SYNERGETIC EFFECTS OF NETTLE (*Urtica parviflora* roxb) POWDER WITH MULTIENZYME ON GROWTH PERFORMANCE OF RAINBOW TROUT (*Onchorhynuss mykiss*, WALLABAUM, 1792)

Soniya Maharjan

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Nepal

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DECLARATION

I hereby declare that the work presented in this thesis has been done by myself, and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the authors.

Date 7/12/2077

Signature

Soniya Maharjan

त्रिभुवन विश्वविद्यालय
TRIBHUVAN UNIVERSITY
प्राणी शास्त्र केन्द्रीय विभाग
CENTRAL DEPARTMENT OF ZOOLOGY
कीर्तिपुर, काठमाडौं, नेपाल
Kirtipur, Kathmandu, Nepal
फोन नं. ०१-४३३१८१६
01-4331896
Email: info@cdztu.edu.np
URL: www.cdztu.edu.np

पत्र संख्या :-
च.नं. Ref.No.:-

Central Department of Zoology T.U., Kirtipur

RECOMMENDATIONS

This is to recommend that the thesis entitled "Synergetic effects of nettle (*Urtica parviflora roxb*) powder with multienzyme on growth performance of rainbow trout (*Onchorhynuss mykiss*, Wallabaum, 1792) has been carried out by Soniya Maharjan for the partial fulfilment of Master's Degree of Science in Zoology with special paper Fish Biology and Aquaculture

This is her original work and has been carried out under my supervision. To the best of my knowledge, this thesis work has not been submitted for any other degree in any institutions.

Date: 11/12/2077

(Signature)

Assoc. Prof. Dr. Archana Prasad

Central Department of Zoology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

त्रिभुवन विश्वविद्यालय
TRIBHUVAN UNIVERSITY
प्राणी शास्त्र केन्द्रीय विभाग
CENTRAL DEPARTMENT OF ZOOLOGY
कीर्तिपुर, काठमाडौं, नेपाल ।
Kirtipur, Kathmandu, Nepal
फोन ०१-४३३१८९६
01-4331896
Email: info@cdztu.edu.np
URL: www.cdztu.edu.np

पत्र संख्या :-
च.नं. Ref.No.:-

Central Department of Zoology T.U., Kirtipur

LETTER OF APPROVAL

On the recommendation of supervisor "Assoc. Prof. Dr. Archana Prasad" this thesis submitted by Miss Soniya Maharjan entitled "Synergetic effects of nettle (*Urtica parviflora roxb*) powder with multienzyme on growth performance of rainbow trout (*Onchorhynuss mykiss*, Wallabaum, 1792)" is approved for the examination in partial fulfilment of the requirements for Master's Degree of Science in Zoology with special paper

त्रिभुवन विश्वविद्यालय
TRIBHUVAN UNIVERSITY
प्राणी शास्त्र केन्द्रीय विभाग
CENTRAL DEPARTMENT OF ZOOLOGY
कीर्तिपुर, काठमाडौं, नेपाल।
Kirtipur, Kathmandu, Nepal
फोन ०१-४३३१८९६
01-4331896
Email: info@cdztu.edu.np
URL: www.cdztu.edu.np

पत्र संख्या :-
च.नं. Ref.No.:-

Central Department of Zoology T.U., Kirtipur

CERTIFICATE OF ACCEPTANCE

This thesis work submitted by entitled "Synergetic effects of nettle (*Urtica parviflora roxb*) powder with multienzyme on growth performance of rainbow trout (*Onchorhynuss mykiss*, Wallabaum, 1792)" has been accepted as a partial fulfilment for the requirements of Master's Degree of Science in Zoology with special paper Fish biology and aquaculture

EVALUATION COMMITTEE

Supervisor

Assoc. Prof. Dr. Archana Prasad
Central Department of Zoology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Head of Department

Prof. Dr. Tej Bahadur Thapa
Central Department of Zoology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

External examiner

Mr. Dharani Man Singh

Internal examiner

Prof. Dr. Kumar Sapkota
Central Department of Zoology
Tribhuwan University
Kirtipur, Kathmandu, Nepal

Date of examination: July 7, 2021

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Sincerely,

Soniya Maharjan

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LIST OF ABBREVIATIONS

Abbreviated form	Details of Abbreviations
HUFA	Highly Unsaturated Fatty Acid
PUFA	Polyunsaturated fatty acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
FAO	Food and Agriculture Organization
ADB	Asian Development Bank
UNDP	United Nations Development Program
NFRC	National fisheries research center
FRS	Fisheries research station
Cal/gm	Calories/gram
ppm	parts per million
SR	Survival Rate
SGR	Specific Growth Rate
CF	Conditioning Factor
FCR	Feed Conversion Ratio
PER	Protein Efficiency Ratio
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
DMRT	Duncan's Multiple Range Tests
SPSS	Statistical Package for Social Sciences
EIFAC	European Inland Fisheries Advisory Commission
OECD	Organization for Economic Co-operation and Development
AOAC	Association of Official Agricultural Chemists

