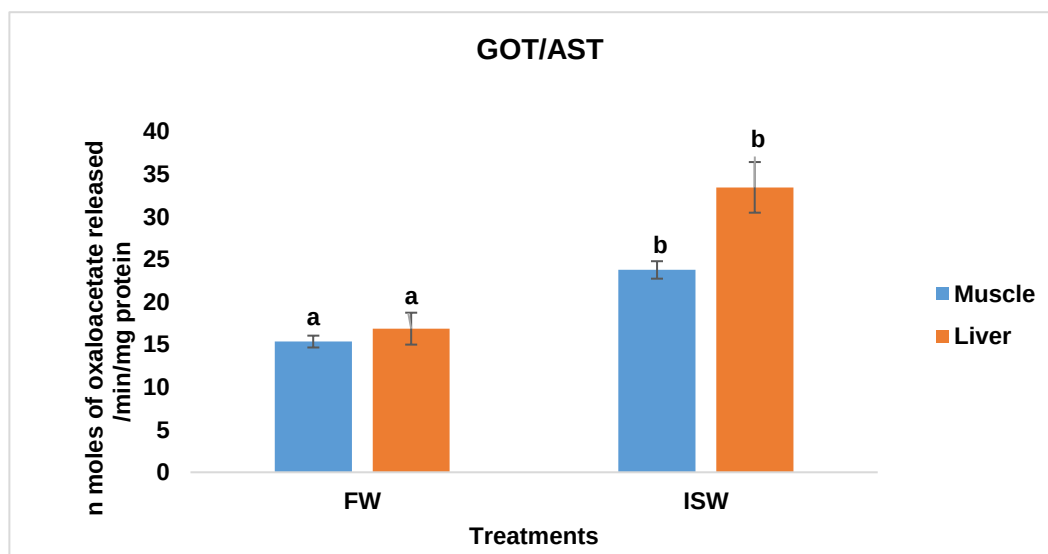


Fig 4. GOT/ AST activity of *Cyprinus carpio* reared in ISW

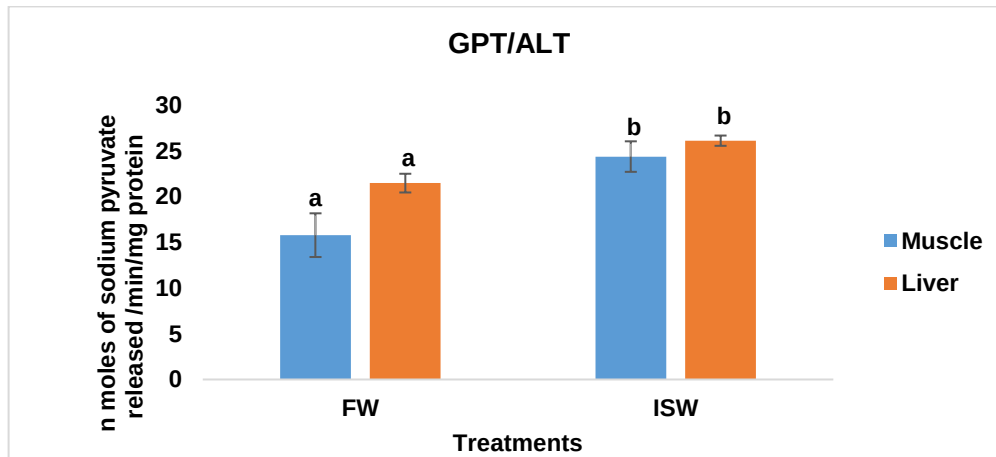


Fig 5. GPT/ ALT activity of *Cyprinus carpio* reared in ISW

4.4.6.2. Enzymes of carbohydrate metabolism (LDH & MDH)

The LDH and MDH activities of the two experimental groups are given in Table 14. There was no significant difference ($P>0.05$) observed in LDH activity of muscle of different experimental groups. However, liver LDH activity varied significantly ($P<0.05$) with significant increase ($P<0.05$) in the ISW group. The MDH activity of both muscle and liver did not show any significant difference ($P>0.05$) between the experimental groups.

Table 14: LDH and MDH activity of *Cyprinus carpio* reared in ISW

Treatments	LDH (Muscle)	LDH (Liver)	MDH (Muscle)	MDH (Liver)
FW	4.58±0.65	15.09 ^a ±0.49	0.25±0.03	2.13±0.24
ISW	6.24±0.89	17.59 ^b ±0.47	0.25±0.09	2.33±0.46
P value	0.117	0.035	0.900	0.712

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean ± SE (n=12). Units

4.4.6.3. Glucose-6 phosphatase (G-6-Pase)

Glucose 6 phosphatase activity in the liver of different experimental groups is given in Table 15 and Fig 6. Significant difference ($P<0.05$) in the liver glucose 6 phosphatase activity was observed between the different experimental groups, with significantly higher ($P<0.05$) activities in the ISW group.

4.4.7. ATPase activity

ATPase activity in the gills and liver of different experimental groups are given in Table 15. There was no significant difference ($P>0.05$) observed in the ATPase activity of gills of the two treatment groups, however significant difference ($P<0.05$) between the experimental groups was observed in the ATPase activity of liver.

Table 15: ATPase and G-6-Pase activity of *Cyprinus carpio* reared in ISW

Treatments	¹ G-6-Pase (Liver)	² ATPase (Gills)	² ATPase (Liver)
FW	2.64 ^a ±0.59	0.02±0.004	0.02 ^a ±0.003
ISW	6.39 ^b ±1.10	0.03±0.003	0.03 ^b ±0.002
P value	0.040	0.150	0.015

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean ± SE (n=12). ¹G-6-pase- µg phosphorous released/min/mg protein at 37°C; ²ATPase- µg phosphorius released/min/mg protein at 37°C

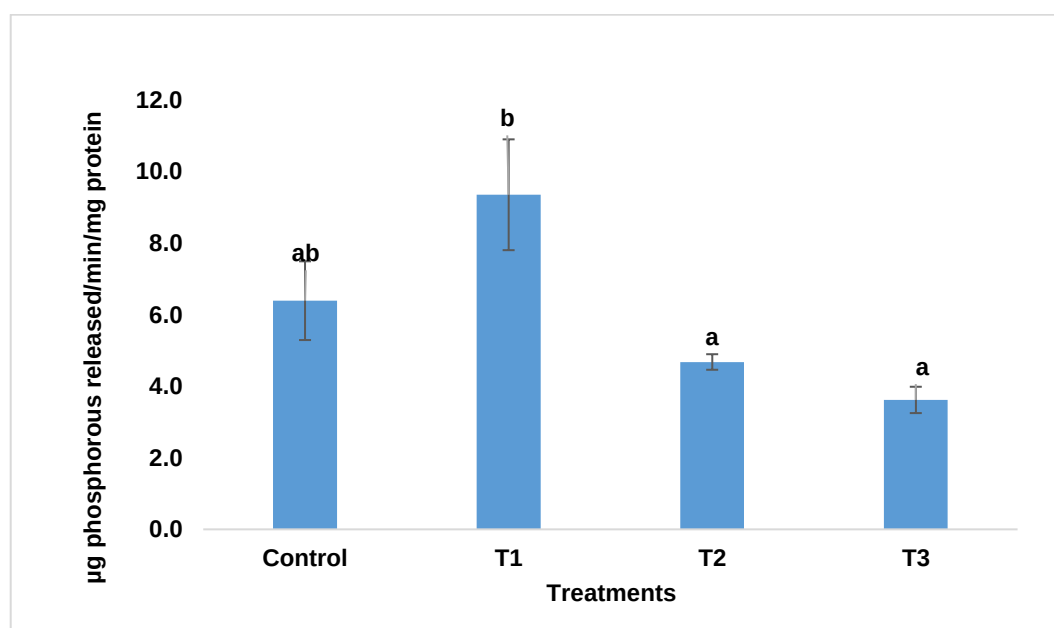


Fig 6. Glucose-6 phosphatase activity in *C. carpio* reared in ISW

4.4.8. Enzymes of oxidative stress

4.4.8.1. Superoxide dismutase (SOD)

The SOD activity in the gills and liver of different experimental groups is represented in the Table 16. The branchial and hepatic SOD activity varied significantly ($P < 0.05$) between the experimental groups. The SOD activities of gills and liver were significantly higher ($P < 0.05$) for the ISW group compared to the FW group.

Table 16: Superoxide dismutase (SOD) activity of *Cyprinus carpio* reared in ISW

Treatment	SOD (Gills)	SOD (Liver)
FW	31.95 ^a ±0.62	38.84 ^a ±0.92
ISW	37.95 ^b ±1.26	60.02 ^b ±0.08
P value	0.013	0.001

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean $\pm$ SE (n=12). SOD- Superoxide dismutase, amount of protein in mg required to give 50% inhibition of epinephrine auto oxidation/min.

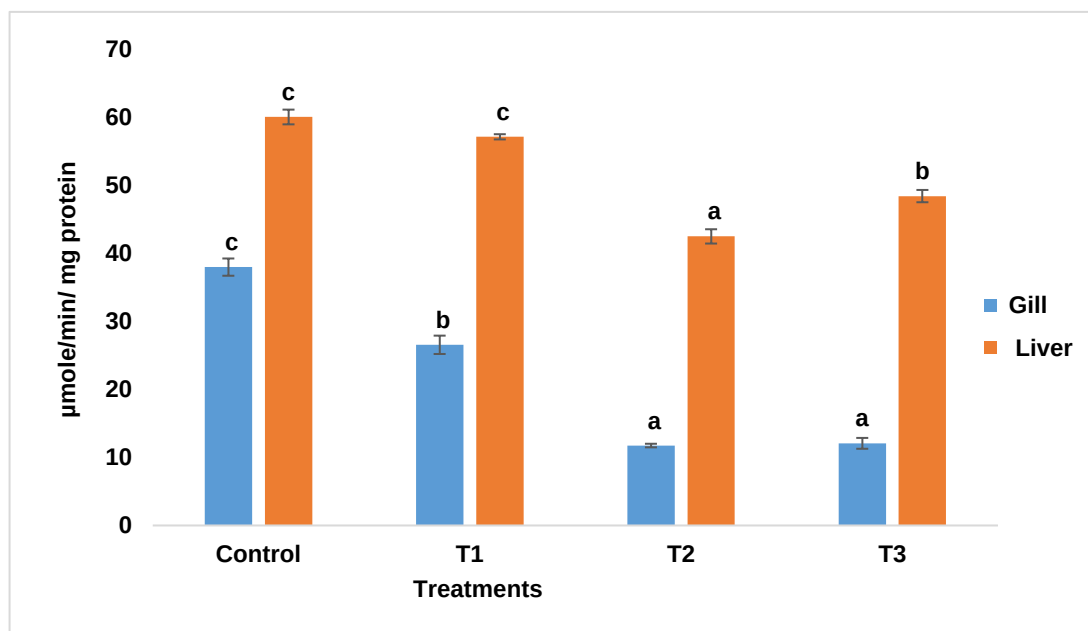


Fig 7. SOD enzyme activity in *C. carpio* reared in ISW

4.4.8.2. Catalase (CAT)

The catalase activity in the gills and liver of the different experimental groups is represented in the Table 17 and Fig 8. There was no significant difference observed in branchial catalase activity whereas hepatic SOD activity varied significantly ($P < 0.05$) between the experimental groups, with the ISW group bearing the significantly higher ($P < 0.05$) activities.

Table 17: Catalase activity of *Cyprinus carpio* reared in ISW

Treatments	Catalase ¹ (Gills)	Catalase ¹ (Liver)
FW	3.49 ^a ±0.27	23.83 ^a ±2.60
ISW	6.15 ^b ±0.75	39.09 ^b ±3.58
P value	0.59	0.026

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean $\pm$ SE (n=12). ¹Catalase- $\mu\text{mol H}_2\text{O}_2$ decomposed/min/mg protein

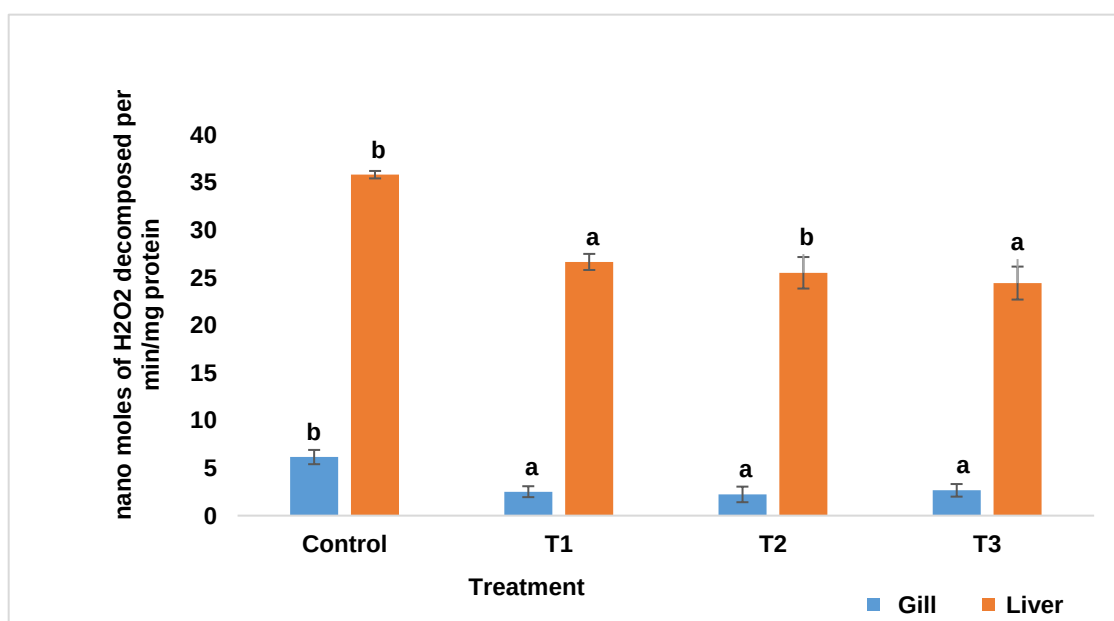


Fig 8. Catalase activity activity of *Cyprinus carpio* reared in ISW

4.4.8.3. Glutathione peroxidase (GPx) activity

The GPx activity in the gills and liver of different experimental groups is represented in the Table 18. There was no significant difference observed between the experimental groups in the GPx activities of gills and liver.

Table 18: Glutathione peroxidase (GPx) activity of *Cyprinus carpio* reared in ISW

Treatments	GPx (Gills)	GPx (Liver)
FW	0.44±0.07	0.46±0.10
ISW	0.54±0.16	0.74±0.20
P value	0.591	0.240

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean $\pm$ SE (n=12). GPx- Glutathione peroxidase (mmol glutathione converted/min/mg protein)

4.4.8.4. Glutathione-S transferase (GST)

The GST activity in the intestine and liver of different experimental groups is represented in the Table 19. There was no significant difference observed between the experimental groups in the GST activity of intestine and liver.