ABSTRACT

The use of medicinal plants such as nettle can be safer, less toxic and ensure biosecurity in comparison to the chemical products. But plant or its products can contain anti-nutritional factors which limit nutrition digestibility and fish growth. Thus, exogenous multienzymes were added on aquafeed to decrease anti-nutritional factors and ensure availability of digestible nutrients. So, this study was conducted to evaluate the use of nettle powder as feed additive along with multienzyme on growth performance and survival rate of rainbow trout. Five isonitrogenous diet (35% CP) i.e., Control diet with 0.05 gm/kg multienzyme, control diet without multienzyme, diet with 1.5% nettle powder and 0.05 gm/kg multienzyme; diet with 3% nettle powder and 0.05 gm/kg multienzyme and diet with 5% nettle powder and 0.05 gm/kg multienzyme were formulated. The nettle supplementation enhanced fish growth over the control diet; the highest fish growth and better feed utilization was obtained when fish fed on a diet containing diet with 1.5 % nettle powder. There were no significant changes in fish survival among the different treatments and its range was 98.4–100% suggesting that nettle had no toxic effect.

1. Introduction

1.1 Background of the study

Fish make an irreplaceable contribution to food and nutrition security in many Asian and African countries where large numbers of people are poor and undernourished (Kent 1987). Fish are a rich source of high-quality digestible protein (Tacon and Metian 2013), key source of vital micronutrients required in trace amounts for normal growth and development such as calcium, vitamin A, iron, and zinc (Kawarazuka and Béné 2011) and excellent dietary sources of highly unsaturated fatty acid (HUFA) and polyunsaturated fatty acid (PUFA) especially the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (USDA 2015). With increasing human population, food fish consumption also increased from 9.0 kg (live weight) in 1961 to 20.3 kg in 2017 and also fish accounted for about 17 percent of total animal protein, and 7 percent of all proteins, consumed globally in 2017 (FAO 2020).

Human relationship with fish has long history from prehistoric times when ancestors capture fish from river, lakes and ocean to consume and later to exchange with valuable goods and services (Pitcher and Lam 2015). With development of advanced fishing technology (gears), wild fish were harvested from natural aquatic systems beyond the regenerating capacity of fish which results overexploitation and depletion of ecologically and economically important wild fish stocks (Hilborn et al. 2015). Approximately 90% of global fishery resources were either overfished or fished to maximum capacity by 2013 which shows global capture fisheries have been under tremendous fishing pressures (FAO 2016). Therefore, Sustainable supply of fish from capture fisheries will not be able to meet the increasing global demand for aquatic food (Subasinghe et al. 2009). Due to these trends in capture fisheries, alternative source of fish production i.e., aquaculture should be more focused to expand fish supply, improve global food security, increase economic activity, reduce fishing pressures and maintain fish conservation and management (OECD 2010). Although anthropogenic activities are involved in aquaculture, the ecological threats caused due to aquaculture is much lower than continuing supply of majority of fish from wild capture (Tidwell and Alan 2001). Ecologically speaking, aquaculture is expected to have significant potential for developing sustainable and secure food systems (Troell et al. 2014).

Global aquaculture production has increased dramatically over the past few decades to around 82.1 million tons (114.5 million tons including aquatic plants) in live weight in 2018 worth USD 250 billion (263.6 billion including aquatic plants) accounting for 46 percent of the total fish production and 52 percent of fish for human consumption (FAO 2020). At the global level, since 2016, aquaculture has been the main source of fish available for human consumption as aquaculture has expanded fish availability to regions and countries with limited or no access to the capture fish species, often at cheaper prices, leading to improved nutrition and food security (FAO 2020).

1.1.1 Rainbow trout (*Oncorhynuss mykiss*)

Rainbow trout (*O. mykiss*), salmonid fish species belonging to the family Salmonidae and order salmoniformes was originally named by Walbaum in 1792 (Wikipedia 2020). Previously, one of specimen of Rainbow trout was named *Salmo gairdneri*, but the genus name was changed from *Salmo* to *Oncorhynchus* in 1992 as DNA studies revealed that rainbow trout is genetically closer to Pacific salmon (*Onchorhynchus* species) than to brown trout (*Salmo trutta*), Atlantic salmon (*Salmo gairdneri* as the species name as *Onchorhynuss gairdneri* is biological name for Kamchatka trout which is different fish species than rainbow trout (Gall and Crandell 1992). Therefore, rainbow trout was renamed with *Oncorhynchus* as genus which refers to "hooked nose", and *mykiss* as species which refers to a Siberian word for the species (Behnke and Williams 2007).