Table 19: Glutathione S transferase (GST) activity of *Cyprinus carpio* reared in ISW

Treatment	GST (Intestine)	GST (Liver)
FW	1.16±0.40	0.28±0.01
ISW	1.49±0.30	0.50±0.10
P value	0.501	0.100

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean $\pm$ SE (n=12)

4.4.8.5. Reduced glutathione (GSH) content

The GSH content in the brain and liver of different experimental groups is represented in the Table 20. The GSH content of brain and liver varied significantly ($P<0.05$) between the experimental groups. The GSH content of brain and liver in the FW reared group is higher than that of the ISW group.

Table 20: GSH content of *Cyprinus carpio* reared in ISW

Treatment	GSH ¹ (Brain)	GSH ¹ (Liver)
FW	20.30 ^b ±0.26	20.94 ^b ±1.44
ISW	18.82 ^a ±0.43	18.16 ^a ±0.58
P value	0.042	0.025

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean $\pm$ SE (n=12). ¹Units-mg/g tissue

4.4.9. Hemato-immunological parameters

4.4.9.1. Blood glucose

The blood glucose values of the experimental groups are represented in the Table 21. The blood glucose level varied significantly ($P<0.05$) between the experimental groups and the significantly ($P<0.05$) lower glucose level was observed in the FW group compared to the ISW group.

4.4.9.2. Serum cortisol

The serum cortisol values of the experimental groups are represented in the Table 21. There was a significant difference in the cortisol level between the different experimental groups. The significantly ($P<0.05$) highest cortisol level was observed in ISW group.

4.4.9.3. Serum creatinine

The serum creatinine content of the different experimental groups are represented in the Table 21. There was no significance difference ($P>0.05$) in the creatinine level between the experimental groups.

Table 21: Serum Glucose, Cortisol and Creatinine content of *Cyprinus carpio* reared in ISW

Treatments	Glucose (mg/dl)	Cortisol (ng/ml)	Creatinine (mg/dl)
FW	26.08 ^a ±1.78	1.52 ^a ±0.08	2.64±0.39
ISW	38.87 ^b ±1.02	4.38 ^b ±0.30	1.50±0.22
P value	0.033	0.001	0.06

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean ± SE (n=12).

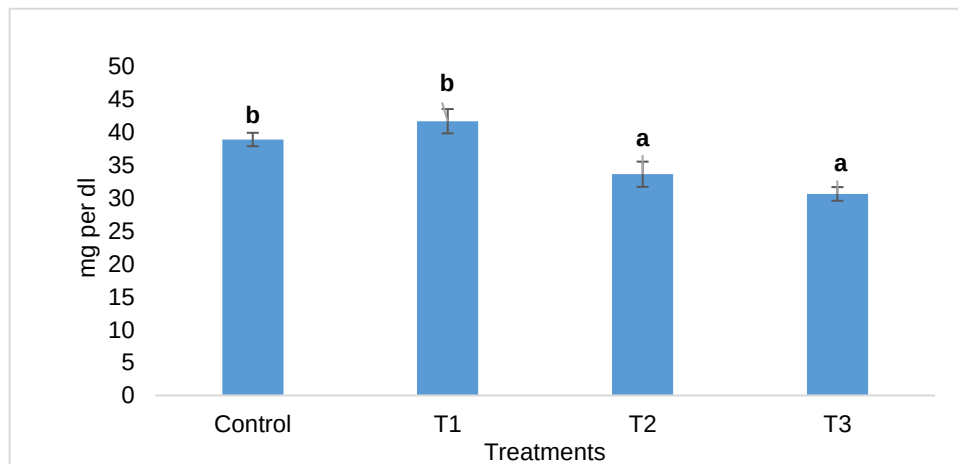


Fig 9. Blood glucose level in *C. carpio* reared in ISW

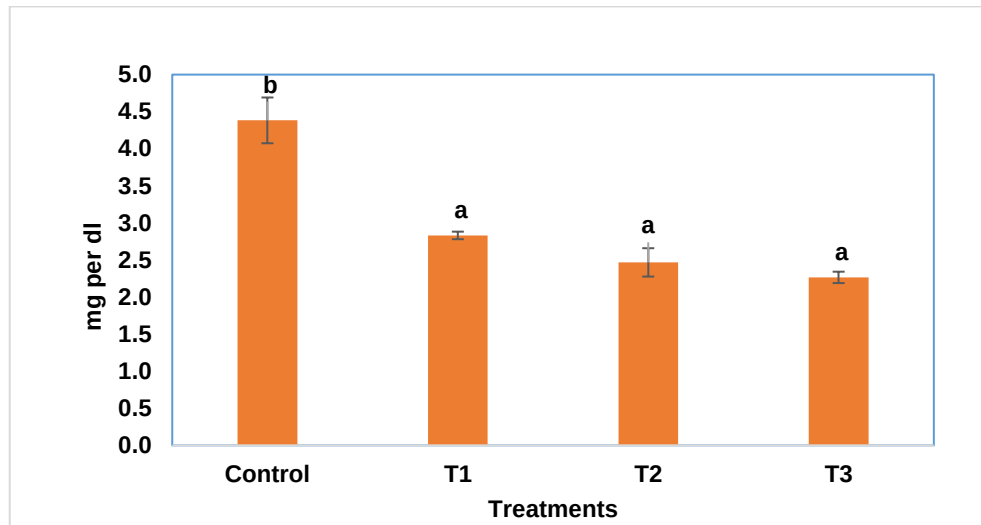


Fig 10: Cortisol level in *C. carpio* reared in ISW

4.4.9.4. Serum lipid profile

The serum lipid parameters of the *Cyprinus carpio* fingerlings of different experimental groups are shown in Table 22.

4.4.9.4.1. Cholesterol

The serum cholesterol level varied significantly between the two experimental groups. The significantly higher ($P < 0.05$) cholesterol levels were recorded in the FW group.

4.4.9.4.2. Triglycerides

The serum triglycerides content varied significantly ($P < 0.05$) between the two experimental groups. The significantly higher ($P < 0.05$) triglyceride levels were recorded in the FW group.

Table 22: Serum lipid profile of *Cyprinus carpio* reared in ISW

Treatment	Cholesterol (mg/dl)	Triglycerides (mg/dl)
FW	147.68 ^b ±2.80	150.13 ^b ±15.11
ISW	125.23 ^a ±6.67	111.29 ^a ±10.62
P value	0.01	0.01

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean ± SE (n=12)

4.4.9.5. Total protein, albumin, globulin and A/G ratio

The total protein, albumin, globulin and Albumin: Globulin (A: G) ratio is represented in the Table 23. There was no significant difference observed in the serum total protein between the experimental groups. The albumin and globulin content of serum in the experimental groups varied significantly ($P<0.05$). The albumin content was significantly higher ($P<0.05$) in the ISW group whereas globulin was significantly ($P<0.05$) higher in the FW group. The A: G ratio varied significantly ($P<0.05$) in the experimental groups and the ratio is higher in the ISW group.

Table 23: Serum Total protein, Albumin, Globulin and A:G ratio of *Cyprinus carpio* reared in ISW

Treatment	Total Protein	Albumin	Globulin	A:G Ratio
FW	3.93 $\pm$ 0.42	1.03 ^a $\pm$ 0.05	2.89 ^b $\pm$ 0.40	0.37 ^a $\pm$ 0.05
ISW	3.00 $\pm$ 0.22	1.69 ^b $\pm$ 0.08	1.31 ^a $\pm$ 0.18	1.32 ^b $\pm$ 0.14
P value	0.122	0.0002	0.023	0.004

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean $\pm$ SE (n=12).

4.4.9.6. Haematological parameters (Hb, RBC, WBC and Platelet)

The blood parameters is presented in Table 24. In the present study, there was no significant difference ($P>0.05$) in the Haemoglobin, RBC, WBC and Plate count of the fishes between the experimental groups.

Table 24: Blood parameters (Haemoglobin, RBC, WBC and Platelet count)

Treatments	RBC (X10 ⁶ /cmm)	Hb (g/dl)	WBC (X10 ³ /cmm)	Platelets (X10 ⁵ /cmm)
FW	1.19 $\pm$ 0.23	8.57 $\pm$ 0.81	19 $\pm$ 1.15	1.25 $\pm$ 0.09
ISW	1.38 $\pm$ 0.17	9.23 $\pm$ 0.62	14.28 $\pm$ 0.72	0.89 $\pm$ 0.08
P value	0.751	0.732	0.572	0.889

4.5. Experiment II

4.5.1. Proximate composition of diets

The proximate composition of the diets is shown in table 25. The experimental diets were prepared containing 30% crude protein and 5% lipid. After analysis, it was found that the crude protein of the diets varied from 29.96-30.81%, whereas the ether extract level varied from 4.79 to 5.31%. The ash content of the diets varied from 7.25 to 10.56%. The crude fibre of the diets was estimated in the range of 7.16 to 11.21%. The calculated nitrogen free extract ranged from 34.36 to 39.36%. The calculated digestible energy (DE) varied from 302.07 to 328.47 Kcal/100g feed.

4.5.2. Proximate composition of fish

The proximate composition of the fish is shown in table 26. The moisture content of all the experimental fish sampled for proximate analysis varied from 76.09 to 76.45%. The crude protein content on dry weight basis varied from 13.9 to 15.34%. The ether extract of all the fish was estimated within 2.07 to 2.78%. The total ash content varied from 14.74 to 15.14%. There was no significant difference among the group fed with different level of replacement of rice bran with the EMLM.

Table 25: Proximate composition of experimental diets (% wet matter Basis) for experiment II

Treatments	Moisture (%)	Crude Protein (%)	Ether Extract (%)	NFE (%)	Crude Fiber (%)	Ash (%)	Energy(DE Kcal/100g)
Control	10.11±0.21	30.81±0.26	5.31±0.09	39.36±0.45	7.16±0.18	7.25±0.05	328.47±0.73
T1	9.21±0.30	29.96±0.14	5.21±0.10	38.51±0.49	8.40±0.44	8.71±0.13	320.77±1.42
T2	9.81±0.17	30.58±0.21	4.79±0.70	34.43±0.42	10.30±0.10	10.05±0.11	303.25±1.94
T3	8.70±0.11	30.20±0.23	4.87±0.11	34.36±0.14	11.31±0.23	10.56±0.09	302.07±0.54

All values are expressed as Mean±SE (n=3) DE= Digestible energy

Calculated Digestible energy (Kcal/EE×9) + (% 100g) = (%CP×4) + (%NFE×4) Halver, 1976

Table 26: Proximate composition of whole body of *Cyprinus carpio* fingerlings (%wet weight basis)

Treatment	Moisture	Crude protein	Total Lipids	Total Carbohydrates	Total ash
Control	74.82±0.23	14.91±0.27	3.94±0.09	3.07±0.15	3.29±0.16
T1	74.96±0.68	14.90±0.14	3.42±0.86	2.56±0.24	3.88±0.23
T2	75.17±0.55	15.30±0.15	3.29±0.38	2.81±0.10	3.42±0.36
T3	74.95±0.42	15.34±0.12	3.37±0.18	2.64±0.14	3.67±0.34

All values are expressed as Mean ± SE

4.5.3. Growth performance

The different growth parameters like PWG, SGR, FCR and PER values are shown in the Table 27. Percentage weight gain (PWG), specific growth rate (SGR) and feed conversion ratio (FCR) varied significantly ($P<0.05$) among different experimental groups. The PWG, SGR and PER significantly decreased ($P<0.05$) in the treatment groups (T1, T2 & T3) compared to the C (control) group. The FCR was significantly higher ($P<0.05$) in the T2 and T3 groups compared to the control groups. There was no significant difference ($P<0.05$) between the C and T1 groups in FCR values.

Table 27: Growth parameters of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	PWG (%)	SGR	FCR	PER
C	53.27 ^c ±2.38	0.71 ^c ±0.02	2.71 ^a ±0.07	1.22 ^c ±0.03
T ₁	45.89 ^b ±1.65	0.62 ^b ±0.02	3.06 ^a ±0.12	1.09 ^b ±0.04
T ₂	38.80 ^a ±1.98	0.54 ^a ±0.01	3.59 ^b ±0.17	0.91 ^a ±0.04
T ₃	35.29 ^a ±0.76	0.50 ^a ±0.08	3.84 ^b ±0.05	0.85 ^a ±0.01
P value	0.001	0.001	0.001	0.001

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as mean ± SE.

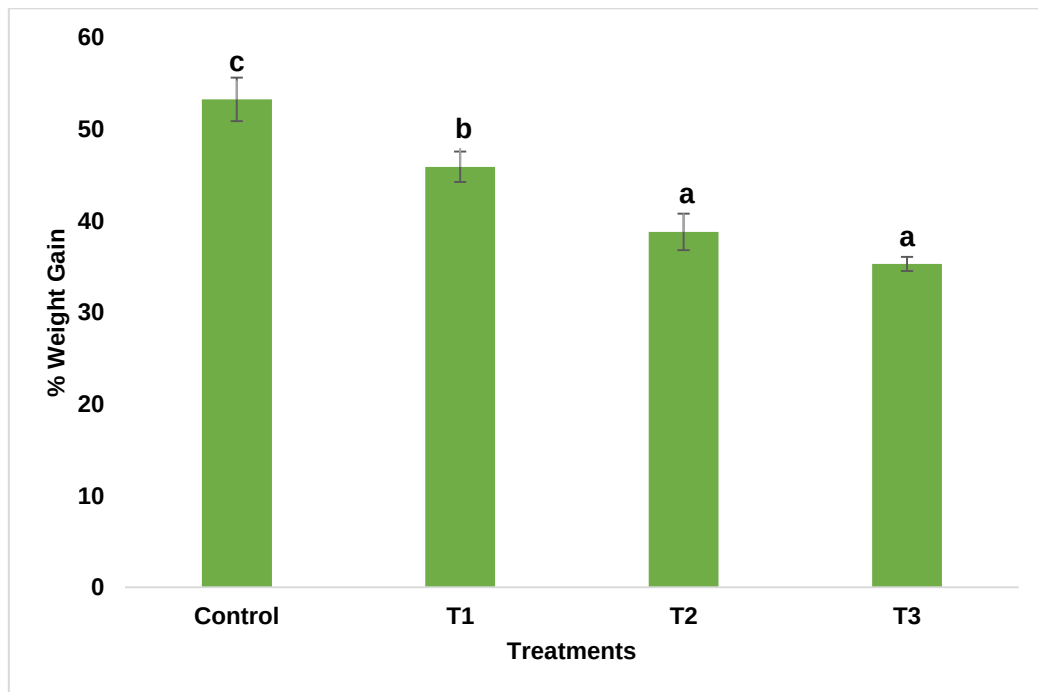


Fig 11: Percentage weight gain of *Cyprinus carpio* fed with EMLM and reared in ISW

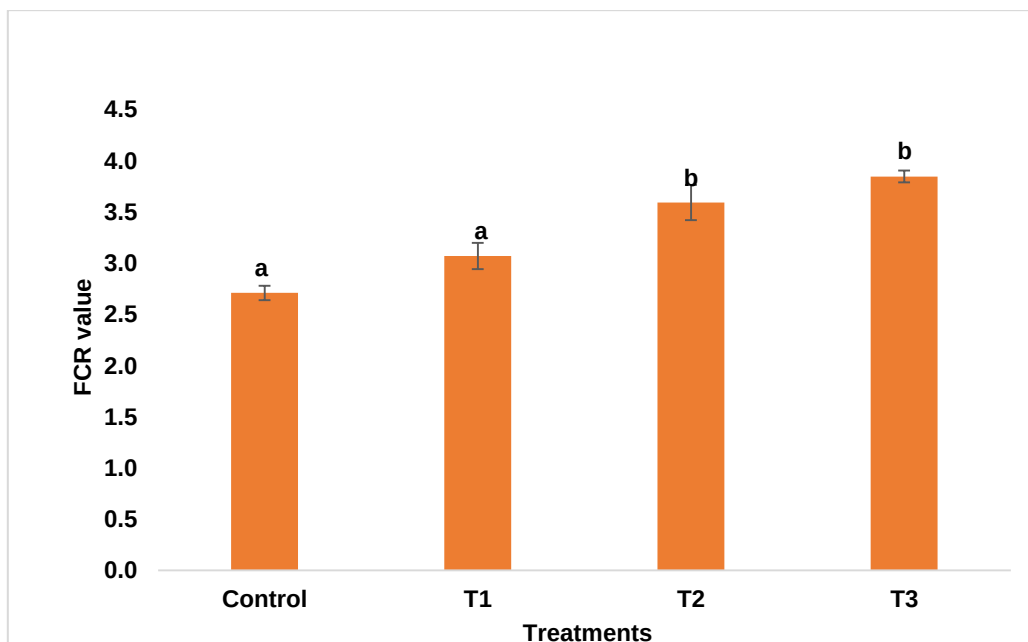


Fig. 12: FCR values of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.4. Body indices

4.5.4.1. Hepatosomatic index (HSI)

The HSI (%) of different treatments is given in given in Table 28. The HSI values of different treatment groups were not significantly different ($P>0.05$) among themselves. The highest value was observed in case of control (1.14 ± 0.13) lowest value was recorded for T3 (1.06 ± 0.03).

4.5.4.2. Gastrosomatic index (GaSI)

The GaSI (%) of different treatments are given in given in Table 28. The GaSI values did not vary significantly among the different experimental groups ($P>0.05$). The highest value was found in the T1 (2.45 ± 0.03) and the lowest value was recorded in the T3 group (2.15 ± 0.05) fed diet.

Table 28: HSI and GaSI of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	¹ HSI (%)	² GaSI (%)
Control	1.14 ± 0.13	3.80 ± 0.09
T1	1.07 ± 0.07	3.95 ± 0.03
T2	1.12 ± 0.11	3.91 ± 0.15
T3	1.06 ± 0.09	4.05 ± 0.25

Different superscripts in the same column signify statistical differences ($P<0.05$). Data expressed as Mean $\pm$ SE (n=9). ¹HSI– Hepato-somatic Index, ²GaSI–Gastro-somatic Index.

4.5.5. DNA, RNA and RNA/DNA ratio

DNA, RNA and RNA/DNA ratio in the muscle of *C. carpio* fingerlings of the different treatment groups is shown in Table 29. There was no significant difference in DNA levels in muscle of fishes belonged to different experimental groups.

There was a significant difference ($P < 0.05$) in the RNA level of muscle among the different experimental groups, with significantly highest value in the C group. The RNA/DNA ratio also varied ($P < 0.05$) significantly among the different experimental groups. The lowest value was recorded in T3 group and highest values in C (control) group.

Table 29: DNA, RNA and RNA/DNA ratio of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	DNA	RNA	RNA/DNA Ratio
C	2.70±0.06	0.65 ^b ±0.04	0.24 ^b ±0.01
T ₁	2.69±0.02	0.61 ^b ±0.03	0.22 ^b ±0.04
T ₂	2.73±0.08	0.58 ^{ab} ±0.03	0.21 ^{ab} ±0.01
T ₃	2.61±0.06	0.50 ^a ±0.01	0.19 ^a ±0.01
P value	0.171	0.048	0.01

Different superscripts in the same column signify statistical differences ($P < 0.05$). Data expressed as Mean ± SE (n = 9). (DNA and RNA units µg/ml)

4.5.6. Digestive enzymes activities

The replacement of rice bran with EMLM significantly ($P < 0.05$) affected amylase protease and lipase activity in the intestine of *Cyprinus carpio* fingerlings in the different experimental groups (Table 30, Fig. 13, 14, 15). The amylase activity ($P < 0.05$) of T2 and T3 groups were significantly higher than that of C and T1 groups. The protease activity ($P < 0.05$) showed significant difference among the experimental groups. It was observed that protease activity was significantly decreased in the T2 and T3 group, compared to the C and T1 groups. The different experimental groups varied significantly ($P < 0.05$) in the lipase activity in the intestine of the fingerlings. The highest activity was recorded in the group C and activity decreased across the treatment groups.

Table 30: Digestive enzyme activity of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	¹ Amylase	² Protease	³ Lipase
C	0.27 ^b ±0.02	0.48 ^b ±0.05	0.34 ^c ±0.05
T ₁	0.25 ^b ±0.01	0.37 ^b ±0.07	0.30 ^{bc} ±0.04
T ₂	0.23 ^a ±0.02	0.16 ^a ±0.02	0.16 ^{ab} ±0.04
T ₃	0.22 ^a ±0.01	0.12 ^a ±0.01	0.13 ^a ±0.04
P value	0.003	0.002	0.041

Different superscripts in the same column signify statistical differences ($P < 0.05$). Data are expressed as Mean $\pm$ SE ($n = 9$). Activities are expressed as follows: ¹Amylase as micromole of unit per min per mg protein; ²Protease as micromole of tyrosine released per min per mg protein; ³Lipase activity as units per hour per mg protein.

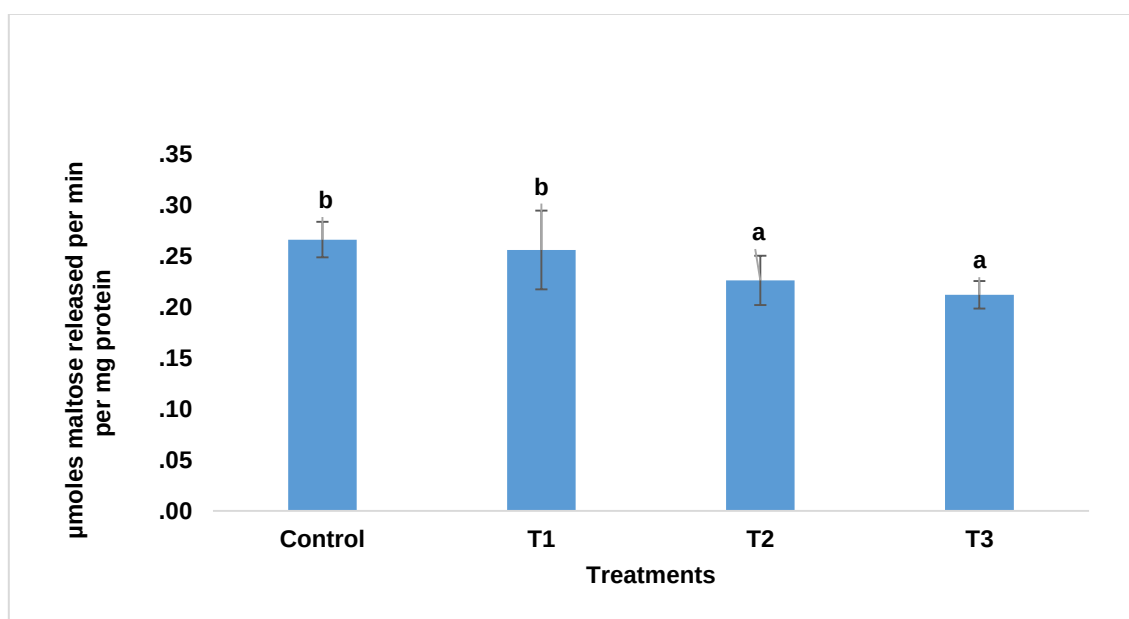


Fig. 13: Amylase activity of *Cyprinus carpio* fed with EMLM and reared in ISW

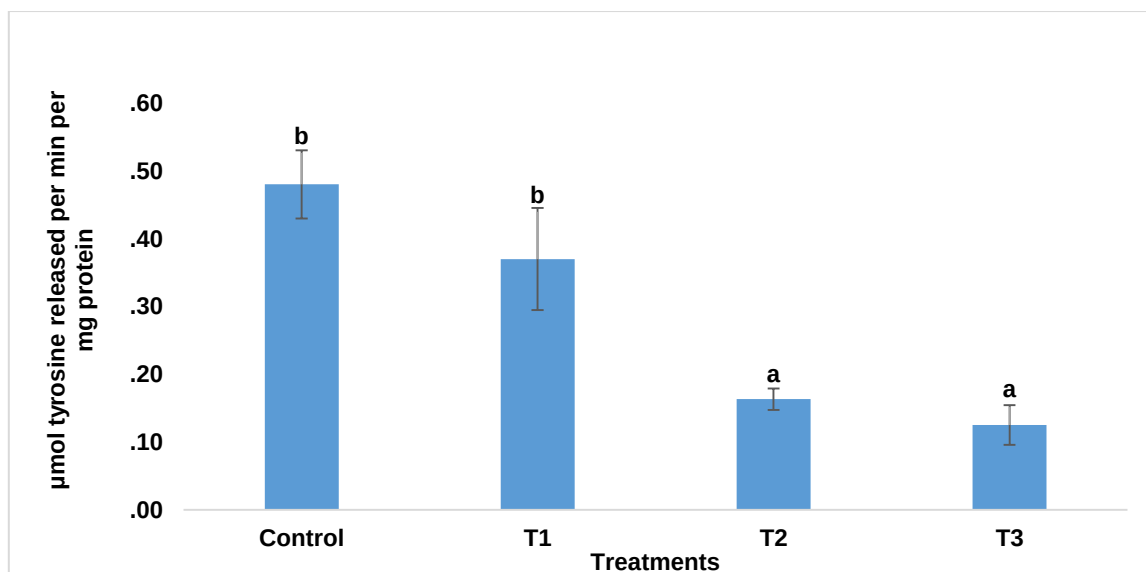


Fig. 14: Protease activity of *Cyprinus carpio* fed with EMLM and reared in ISW

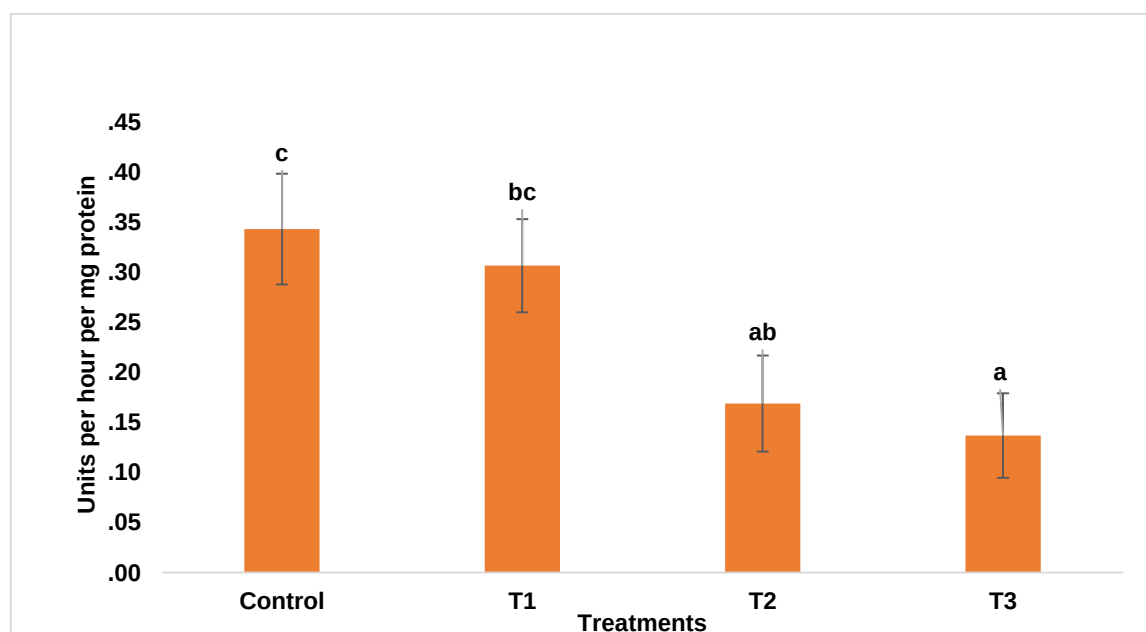


Fig. 15: Lipase activity of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.7. Amino-transferases

4.5.7.1. Aspartate aminotransferase (AST) activity

AST activity in the liver and muscle of *Cyprinus carpio* fingerlings of different experimental groups are presented in the Table 31 and Fig. 16. There was no significant difference ($P < 0.05$) in the hepatic AST activity among the treatment groups. However, the AST activities were significantly different ($P < 0.05$) in muscle among the different experimental groups. The significantly highest activity ($P < 0.05$) was observed in the T3 group ($P < 0.05$) whereas the significantly lowest activity was detected in C group.

4.5.7.2. Alanine aminotransferase (ALT) activity

ALT activity in the liver and muscle of different experimental groups are presented in Table 31 and Fig. 17. There was no significant difference ($P < 0.05$) in the muscle ALT activity. However, hepatic ALT activity among the treatment groups varied significantly ($P < 0.05$). The ALT activity of the treatment groups (T1, T2 and T3) has significantly ($P < 0.05$) increased compared to the C group.

Table 31: Aminotransferase (AST and ALT) activity of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	AST (Muscle)	AST (Liver)	ALT (Muscle)	ALT (Liver)
C	23.76 ^a ±1.02	24.96 ±0.51	24.39±1.66	26.13 ^a ±0.56
T ₁	31.36 ^b ±0.27	28.03±0.39	26.27±2.57	28.8 ^{ab} ±0.86
T ₂	31.57 ^b ±0.77	28.80 ±0.55	25.91±2.93	29.91 ^b ±0.34
T ₃	33.82 ^c ±0.13	30.84±3.7	28.14±1.31	34.70 ^c ±1.47
P value	0.001	0.273	0.705	0.001

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean ± SE (n = 9).

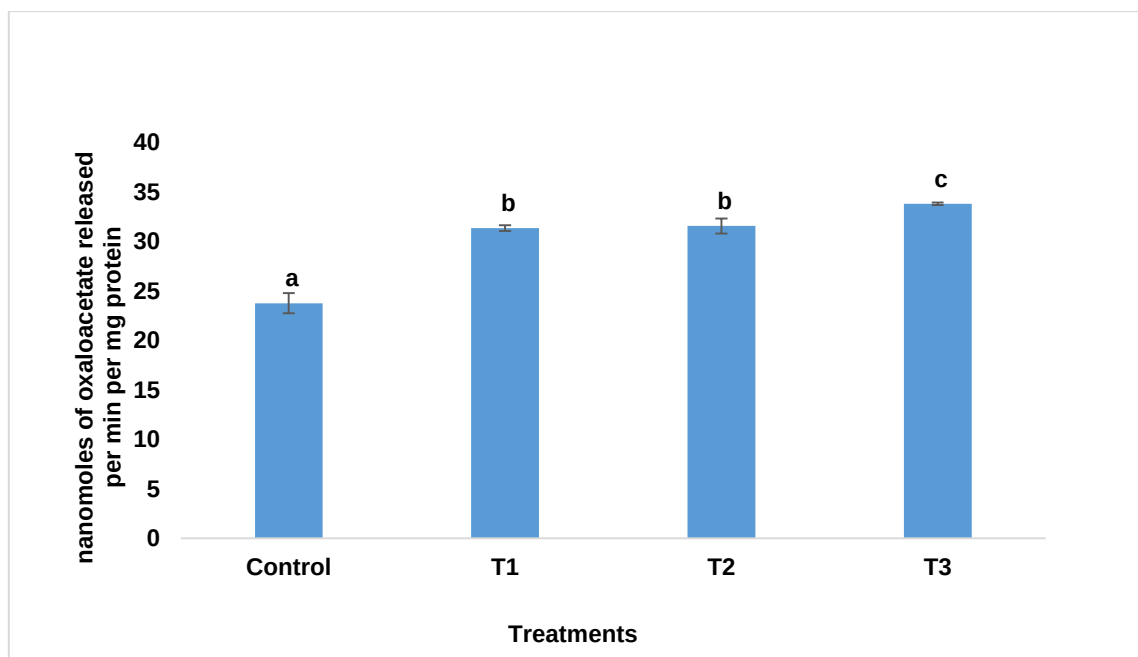


Fig. 16: Aspartate amino transferase activity (AST) in muscle of *Cyprinus carpio* fed with EMLM and reared in ISW

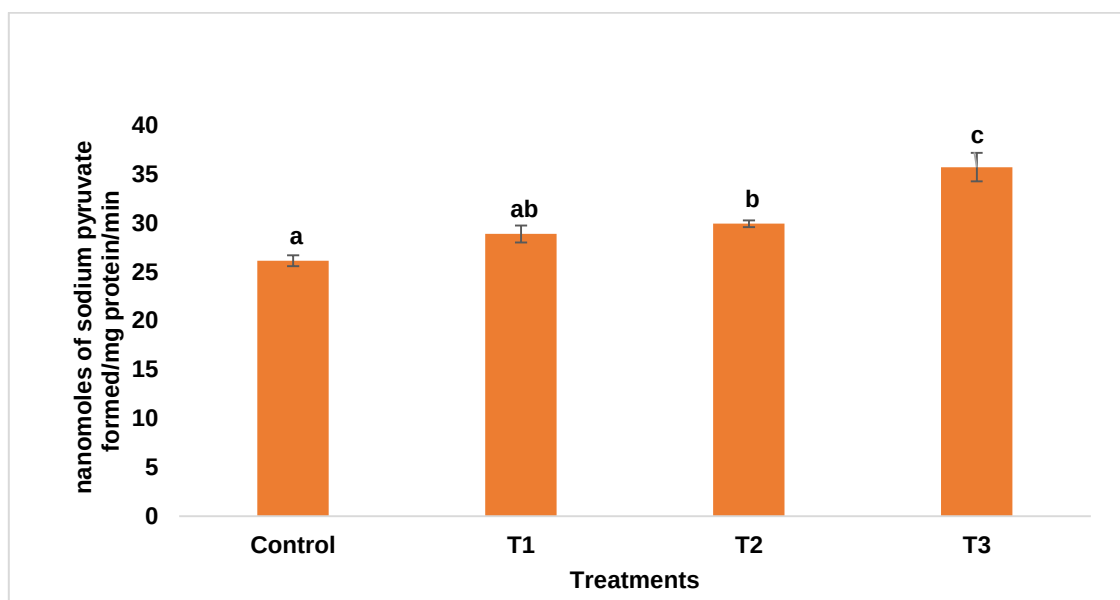


Fig. 17: Alanine amino-transferase (ALT) activity of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.8. Lactate dehydrogenase and malate dehydrogenase

The LDH and MDH activity in liver and muscle of *Cyprinus carpio* fingerlings of different experimental groups is shown in Table 32. It was observed that the MDH activity of the muscle and liver were not significantly different ($P>0.05$) among the different experimental groups fed with different experimental diets and reared in ISW.

Significant difference ($P<0.05$) of liver and muscle LDH activity was observed among the different experimental groups. The muscle LDH activity of T1, T2, T3 group were statistically same and higher than the C group fed with control diet. The liver LDH activity of C (control) group is lower than the treatment groups (T1, T2 & T3).

Table 32: LDH and MDH activity of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	¹ LDH (Muscle)	¹ LDH (Liver)	¹ MDH (Muscle)	¹ MDH (Liver)
C	6.24 ^a ±0.51	17.59 ^a ±0.49	0.25±.08	2.26±0.39
T ₁	13.12 ^b ±1.04	20.51 ^{ab} ±0.62	0.39±.10	1.34±0.16
T ₂	14.29 ^b ±0.46	22.32 ^{bc} ±1.02	0.70±.24	1.41±0.25
T ₃	14.81 ^b ±0.77	25.65 ^c ±1.7	0.34±.24	2.09±0.21
P value	0.001	0.005	0.234	0.084

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean ± SE (n = 9). ¹units- millimoles of NAD released/mg protein/ min at 37°C

4.5.9. ATPase

The ATPase activity in the liver and gills of different experimental groups are presented in the Table 33 and Fig.18. The atpase activities varied significantly ($P<0.05$) in the gills and liver of *Cyprinus carpio* fingerlings among the different

experimental groups. The branchial ATPase activity was significantly highest ($P < 0.05$) in the C group whereas the hepatic ATPase activity was recorded significantly highest ($P < 0.05$) in the T1 group.

Table 33: ATPase activity of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	ATPase (Gills)	ATPase (Liver)
C	0.025 ^c ±0.003	0.031 ^b ±.002
T ₁	0.017 ^b ±0.002	0.045 ^c ±.003
T ₂	0.007 ^a ±0.001	0.017 ^a ±.002
T ₃	0.005 ^a ±0.001	0.019 ^a ±.003
P value	0.001	0.001

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean ± SE (n = 9).

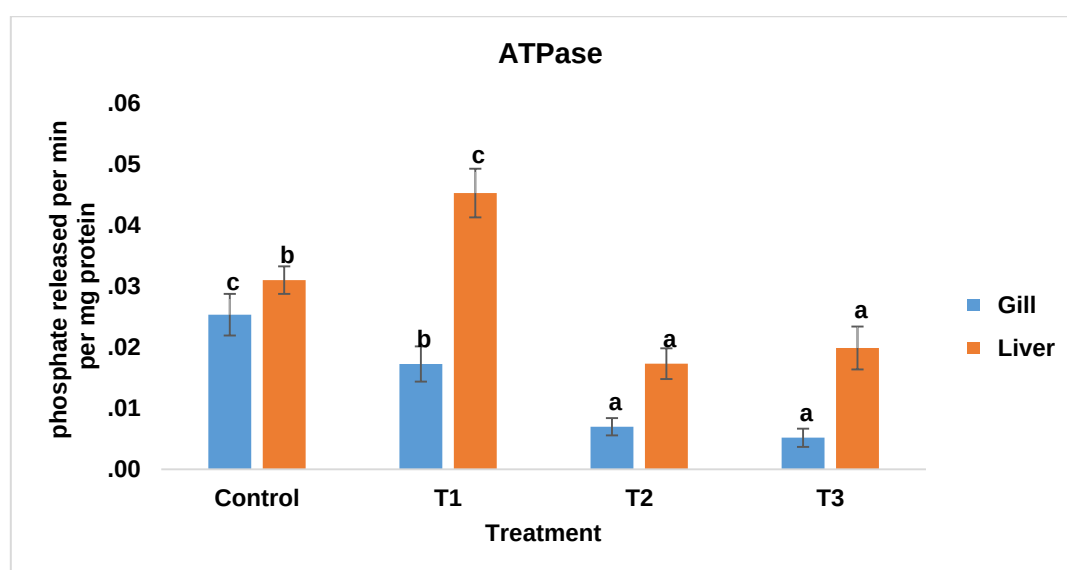


Fig. 18: ATPase activity of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.10. Glucose-6 phosphatase activity

Changes in the glucose-6 phosphatase activity with different treatments are presented in table 34 and fig 19. The liver glucose-6 phosphatase activities varied significantly ($P < 0.05$) among different experimental groups. The glucose-6

phosphatase enzyme activity was significantly lower in the T2 and T3 groups compared to that of T1 group. However, the C group was not significantly different from the treatment groups.

Table 34: Glucose-6 phosphatase activity of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	G-6-Phosphatase (Liver)
C	6.39 ^{ab} ±1.09
T ₁	9.35 ^b ±1.54
T ₂	4.67 ^a ±0.21
T ₃	3.61 ^a ±0.36
P value	0.015

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean $\pm$ SE ($n = 9$).

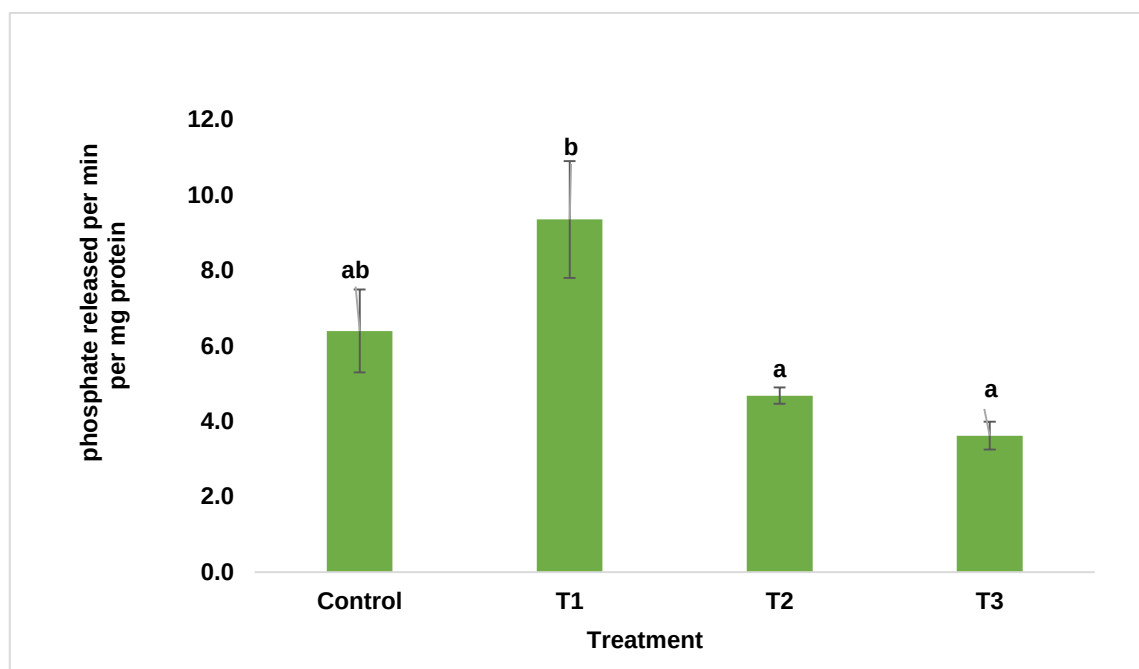


Fig. 19: Glucose-6 phosphatase activity of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.11. Antioxidant enzymes

4.5.11.1. Superoxide dismutase

The SOD activity in gills and liver of the different experimental groups is represented in Table 35 and fig 20. The branchial SOD activity varied significantly ($P<0.05$) among different treatment groups with highest activity in the C (control) group which was significantly different from other groups. The T2 and T3 groups were not significantly different ($P>0.05$), however they differed significantly ($P<0.05$) from T1 and control group. The hepatic SOD activity varied significantly ($P<0.05$) among different groups. The SOD activity is significantly lowest ($P<0.05$) in the T2 group while it was not significantly different ($P>0.05$) between T1 and C groups.

Table 35: SOD activity of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	SOD (Gills)	SOD (Liver)	Catalase (Gills)	Catalase (Liver)
C	37.95 ^c ±1.26	60.02 ^c ±1.08	6.15 ^b ±0.75	35.81 ^b ±.38
T ₁	26.54 ^b ±1.3	57.10 ^c ±0.38	2.52 ^a ±0.99	26.64 ^a ±.84
T ₂	11.73 ^a ±0.26	42.47 ^a ±1.05	2.23 ^a ±0.81	25.50 ^a ±1.65
T ₃	12.06 ^a ±0.79	48.39 ^b ±0.90	2.66 ^a ±0.66	24.42 ^a ±1.73
P value	0.001	0.013	0.013	0.034

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean ± SE (n=9)

4.5.11.2. Catalase (CAT)

The catalase activity in the gills and liver of different experimental groups is given in Table 35 and fig 21. There was a significant difference ($P<0.05$) in the branchial catalase activity among the different experimental groups. The catalase activity decreased significantly in the treatment groups compared to the control. The hepatic catalase activity of the control group was significantly highest ($P<0.05$) compared to the treatment groups.

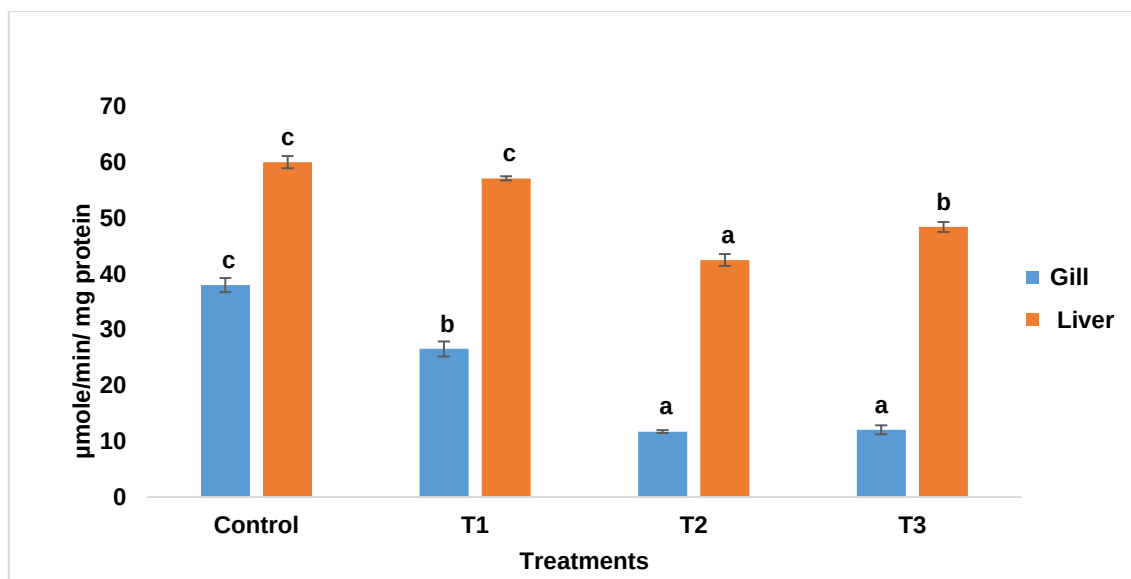


Fig. 20: SOD activity of *Cyprinus carpio* fed with EMLM and reared in ISW

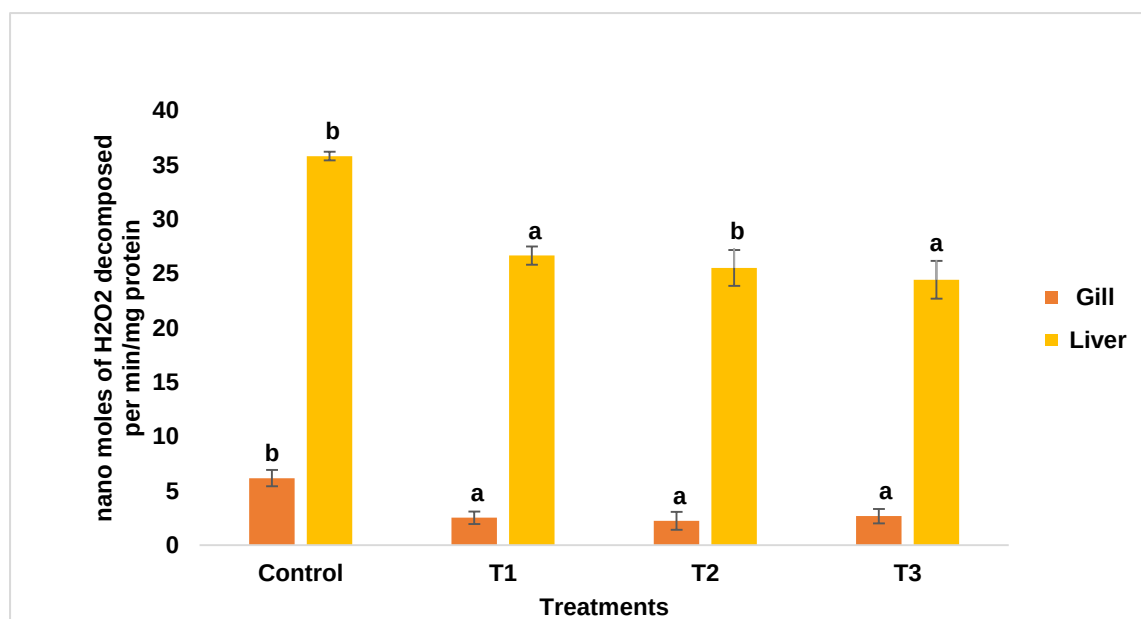


Fig. 21: Catalase activity of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.11.3. Glutathione peroxidase (GPx)

The glutathione peroxidase activity of gills and liver of the different experimental groups are presented in Table 36. The GPx activity of the control group was not significantly different from T1, however it differed significantly ($P<0.05$) from the experimental groups, T2 and T3. There was no significant difference among the different experimental groups in the hepatic GPx activity.

Table 36: Catalase and Glutathione peroxidase activities of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	Catalase (Gills)	Catalase (Liver)	GPx (Gills)	GPx (Liver)
C	6.15 ^b ±0.75	35.81 ^b ±.38	0.56 ^c ±0.09	0.81±0.09
T1	2.52 ^a ±0.99	26.64 ^a ±.84	0.35 ^{bc} ±0.03	0.95±0.09
T2	2.23 ^a ±0.81	25.50 ^a ±1.65	0.23 ^a ±0.07	0.68±0.11
T3	2.66 ^a ±0.66	24.42 ^a ±1.73	0.11 ^a ±0.01	0.61±0.08
P value	0.013	0.034	0.006	0.154

Different superscripts in the same column signify statistical differences ($P<0.05$). Values are expressed as Mean ± SE (n=9)

4.5.12. Glutathione-S-transferase (GST)

The GST activity in the intestine and liver of different groups is given in the Table 37 and fig. 22 There was no significant difference ($P>0.05$) in the intestinal GST activity among the different experimental groups. The liver GST activity among different experimental groups varied significantly ($P<0.05$). The groups, T2 and T3 have shown significantly higher values and were significantly different from control and T1.

Table 37: Glutathione S transferase (GST) activity of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	¹ GST (Intestine)	¹ GST (Liver)
C	1.49±0.29	0.49 ^a ±0.10
T1	1.56±0.10	0.64 ^a ±0.03
T2	1.59±0.15	0.91 ^b ±0.07
T3	1.80±0.09	1.08 ^b ±0.09
P value	0.547	0.004

The value represents the mean ± standard error mean (n = 9). ¹GST activity is expressed as nano moles CDNB conjugate formed/min/mg protein

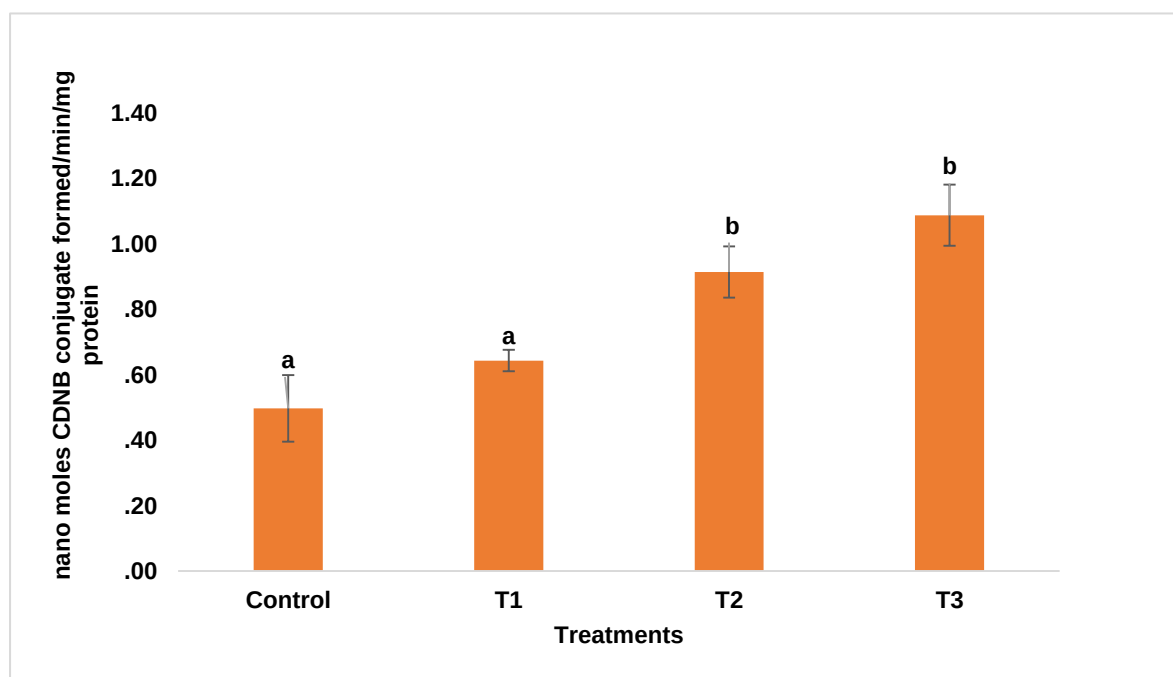


Fig. 22: Glutathione-S-transferase (GST) activity of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.13. Reduced glutathione content (GSH)

The brain and liver GSH contents of *Cyprinus carpio* fingerlings of different experimental groups are shown in the Table 38. The brain and hepatic GST content showed no significant difference among the different experimental groups.

Table 38: GSH content of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	¹ GSH (Brain)	¹ GSH (Liver)
C	18.82±0.42	18.16±0.58
T ₁	16.14±0.88	18.36±0.97
T ₂	17.62±1.26	19.30±1.10
T ₃	19.04±1.17	19.15±0.73
P value	0.225	0.742

Different superscripts in the same column signify statistical differences (P < 0.05). Values are expressed as Mean ± SE (n=9).

4.5.14. Hemato-immunological parameters

4.5.14.1. Serum proteins

4.5.14.1.1. Total serum protein

The data on serum protein levels of different experimental groups are presented in Table 39. There was no significant difference observed in the serum total protein level among the different experimental groups.

4.5.14.1.2. Serum albumin

The data on the total serum protein is presented in table 39. There was no significant difference among the different experimental groups in the serum albumin content.

4.5.14.1.3. Serum globulin

The data on the total serum protein is presented in table 39. There was no significance difference among different experimental groups in the serum globulin levels.

4.5.14.1.4. Albumin/Globulin ratio

The Albumin/Globulin ratio varied significantly (P<0.05) among different experimental groups. The A/G ratio of T1 and T2 was significantly higher than control and T3. The Albumin/Globulin ratio decreased with the increased inclusion level of leaf meal in the diet.

Table 39: Serum protein, albumin, Globulin and A/G ratio of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatment	Total Protein	Albumin	Globulin	A/G Ratio
C	3.00±0.22	1.69±0.07	1.31 ^{ab} ±0.17	1.32 ^a ±.14
T ₁	2.41±0.08	1.95±0.08	0.59 ^a ±0.10	3.62 ^b ±0.87
T ₂	2.78±0.08	1.84±0.15	0.93 ^{ab} ±0.08	2.04 ^{ab} ±0.39
T ₃	3.93±0.92	1.85±0.25	2.09 ^b ±0.64	1.01 ^a ±0.22
P value	0.240	0.715	0.001	0.001

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean $\pm$ SE (n = 9).

4.5.14.2. Serum glucose

The serum glucose levels of different experimental groups is shown in the Table 40 and fig. 23. The glucose values of the fishes in different treatment group varied significantly ($P < 0.05$) and the glucose level was significantly ($P < 0.05$) decreased in the the treatment groups.

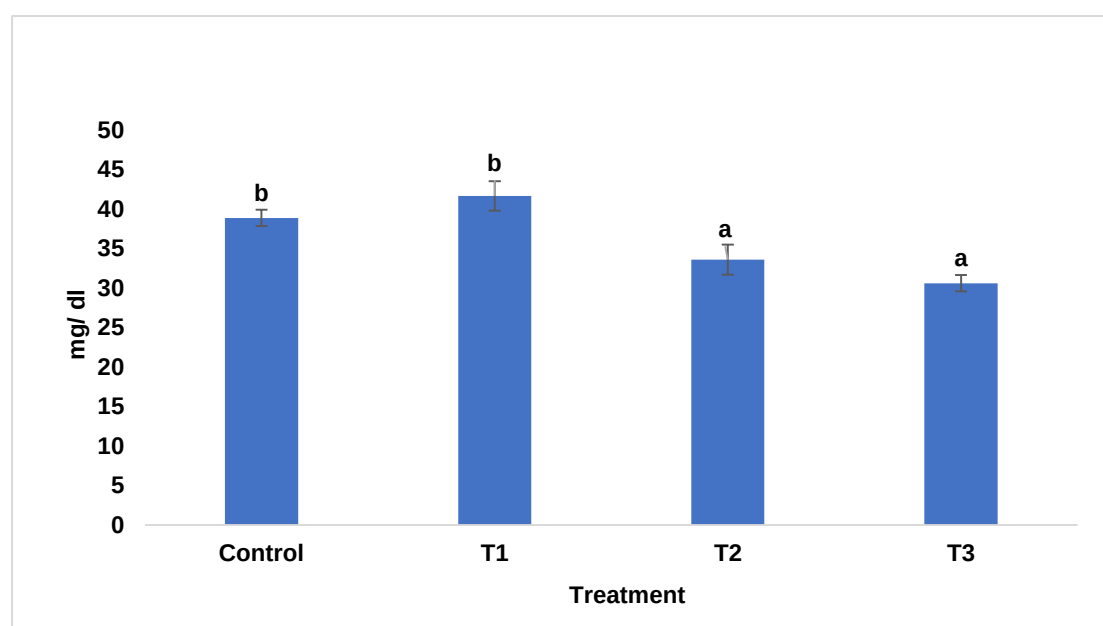


Fig. 23: Serum glucose level of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.14.3. Serum creatinine

The serum creatinine levels of different experimental groups is shown in the Table 40. The serum creatinine levels were significantly different ($P < 0.05$) among the experimental groups. The creatinine values were significantly higher ($P < 0.05$) in the treatment groups compared to the control

4.5.14.4. Serum cortisol

The serum cortisol levels of different experimental groups is shown in the Table 40 and fig.24. The cortisol values of the fishes in different treatment groups varied significantly ($P < 0.05$) and significantly highest value ($P < 0.05$) was observed in the control group.

Table 40: Serum Glucose, Creatinine and Cortisol Levels of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	Glucose	Creatinine	Cortisol
C	38.87 ^b ±1.02	1.50 ^a ±0.22	4.38 ^b ±0.30
T ₁	41.65 ^b ±1.84	3.89 ^{ab} ±1.03	2.83 ^a ±0.15
T ₂	36.60 ^a ±1.91	4.86 ^b ±0.89	2.47 ^a ±0.19
T ₃	30.60 ^a ±1.04	5.75 ^b ±0.64	2.59 ^a ±0.26

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean ± SE (n = 9).

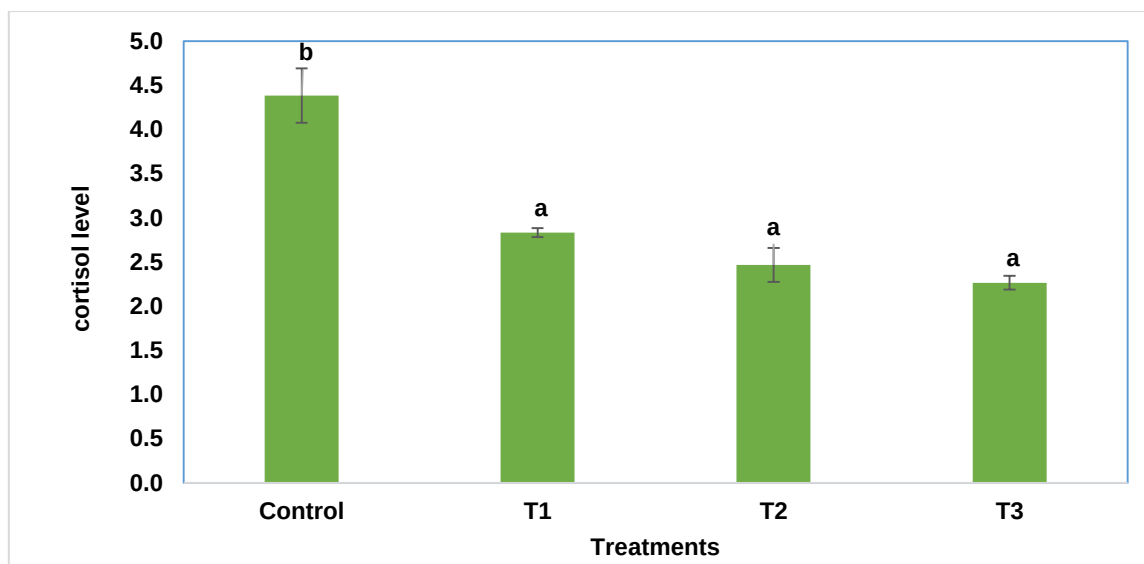


Fig. 24: Cortisol levels of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.14.5. Serum lipids (triglycerides and cholesterol)

The serum cholesterol and triglyceride levels of different experimental groups is shown in the Table 41 and fig. 25. The serum cholesterol level among the different experimental groups varied significantly ($P < 0.05$). The highest level was observed in T2 group which varied significantly ($P < 0.05$) from the control and other treatment groups. The different experimental groups showed significant difference ($P < 0.05$) in the serum triglycerides levels. The C and T1 and groups varied significantly ($P < 0.05$) from the T2 and T3 groups.

Table 41: Serum lipid profile of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	Cholesterol (mg/dl)	Triglycerides (mg/dl)
C	125.23 ^b ±3.85	116.53 ^b ±5.96
T ₁	118.05 ^b ±0.80	118.63 ^b ±7.73
T ₂	102.31 ^a ±1.51	79.26 ^a ±3.44
T ₃	99.76 ^a ±2.05	90.81 ^a ±3.19

Different superscripts in the same column signify statistical differences ($P < 0.05$). Values are expressed as Mean $\pm$ SE ($n = 9$).

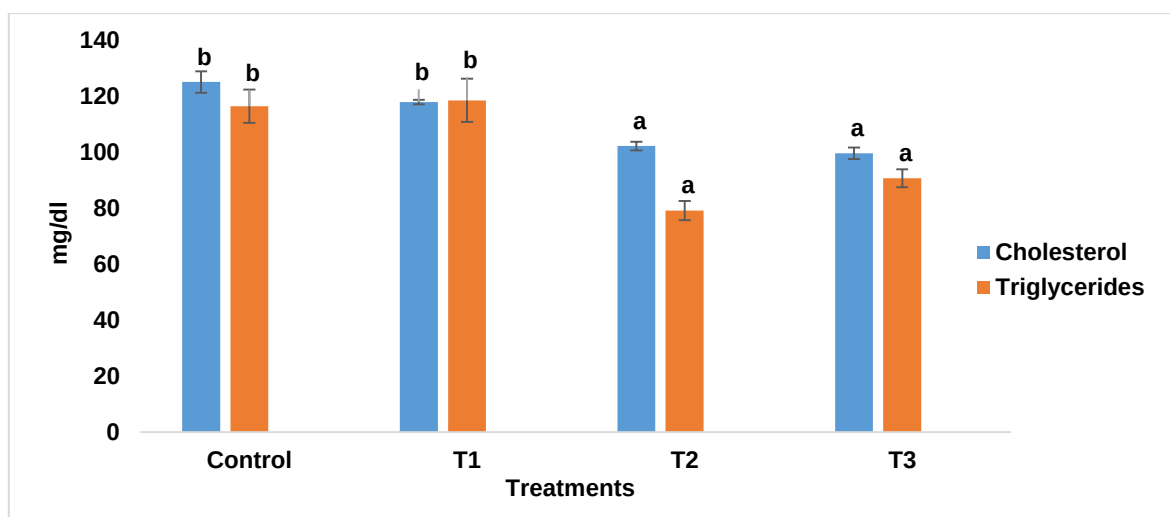


Fig. 25: Serum cholesterol and triglycerides profile of *Cyprinus carpio* fed with EMLM and reared in ISW

4.5.14.6. Blood parameters (Hb, RBC, WBC & platelet count)

The blood parameters (Hb, RBC, WBC and Platelet count) is presented in Table 42. The blood parameters viz. Haemoglobin (Hb), RBC and WBC varied significantly ($P < 0.05$) among the different experimental groups, however in the platelet count no significant ($P > 0.05$) difference was observed in the experimental groups fed with EMLM and reared in ISW.

Table 42: Blood parameters (Hb, RBC, WBC & Platelet count) of *Cyprinus carpio* fed with EMLM and reared in ISW

Treatments	RBC ($\times 10^6/\text{cmm}$)	Hb (g/dl)	WBC ($\times 10^3/\text{cmm}$)	Platelets ($\times 10^5/\text{cmm}$)
C	$0.96^a \pm 0.04$	$7.73^a \pm 0.13$	$14.28^a \pm 0.72$	0.89 ± 0.08
T1	$1.09^{ab} \pm 0.06$	$8.17^{ab} \pm 0.17$	$15.87^{ab} \pm 0.58$	1.00 ± 0.12
T2	$1.21^b \pm 0.03$	$8.60^b \pm 0.06$	$18.04^b \pm 0.71$	0.72 ± 0.04
T3	$1.53^c \pm 0.09$	$9.73^c \pm 0.35$	$22.18^c \pm 1.49$	0.96 ± 0.14
P value	0.001	0.001	0.002	0.267

5. DISCUSSION

5.1. Experiment 1

The first experiment was conducted to delineate the effect of rearing of common carp in inland saline water (ISW) on the growth and stress responses of the fish in comparison to the rearing in freshwater (FW). To this end, 2 experimental groups such as ISW and FW were set up with 4 replicates and the growth trial was conducted for 60 days.

5.1.1. Growth performance

In the present study, the effect of salinity on percentage weight gain, specific growth rate, feed conversion ratio, and protein efficiency ratio was studied in the experimental groups. The fishes reared in inland saline water (ISW) have shown lower PWG, SGR, and PER than the fishes reared in fresh water. The FCR of ISW group was also higher than the FW group. These results conclude that the rearing of common carp in 5 ppt ISW results in significant reduction in growth (approximately 10%) as well as feed conversion. Our results are in well agreement with studies of different authors (Wang *et al.*, 1997; De Boeck *et al.*, 1999) who reported that the growth of *Cyprinus carpio* decreased with increase in salinity. Under salinity stress, the energy cost of fish increases to maintain the osmotic gradient for ion regulation, which is known to negatively affect growth performance (Likongwe *et al.*, 1996; Luz *et al.*, 2008).

5.1.2. Water quality parameters

The physicochemical parameters of water such as temperature, pH, dissolved oxygen, free carbon dioxide, total hardness, alkalinity, ammonia nitrogen, nitrate nitrogen, nitrite nitrogen, and potassium were within the optimum range of requirements for fish as suggested by several authors. Temperature is the main factor determining the growth and the wellbeing of the fish. The fish thrive best in its optimal temperature zone beyond which temperature stress starts to set in eventually reflecting upon growth. Das *et al.* (2005) found that the temperature range of 26–36 °C is not fatal to *Cyprinus carpio* fingerlings. The optimum temperature range for growth was 31–33 °C, which supports the range of temperature observed during the

present feeding trial. The pH of water in all the experimental tanks ranged from 7.2-8.5, which is well within the acceptable range for carps as suggested by Banerjee (1967). The dissolved oxygen (DO) requirement of fish varies with a large number of factors such as water temperature, metabolic rate, biomass density, aeration, etc. In the present study the aeration was provided round the clock and the DO varied between 6-8ppm which was well within the acceptable range as suggested by Ali *et al.*, (2000). Carbon dioxide was found to be negligible, which may be due to low biomass and frequent water exchange during the experimental period. The carbonate hardness was found to be in the range of 229-246 mg L⁻¹ which lies within the optimum range as suggested by APHA (1998). The ammonia values were in the range of 0.07-0.1 mg L⁻¹. Jhingran (1991) suggested that the ammonia concentration of water must be in the range of 0.05-0.1 mg L⁻¹. The ammonia concentration was within the acceptable limits during the entire experimental period. The nitrite concentration was recorded in the range of less than 0.1 mg L⁻¹ which is well within the permissible limits of pond culture (Boyd and Tucker, 1998). Nitrate–N level in a pond can be within 0.1-5 mg L⁻¹ (Boyd and Tucker, 1998). In the present study, the nitrate –N was 0.03-0.07 mg L⁻¹, which is well below the toxic level and hence did not affect the fish adversely.

5.1.3. RNA DNA ratio

As the RNA content is directly related to the rate of protein synthesis, the RNA/DNA ratio can be used as an index of growth (Smith and Buckley, 2003; Akhtar *et al.*, 2012). The nucleic acids viz. DNA, RNA and RNA/DNA ratio did not vary significantly among the experimental groups suggesting the same rate of protein synthesis in the fishes fed with EMLM and DORB. Although, the nucleic acid levels were similar, growth was lower in ISW group which implies that the synthesis of protective stress proteins might have taken place due to increased salinity. These protein synthesis might have compensated the lowering of the structural protein synthesis.

5.1.4. Digestive enzyme activity

Breakdown of large nutrients into small absorbable subunits in the digestive tract of the animal depends largely on the available enzymes (Cho, 1987). In the present study, a significant increase in digestive enzyme activities of *Cyprinus carpio* fingerlings was observed in ISW reared fishes than in FW. The activation of

digestive enzyme activities of fishes in this study suggests that enhanced digestive activities occur at higher salinities (Li *et al.*, 2008). The increase in digestive enzyme activity provides direct evidence of deriving extra food energy to compensate the energy loss for osmoregulation when ambient salinity sways from the optimum physiological salinity.

5.1.5. Metabolic enzyme activity

Aspartate aminotransferase (AST) or glutamate oxaloacetate transaminase (GOT) and alanine aminotransferase (ALT) or glutamate pyruvate transaminase (GPT) are enzymes involved in the transfer of amino groups from one specific amino acid to another. Therefore, higher values indicate a greater transfer of amino groups or the greater metabolic waste of amino acids in the tissue. Besides lactate, amino acids are the preferred substrates for gluconeogenesis in fish. Fish utilize protein and lipid sources rather than carbohydrate for energy fulfilment (Demeal, 1978). Teleosts are known for their ability to convert amino acids into glucose. Therefore, the higher activity of AST and ALT indicates the mobilization of aspartate and alanine via gluconeogenesis for glucose production to cope with stress (Knox and Greengard, 1965; Chatterjee *et al.*, 2006). It is also reported that elevated level of transaminase activity during stress would lead to increased feeding of ketoacids into TCA cycle, thereby affecting oxidative metabolism. In the present experiment, the observed increases in the activities of aspartate aminotransferase and alanine aminotransferase suggest that the observed proteolysis is intended to increase the role of proteins in the energy production during salinity stress.

LDH converts pyruvate to lactate in the presence of coenzyme NADH, which is converted to NAD⁺. Lactate dehydrogenase is the terminal enzyme of the glycolysis pathway. Hence, lactate dehydrogenase helps in maintaining the glycolysis cycle by supplying NAD⁺. In the presence of enough oxygen pyruvate enters the Krebs cycle, but when there is an oxygen shortage in the tissue, pyruvate is converted to lactate. (Murray *et al.* 2000). In the present study, there is no significant difference in the LDH activity in muscle, but the LDH activity in the liver of ISW group fishes have higher activity than the FW group which is due to salinity stress. Generally, LDH activity increases during stress (Vijayaraghavan and Rao, 1986).

The glucose-6 phosphatase activity significantly increased in the liver of ISW group than the FW group. A higher glucose-6 phosphatase activity indicated that the increased formation of free glucose in liver from glycogenolysis and gluconeogenesis pathway, both of which comprise glucose-6 phosphatase as terminal enzyme. The increased glucose-6 –phosphatase of ISW in the present study indicates the enhanced production of glucose to meet increased energy demand during stress, as is clear from the enhanced glucose levels in ISW.

5.1.6. Total ATPase activity

In the present study, it was observed that ATPase activity of liver was increased in the ISW reared fishes. ATPase are suggested to be among the sensitive biomarkers used for the assessment of the membrane fragility of the gills (Stagg *et al.*, 1992). ATPase are the membrane-bound enzymes responsible for the transport of ions through biological membranes and thus regulate, e.g., cellular volume, osmotic pressure, and membrane permeability (Sancho *et al.*, 2003). Na⁺ K⁺-ATPase is found in the basolateral membrane of gill epithelial cells and is involved in the active electrolyte transport across the gills. Mg²⁺-ATPase plays an important role in oxidative phosphorylation and ionic transport (Gerenscer and Lee, 1983). The fish gill H⁺-ATPase has been reported to have a central role in ionic uptake (Evans, 2002). Thus, the most likely explanation of the enhanced ATPase levels in ISW is there may be higher activation of membrane bound channel ATPase such as Na⁺,K⁺-ATPase and Mg²⁺-ATPase due to the ionic alterations in ISW.

5.1.7. Oxidative stress enzymes

Under normal physiological conditions, animal cells produce reactive oxygen species (ROS) such as H₂O₂, O₂⁻ and OH⁻ that may damage most cellular components leading to cell death and the living organisms are protected from reactive oxygen species by several defense mechanisms including antioxidant enzymes. Oxidative stress comes when the antioxidant defences are overcome by pro-oxidant forces and reactive oxygen species are not adequately removed (Sies *et al.*, 1986). Living organisms are protected from the deleterious effects of ROS by several defense mechanisms including antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and non-enzymatic antioxidants such as vitamin E, vitamin C etc. (Birben *et al.*, 2012).

In the present study, increase in superoxide dismutase, catalase and glutathione peroxidase were observed in both gill and liver of ISW reared fishes i.e. there was a net effect of salinity on the activities of antioxidant enzymes. It can be inferred that these enzymes work as antioxidant defense system and protects the cell from ROS, generated due to salinity stress. The stress induced by salinity change has been associated with enhanced ROS generation, causing oxidative damage (Liu *et al.*, 2007). For example, when shrimp *Litopenaeus vannamei* were subjected to acute salinity change, the activities of SOD, catalase, glutathione peroxidase and Na⁺ K⁺-ATPase were enhanced.

GST is a multicomponent enzyme involved in the detoxification of many xenobiotics, which plays an important role in protecting tissues from oxidative stress (Fournier *et al.*, 1992). The GST activity in intestine and liver of the experimental groups were not significantly different, indicating the absence of toxic or xenobiotics in the body. GSH, in addition to being a necessary cofactor for GPx and GST activity, is itself an effective protectant capable of quenching oxyradicals (Ross, 1988). The GSH plays an important role in the detoxification of electrophiles and prevention of cellular oxidative stress (Hasspieler *et al.*, 1994; Sies, 1999). In the present study, the rearing of fishes in ISW showed a significant decrease in the GSH content of brain. This decrease in GSH levels indicates the induction of stress in fishes due to salinity, which caused the oxidation of GSH. During a moderate oxidative stress, the GSH levels can increase as an adaptive mechanism by means of an increased synthesis. However, a severe oxidative stress may suppress GSH levels due to the impairment of the adaptive mechanisms (Zhang *et al.*, 2004). According to Elia *et al.*, (2003) GSH depletion may reduce the cellular ability to scavenge free radicals raising the general oxidative potential in the cells.

5.1.8. Immuno-haematological parameters

5.1.8.1. Serum glucose

Serum glucose of fishes is used as an indicator of stress (Mock and Peters, 1990). The elevation of blood sugar levels in fish by both corticosteroids and catecholamines makes it the ideal parameter to study the secondary stress response on activation of direct sympathetic (chromaffin tissue) as well as humoral (interrenal tissue) pathways (Wedemeyer and Mcleay, 1981). A number of literature is available

on the rise of blood glucose level on exposure to various stresses like handling (Wedemeyer, 1972), thermal stress (Manush *et al.*, 2005). In order to provide energy during stress process, blood glucose would abruptly rise (Barton and Iwama, 1991; Pottinger, 1998; Grutter and Pankhurst, 2000; Rotllant *et al.*, 2003; Hsieh *et al.*, 2003), therefore blood glucose could be considered as an important index of fish under stress (Hsieh *et al.*, 2003). In the present study, blood glucose level varied significantly with a higher value in the fishes reared in inland saline water. This hyperglycemia in ISW group indicates that rearing in ISW induces stress in fish. Stress-induced elevation of plasma cortisol may be due to the activation of HPI axis, which induces the release of corticosteroid hormones. It was reported that cortisol could enhance liver gluconeogenesis and glycogenesis when fish were subjected to suboptimum or stressful conditions (Auperin *et al.* 1997; Barton 2002; Bonga 1997; Laiz-Carrión *et al.* 2002). Thus, plasma glucose levels increased consistent with increased cortisol in this study.

5.1.8.2 Cortisol

The primary stress response in teleost fish includes increases in circulating cortisol (Wendelaar-Bonga, 1997). It is known that stress-induced hormonal responses, such as elevated levels of plasma cortisol and catecholamines, lead to osmotic imbalances in fish exposed to hypertonic and hypotonic environments (Mazeaud and Mazeaud, 1981; Pickering and Pottinger, 1995). In our study the plasma cortisol level in ISW group is significantly higher than FW group which infers that the cortisol is produced during salinity stress and helps in adaptation of fish to salinity. These results are consistent with the study of Luz *et al.* (2008) in gold fish, the plasma cortisol increased with increase in salinity. The rise of hormone level such as cortisol etc. would cause glycogenolysis and release of glucose to blood (Laiz-Carrión *et al.*, 2002).

5.1.8.3 Serum proteins

Among the serum proteins the albumins and globulins are the major proteins, which play an important role in the immune response. The liver is responsible for synthesizing the albumin proteins, which creates an osmotic force that maintains the fluid volume of the vascular space. The serum globulins consist of several components like alpha, beta and gamma. The gamma globulin fraction is the source of the all

immune proteins in the blood. Gamma globulins are absolutely essential for the good immune status of the fish. In the present study, the serum protein, albumin, globulin was analysed. Globulin level was found significantly lower in the ISW group and the A: G ratio was higher in the ISW group which indicated immunosuppression in the fishes reared in ISW.

5.2. Experiment II

5.2.1. Growth performance

In the present study, evaluation of extracted *Mentha arvensis* leaf meal (EMLM) in the diet of *Cyprinus carpio* reared in inland saline water, the experimental groups showed significant difference in percentage weight gain, specific growth and feed conversion ratio. It was observed that control group (0% leaf meal, 30% DORB in diet) have higher PWG, SGR and low FCR than the treatment groups (T1, T2 and T3 with 10%, 20% and 30% leaf meal replacing 33%, 66% and 100% of DORB respectively in the diet). To date, there is no published information available on the incorporation of extracted *Mentha arvensis* leaf meal in the fish diets as a replacer of deoiled rice bran. However, it was found that Ipil-ipil, *Leucaena leucocephala* leaf meal in the diets of *Oreochromis niloticus* at 12.5% inclusion did not affect growth, however, at higher levels of inclusion, 25% or more, the growth of *O. niloticus* was adversely affected (Santiago *et al.*, 1988). The growth and nutrient utilization was decreased with the increase in inclusion of EMLM in the diets. This observation concur with the findings of Richter *et al.* (2003), who showed that higher substitution of *M. oleifera* leaf meal with fish meal had a negative impact on growth performance because of the presence of anti-nutrients such as phenol, tannins, phytates and saponins. Wee and Wang (1987) reported that reduced growth performance and feed utilization efficiency was observed with increase in *Leucaena* leaf meal incorporation. In the present study, the reduced growth in EMLM incorporated diets may be due to the presence of excess crude fibre (20%) and anti-nutritional factors such as phytate, oxalate and alkaloids.

The decrease in the values of specific growth rate (SGR) could be due to difference in the extracted *M. arvensis* leaf meal levels, which decreased at increasing level of EMLM in the diet. Anti-nutritional factors contained in the leaf meal based diet were probably responsible for retarded growth response in the EMLM fed fishes. The

PER values also decreased with the increase in inclusion of EMLM in the diet. These results seem to have direct link with palatability of the diet which causes reduced feed intake (Faturoti, 1989). The FCR was better in control group, and there was a decreasing trend across the treatments. The reason for this present observation could be the presence of high fibre content of EMLM.

5.2.2. Hepatosomatic index and gastrosomatic index

The HSI values of the different experimental groups were found to be significantly not different. There are no parallel literature available with respect to the effect of different level of rice bran replacement with EMLM on the HSI values of the fish. Also, there was no significant difference in the GaSI values of the different experimental groups. From these results, it is possible to conclude that the inclusion EMLM did not affect the gut morphology and liver.

5.2.3. RNA DNA ratio

Quantitative analysis of nucleic acid provides simple and promising tool for analyzing growth rate in fish. RNA directly controls the protein synthesis and it is expected to exhibit an increasing level with subsequent growth of somatic tissues (Bulow 1987). DNA, a genetic information carrier molecule, remains constant in somatic tissue and primarily used as an index of cell number or biomass (Regnault and Luquet, 1974). In the present study, RNA levels and RNA/DNA ratio showed a decline with the increase in EMLM in the diet, and having resemblance with data about the percentage weight gain and SGR. To the best of our knowledge, there is no literature available on effect of deoiled rice bran replacement with EMLM on RNA and RNA/DNA ratio in *Cyprinus carpio* to substantiate our findings.

5.2.4. Digestive enzyme activity

In the present study, a significant decrease in digestive enzyme activities of *Cyprinus carpio* fingerlings were observed. The group fed with higher levels of EMLM had significantly lowered amylase, protease, and lipase activity. In the EMLM fed groups, the proteins will be complexed by phytate, which results in the decreased activation of proteases in intestine, resulting in decreased protease activity. It is reported earlier that phytate forms complexes with minerals (Sugiura, 1999), and proteins (Moyano *et al.*, 1999) exhibited reduced protein digestibility, which reflects

the reduced growth performance of animals. The amylase activity also decreased in the treatment groups which may be due to the lower level of carbohydrate or NFE in the diet (Deshpande and Cheryan, 1984) with increase in EMLM

5.2.5. Enzymes of amino acid metabolism

Transaminases (AST and ALT) are the enzymes that redistribute amino nitrogen among the amino acids, forming new amino acids with the amino group of the pre-existing ones. Both the aspartate and alanine aminotransferase act as a link between carbohydrate and protein metabolism by catalysing the interconversion of compounds like α -ketoglutarate and alanine to pyruvic acid and glutamic acid, respectively (Knox and Greengard, 1965). The pyruvate and oxaloacetate thus formed are responsible for the synthesis of non-essential amino acids like alanine, asparagine and glutamine which in turn helps in protein synthesis and hence the growth of the animal. Apart from this the amino acids are deaminated or transaminated to produce TCA cycle intermediates also helping in energy production in the form of ATP (De Silva and Anderson, 1995). In the present study, AST and ALT activity of muscle and liver are studied and significant differences were observed between the experimental groups. There is no significant difference in the AST activity of liver and ALT activity of muscle. Significant difference was observed in AST (muscle) and ALT (liver) with an increasing trend from control to T3 group, indicating enhanced metabolism of protein in the muscle and liver to produce the TCA cycle intermediates. The excess amino acid catabolism helps to meet the enhanced energy demand caused by the metabolic stress imparted by EMLM inclusion. Therefore, the higher activity of ALT and AST indicates the mobilization of alanine and aspartate via gluconeogenesis for glucose production to cope with stress. Knox and Greengard (1965) and Chatterjee *et al.*, (2006) also reported that elevated level of transaminase activity during stress would lead to increased feeding of keto acids (pyruvate) into TCA cycle, thereby affecting oxidative metabolism.

5.2.6. Lactate dehydrogenase and malate dehydrogenase

LDH converts pyruvate to lactate in the presence of coenzyme NADH, which is converted to NAD⁺. Lactate dehydrogenase is the terminal enzyme of the glycolysis pathway. Hence, lactate dehydrogenase helps in maintaining the glycolysis cycle by supplying NAD⁺. In the presence of enough oxygen pyruvate enters the Krebs

cycle, but when there is an oxygen shortage in the tissue, pyruvate is converted to lactate. (Murray *et al.*, 2000). The reversible conversion of oxaloacetate and malate is catalysed by malate dehydrogenase (MDH), an enzyme present in both mitochondria and cytosol. The level of LDH and MDH enzymes increases under different stress conditions like high temperature (Gupta *et al.*, 2010) and high density transportation (Chatterjee *et al.*, 2006) starvation stress (Vijayaraghavan and Rao, 1986). In the present study, the LDH activity in muscle and liver significantly increased in the EMLM fed groups, which may be due the anti-nutritional factors present in the EMLM. Whenever the organism encounters toxic substances, liver cells try to detoxify it and excrete it. Exposure to toxin increased the activity of aryl hydrocarbon hydroxylase (AHH), a monooxygenase enzyme system in animals which helps to detoxify the toxic hydrocarbon chemical which requires molecular oxygen (Raunio and Pelkonen, 1983). This detoxification system compete for oxygen with the electron transport chain (ETC), which may lead to reduced NAD⁺ availability from ETC. The low level of NAD⁺ can lead to LDH activity in liver. In the present study, no significance difference was observed in MDH activity of muscle and liver. The MDH activity is a direct indication of Kreb cycle operation, which seems to be constant irrespective of the EMLM inclusion. So far, the significance of this finding is not clear.

5.2.7. ATPase activity

ATPase hydrolyses the high-energy phosphate (ATP) and utilizes that energy to maintain ionic gradient across the plasma membrane (Das, 2002). Adenosine triphosphatases (ATPases) are a group of enzymes which play an important role in intracellular functions and critical for cellular viability because they control many essential cellular functions, and are considered to be a sensitive indicator of toxicity. Na⁺K⁺-ATPase pumps 3Na⁺ and 2K⁺ ions against their concentration gradient through proper function; maintains transmembrane gradients for the ions, and produces a convenient driving force for the secondary transport of metabolic substrates such as amino acids and glucose (Koksoy, 2002). In the present study, the ATPase activity significantly decreased at higher inclusion levels of EMLM in the diet which may be due to the presence of phytate in the diet. Phytic acid has a strong ability to chelate multivalent metal ions, specially zinc, calcium and iron. The binding can result in very insoluble salts with poor bioavailability of minerals (Zhou and Erdman,

1995). The increase in phytic acid in the diet reduces the ATPase activity due to decreased availability of divalent cations (Zishan *et al.*, 2016).

5.2.8. Glucose-6 phosphatase activity

Glucose-6 phosphatase is an enzyme that hydrolyses glucose-6 phosphate resulting in the formation of a phosphate group and free glucose. The glucose is then transported from cell via glucose transporter proteins. The Glucose-6 phosphatase catalysis completes the final step in gluconeogenesis and therefore plays a key role in homeostatic regulation of glucose levels. In the present study, the Glucose-6 phosphatase activity significantly decreased with the inclusion of EMLM in the diet. The glucose levels in serum is in concurrent with the glucose-6 phosphatase enzyme activity.

5.2.8.1. Oxidative stress enzymes

Fishes possess defence mechanisms against free radical-induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) etc. Non-enzymatic anti-oxidants are ascorbic acid (vitamin C), α -tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, etc. SOD catalyses the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. Subsequently, catalase (CAT) activity decomposes this hydrogen peroxide into oxygen and water and this constitutes a crucial part of the cellular antioxidant defence mechanism. In general, stress causes oxidative damage to cells, which overcomes that effect through the increased activity of anti-oxidative enzymes.

In the present study the SOD activity had decreased significantly with the increasing level of dietary EMLM. This indicates that the free radicals were effectively scavenged by the multiple compounds present in EMLM. It may be due to the presence of phenolic compounds, flavonoid and tannins, which act as a powerful antioxidant and had been shown to scavenge free radicals and protect the lipid membrane and other compounds being oxidized or destroyed (Popeskovic *et al.*, 1980). The catalase activity in gill and liver of different experimental groups were analysed, it was observed that the control group had significantly higher activity than

the treatment groups. The catalase activity decreased with increase in inclusion of EMLM in the diet but it was not significantly different.

GPx catalyse the reduction of H₂O₂ and lipid peroxides (Uner *et al.*, 2006). In the present study, the GPx activity in gill and liver among different experimental groups did not vary significantly. This indicate the no effect of the EMLM on the GPx activity and the antioxidant activity of GPx was not hampered due to EMLM inclusion in the diet.

The decrease in the SOD and Catalase activity can be supported by the study of Lygren *et al.* (1999) and Gopan *et al.* (2018) who reported that when there are high levels of fat-soluble antioxidants, such as astaxanthin and vitamin E, there is a reduced need for endogenous antioxidants, such as catalase and SOD. Similar results also have been found in the body tissue as well as plasma of ornamental fish Characins (Wang *et al.*, 2006) and farmed fish juvenile olive flounder *Paralichthys olivaceous* (Pham *et al.*, 2014) where the antioxidant enzyme activity was decreased with increase in dietary antioxidant like astaxanthin. The antioxidant and antimicrobial properties of *Mentha* species was reported by Yadegarinia *et al.* (2006). Hence this findings may suggest that the antioxidants present in the EMLM helped in scavenging the free radicals and ROS and due to which the activity of SOD and catalase was less.

5.2.8.2. Glutathione-S-transferase activity

The major role of the GST is a cellular defense against chemically induced toxicity (Blanchette *et al.*, 2007). According to Hisar *et al.* (2012) higher GST activities in liver and muscle signifies the enhanced antioxidant protection of fish which supports the present findings of increased level of GST with the increased level of the EMLM. These findings suggest that EMLM in diet provided enhanced antioxidant protection in fish.

5.2.8.3. Non enzymatic antioxidant assay

Reduced glutathione (GSH) is an indicator of antioxidant status, as it will be utilized for neutralising harmful free radicals by the natural antioxidant defences in the body (Urso and Clarkson, 2003). Thiol groups act as intracellular antioxidants by scavenging free radicals and through enzymatic reactions. The water soluble thiol group protects biological membrane (Moscio *et al.*, 1994). In the present study, there

was no significant difference in GSH content in brain and liver of the experimental groups but a slight increasing trend is seen. The increased GSH in EMLM, fed fish compared with the control indicates better antioxidant status. Moreover, the increased concentration of thiol groups could explain the reduced lipid peroxidation, as these are known to play greater protective roles in preventing lipid peroxidation of membranes.

5.2.8.3. Hemato- immunological parameters

Haematological investigations have proven valuable for fisheries biologists for quick detection of changes in fish health and these changes may precede changes in fish behaviour and visible lesions. Haematological components of blood are also valuable in monitoring feed toxicity especially with feed constituents that affect the formation of blood in culture fisheries (Oyawoye and Ogunkunle, 1998).

5.2.8.3.1. Serum glucose

Glucose is the end product of carbohydrate digestion and is the primary source of energy for the body. Blood glucose is one of the most commonly measured indicators of stress response in fish due to its low cost and easy measurement (Wedemeyer, 1990). In the present study, blood glucose level varied significantly among different experimental groups. The inclusion of EMLM beyond 20%, caused a decrease in blood glucose level. These results indicated the stress mitigation property of extracted *Mentha arvensis* leaf meal. Our results are consistent with the reports which demonstrated the use of *Mentha piperita* as supplemented diet can cause the decrease of glucose and triglyceride in common carp and Asian seabass (Heidarieh *et al.*, 2013; Abasali and Mohamad, 2010). The important implication of these finding is that the glucose levels maintained at minimal levels in spite of the increase in amino acid metabolism. The reason for the discrepancy could be due to the low energy requirement for antioxidant defence due to the presence of intrinsic antioxidant compounds in *Mentha*.

5.2.8.3.2. Cortisol

One of the main fish responses to adverse situations is the production of catecholamines and corticosteroids; these are responsible for physiological and biochemical changes and are usually characterized as stress responses (Barton and Iwama, 1991). The secretion of cortisol is dependent on the severity and the precise

nature of the stress that is applied (Sumpter *et al.*, 1985). The cortisol level is significantly lower in the treatment groups than the control due to the stress alleviating property of EMLM.

5.2.8.3.2. Creatinine

Serum creatinine levels were increased with increase of EMLM inclusion in *Cyprinus carpio*. Creatinine is a breakdown product of creatine phosphate in muscle, and is usually produced at a fairly constant rate. Any increase in creatinine level indicate renal dysfunction, i.e. failure to excrete the creatinine through urine causes its increase in the serum. Here, increased level of creatinine due to EMLM feeding beyond 20% indicated that kidneys are affected due to feeding EMLM.

5.2.8.3.2. Serum proteins

Among the serum proteins the albumins and globulins are the major proteins. The liver is responsible for synthesizing the albumin proteins, which creates an osmotic force that maintains the fluid volume of the vascular space. Globulin contents are recognised prime factors for retaining a healthy immune system and immune functions in the blood and are well known to reflect strong innate immunity (Nya and Austin, 2009). The serum globulins consist of several components like alpha, beta and gamma. The gamma globulin fraction is the source of the all immune proteins in the blood. Gamma globulins are absolutely essential for the good immune status of the fish. Albumin is the most abundant protein in the blood plasma of vertebrates and functions as a carrier for a variety of nutrients, metabolites and xenobiotics. Increase in the total protein, albumin and globulin levels is thought to be associated with a stronger innate response in fish (Wiegertjes *et al.*, 1996). In the present study there is no significant difference observed in the total protein and albumin level but the globulin level and albumin: globulin ratio varied significantly. The globulin level significantly increased with the increase in inclusion level of EMLM in the experimental diets. The A: G ratio decreased with increase in EMLM inclusion. The A: G ratio was decreased due to the increase of globulin level. The results of the present study are in well agreement with the findings of Abasali and Mohamad (2010) who found an increase globulin contents in *C. carpio* after feeding with extract of *M. piperita*. Similarly, the study of Talpur *et al.* (2014) in *Lates calcarifer* also revealed that feeding *M. peperita* decreases AG ratios. In conclusion, it is evident that higher inclusion of Mentha lead

to increased immune response, which may be due to the presence of flavonoids in the EMLM.

5.2.8.3.2. Serum lipid profile

Excess food calories are converted to triglycerides and stored by the body for later use. Triglycerides are needed to supply energy in the fasting state or when there is a deficiency in supply of energy through other nutrients (Swinburn Ravussin, 1993). Serum triglycerides and cholesterol levels are indicative of energy requirement and mobilized energy stores. In the present study, the cholesterol and triglycerides level varied significantly. The inclusion of EMLM beyond 20% decreased the level of cholesterol and triglycerides in the blood. The present study revealed that feeding EMLM to *C. carpio* led to decreased triglycerides and cholesterol content, which is in concurrence with the findings of Talpur and Ikhwanuddin (2012, 2013), who reported that feeding of garlic and neem to *L. calcarifer* reduced triglycerides content. In addition, it is also revealed that the use of *Mentha piperita* in the diet can cause the decrease of glucose and triglyceride in common carp and Asian seabass (Heidarieh *et al.*, 2013; Abasali and Mohamad, 2010).

5.2.8.3.3. Haematology

Haematological components of blood are valuable in monitoring feed toxicity especially with feed constituents that affect the formation of blood (Oyawoye and Ogunkunle, 1998). All the haematological parameters measured in this study were within the recommended physiological ranges reported for *Cyprinus carpio*. The haemoglobin, RBC and WBC content varied significantly whereas in platelet count there was no significant difference observed in the experimental groups fed with EMLM in the diet. The effects of peppermint extracts on immune cells and blood biochemical parameters of broilers (Fallah *et al.*, 2013) and humans (Cosentino *et al.*, 2009) have been evaluated and they affect the functional responses of granulocytes. In the present study, red and white blood cells, haemoglobin and hematocrit were also increased with the increasing inclusion level of EMLM in the diets being the highest increments recorded in fish fed the highest EMLM.

6. SUMMARY

Aquaculture in India, is a progressively growing sector with varied resources and water availability is a limiting factor in aquaculture production. At present scenario of global warming and climatic change, shortage of water made the future of aquaculture unpredictable in many parts of the world. On other hand, land salinization is a growing problem, presenting great threat to the agriculture sector. In this situation, the farmers need to be educated about making economic use of saline water and land areas, and it's possible by adopting inland saline aquaculture. Therefore, there is a vital need for exploring the productivity of inland ground saline water and suitability of species to be farmed in such waters. Inland ground saline water is being successfully used to grow marine fishes, shrimps etc. and many other organisms. The huge demand for fish has put strains on aquaculture industry which relates to resources of aqua feed formulation. The intensification of aquaculture practices with farming priorities has shifted to formulate feeds based culture. Ultimately the feed based aquaculture has lead more demand and high price of almost all feed ingredients. Plant based sources are widely available, renewable and many are already being used for aqua feeds. The most commonly used ingredient is ricebran; however, a shortage in its availability in future is foreseen. The long-term availability of the plant based ingredients can be ensured by exploring plentifully available cheap sources, such as leafmeals. With these backdrops, the present study aimed to find a plentifully available alternative for ricebran and explore the inland saline water for fish rearing alongside.

Experiment 1

In the present study, a 60 days trial was conducted with two experimental groups (FW & ISW) in four replicates and fed with normal diet containing 30% protein in order to evaluate the stress response and growth of *Cyprinus carpio* in inland saline water (ISW). At the end of the feeding trail, the experimental fishes were subjected to analysis of growth, metabolic and stress parameters. The metabolic enzymes include amino-transferases, LDH and MDH and stress parameters viz. SOD, CAT, GPx, GSH and other haematological parameters. The results of the study revealed that the growth parameters percentage weight gain, specific growth rate and protein efficieny

ratio were negatively affected due to rearing of fishes in ISW. There was a reduction in growth as revealed by a decrease in the percentage weight gain, SGR and PER for the fishes reared in ISW. The FCR was also affected negatively it was increased in the fishes reared in ISW. Significant difference was observed in the digestive enzyme activity of the fishes with higher activity in the ISW group. Among the metabolic enzymes the AST, ALT activity in both muscle and liver were significantly higher in the ISW group. LDH activity was not significant in muscle but in liver the activity was higher in ISW. MDH enzyme activity in muscle and liver were not significant between the experimental groups. The ATPase and Glucose-6 phosphatase activity in liver increased in the ISW compared to FW group. The metabolic enzyme activities were comparatively higher in fishes reared in ISW indicating higher energy cost for osmoregulation and body maintenance. The activity of oxidative stress enzymes (SOD and Catalase) in both gill and liver was significantly higher in the fishes reared in ISW than the fishes reared in FW. The GPx and GST enzyme activities were not significantly different between the experimental groups. Significant difference was observed in the GSH content in brain and liver with a lower content in the ISW. The increased activity of stress enzymes infers higher production of free radicals due to salinity stress. The blood glucose and cortisol levels were significantly higher in the fishes reared in ISW. The serum cholesterol and triglycerides level were also found to be significantly higher in ISW group. The serum total protein did not vary significantly but albumin, globulin and A: G ratio significantly varied. The higher A: G ratio in ISW group indicates the immunosuppression in the fishes reared in inland saline water. Summing up the results, it can be concluded that there is a 10% reduction of growth in 5 ppt ISW. In addition, rearing in ISW has potentiated stress responses and impaired immunity of *C. carpio*.

Experiment 2

A growth trial of 60 days was conducted exclusively in ISW with 4 experimental groups, each with 0% EMLM and 30% DORB in the diet (control), EMLM replacing 33% DORB (T1), 66% DORB (T2), 100% DORB (T3). And, each group have contained EMLM at different levels, such as 0%, 10%, 20% and 30% and the diets were isonitrogenous and isocaloric containing 30% protein. The fishes were fed with different experimental diets twice daily and they were reared in ISW of salinity 5ppt without any fortification with minerals. Different response parameters were evaluated

to understand the stress response and growth of *Cyprinus carpio* fed with EMLM and reared in inland saline water (ISW). It was found that EMLM had a good protein content (18.64%); however, the in vitro digestibility of EMLM was 32.37% only. This low digestibility can be attributed to the presence of crude fibre (20.83%) and several antinutritional factors viz. tannins (1.26%), total oxalate (0.032%), phytic acid (4.43%) and alkaloid (0.68%). Many of the antinutritional factors such as phytate and tannins can bind to protein and reduce the digestibility.

The growth parameters like percentage weight gain, specific growth rate, protein efficiency ratio and FCR varied significantly among the experimental groups. The replacement of DORB with EMLM in ISW had a negative effect on growth and food conversion ratio. There was no significant difference observed in the body indices viz. HSI and GaSI which indicated that there was no changes in the intestine and liver of the fishes due to inclusion of EMLM. The DNA level was not significantly different but the RNA level and RNA/DNA ratio in the muscle of fishes significantly reduced at higher inclusion levels of EMLM in diet. The digestive enzyme activity was also significantly decreased in the fishes at higher inclusion levels. The AST activity in muscle and ALT activity in liver significantly increased indicating metabolic stress in the fishes. The LDH activity in the muscle also increased significantly which may be due to anaerobic production of energy in the fishes to meet the energy demand. The ATPase and Glucose-6-phosphatase activity significantly decreased among the experimental groups. The antioxidant stress enzymes (SOD, CAT) activity significantly decreased among the treatment groups compared to control, which may be due to the free radical scavenging activity of EMLM. The GSH content of brain and liver showed no significant difference among the experimental groups. At higher inclusion levels of EMLM in diet, the cortisol and blood glucose level decreased which indicates the stress relieving property of EMLM. There was no significant difference observed in serum protein and albumin level. However, the globulin level increased in the EMLM fed groups and A/G ratio decreased significantly with EMLM inclusion indicating the immuno-stimulatory property of EMLM.

Overall, it can be concluded that the EMLM cannot replace rice bran in the diet of *Cyprinus carpio* reared in ISW. However, it could be possible that the stress due to rearing of fishes in 5 ppt and the ionic imbalance might have reduced their tolerance to EMLM. Thus, further studies could be undertaken with EMLM in

freshwater to enhance the utilisation of this plentifully available leafmeal. As there is a decrease in digestibility and digestive enzyme activities, the supplementation of exogenous enzymes may help to increase the utilisation of EMLM. In addition, the stress ameliorating and immune stimulating properties of EMLM could be considered while formulating feed including EMLM.

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APPENDIX

ABBREIVATION:

μg	Microgram
μl	Microliter
μM	Micromole
ANOVA	Analysis Of Variance
AST	Aspartate Amino Transferase
BHT	Butylated Hydroxytoluene
CAT	Catalase
CF	Crude Fiber
CMC	Carboxymethyl Cellulose
CP	Crude Protein
CRD	Completely Randomized Design
DE	Digestible Energy
DM	Dry Matter
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DORB	Deoiled Rice Bran
EDTA	Ehtylenedye Tetra Acetic Acid
EE	Ether Extract
EMLM	Extracted <i>Mentha Arvensis</i> Leaf Meal
FCR	Feed Conversion Ratio
FER	Feed Efficiency Ratio
FW	Fresh Water
g	Gram
GaSI	Gastrosomatic Index
GDP	Gross Domestic Product
GE	Gross Energy
GOT	Glutamate Oxaloacetate Transaminase
GPT	Glutamate Pyruvate Transaminase
GPx	Glutathione Peroxidase

GSH	Reduced Glutathione
GST	Glutathione-S Transferase
Hb	Hemoglobin
HSI	Hepatosomatic Index
IMC	Indian Major Carp
ISW	Inland Saline Water
Kcal	Kilo Calorie
LDH	Lactate Dehydrogenase
LM	Leaf Meal
MDH	Malate Dehydrogenase
Mg	Milligram
mha	Million Hectares
MI	Millilitre
n mole	Nano Mole
ND	Not Detected
NDF	Neutral Detergent Fiber
NFE	Nitrogen Free Extract
PER	Protein Efficiency Ratio
Ppm	Parts Per Million
PWG	Percentage Weight Gain
RBC	Red Blood Cells
RNA	Ribonucleic Acid
RNA	Ribonucleic Acid
RPD	Relative Protein Digestibility
Rpm	Revolutions Per Minute
SE	Standard Error
SGR	Specific Growth Rate
SOD	Superoxide Dismutase
TA	Total Ash
TC	Total Carbohydrate
TL	Total Lipid
WBC	White Blood Cells