Rainbow trout is anadromous fish species native to cold water tributaries of Pacific Ocean in Asia and North America which has been domesticated and introduced worldwide to gain profit through commercial aquaculture (Parisi 2013).

Rainbow trout *(O. mykiss)* is a fast-growing fish which is capable of occupying many different habitats such as gravel-bottomed, fast-flowing, well oxygenated rivers and streams, cold headwaters, creeks, and lakes tolerating a wide range of environment and temperature (0-27˚C) (Coombs 1999). The optimum water temperature for rainbow trout culture is below 21 ˚C as it cannot survive in water reaching summer temperatures above 25˚C with very low oxygen concentrations and favourable temperature range for spawning and growth is between 9˚C to 14˚C (Hardy 2000). The range of optimal pH is narrow for culture of rainbow trout i.e., 6.5-8. Salmonids like rainbow trout have the more dissolved oxygen demand as their optimum dissolved oxygen requirements is 8–

10 ppm, and if the level declines below 3 mg per litre they begin to show signs of suffocation (Svobodova 1993). In natural condition, rainbow trout feeds on aquatic and terrestrial insects, molluscs, crustaceans, fish eggs, minnows, freshwater shrimps and other small fish and aquaculture feeds for rainbow trout mimic the carnivorous diet that wild rainbow trout consume with 35 - 40% animal protein especially derived from fish meal and also aqua feed supplied for grower rainbow trout must contain 12% ash, minimum 3.58 cal/gram digestible energy, maximum 10% moisture and maximum 21% crude lipid (Woynarovich et al. 2011).

Due to great taste and attractive culinary properties, rainbow trout have high sales value (upto USD 10.20 per kilogram) and demand than warm water fish species (Bhandari and Parajuli 2016). Generally, consumers, hotels and restaurants directly visit rainbow trout farms to get fresh rainbow trout while some of farms have also established rainbow trout restaurants and food courts as supporting business (Bhandari and Parajuli 2016).

1.1.4 Growth promoters used in aquaculture

Growth promoters are mainly used in farmed animals with the purpose to promote growth, improve distribution of fat and protein and increase the feed to muscle conversion rate (Toldra and Reig 2016). The most commonly used approach in aquaculture for enhancing efficacy of feed, growth performance and fish culture productivity is chemotherapy i.e., the use of chemical-based hormones, antibiotics and other chemical products (Makkar et al. 2007). These chemical products can have residual effects in the fish muscles which can develop carcinogenic antimicrobial resistant strains (Ahmad and Abdel-Tawwab 2010) as well as emissions of chemicals used in the aquaculture directly or indirectly exploits environmental health (Pullin et al. 2007). Since the European Union has banned in the use of all sub-therapeutic antibiotics in (Regulation 1831/2003/EC), intensified efforts were made to identify and develop safe dietary supplements and additives for aquafeed that can increase the growth performance of farmed fish by enhancing their life activity, health, immune system and gut microbiota (Shim et al. 2009).

Functional feed additives such as probiotics, prebiotics, phytogenic (phytochemical) substances, exogenous enzymes and organic acids have been developed for aquafeeds to combat threats due to use of antibiotics and chemical products (Encarnação 2016).

These functional feed additives develop emerging new pattern of fish growth by enhancing health status of digestive system and its functionality as feed transformation into biomass gain begins in the digestive system of the animal (Encarnação 2016) as well as modulating the immune system of fish to produce disease resistant fish (Denev 2008). Although dietary probiotics and prebiotics can promote the growth of fish, they can be responsible for some adverse impacts such as gene transfer, excessive immune stimulation, systemic infections and deleterious metabolic action in susceptible fish and host (Amenyogbe et al. 2019). The dietary supplementation of probiotics and prebiotics in aquafeeds also lead to extra costly expenditure in feed production as expensive methodologies were used by the industries in production of prebiotics and probiotics for the harmless and excellence products (Ayisi et al. 2017). However, phytogenic feed additives derived from plants are safe to use, locally available, cost effective which provide valid alternative to synthetics in aquaculture (Chakraborty et al. 2013).

1.1.5 Plants as growth promoter in aquaculture

Phytochemicals are a large group of plant-derived compounds that are commonly found in fruits, vegetables, beans, cereals and plant-based beverages such as tea and wine (Arts and Hollman 2005). The phytogenic or phytochemical feed additives possess active components, secondary metabolites of medicinal plants known as phytobiotics or botanicals in such a way that they have positive impacts on animal health and performance (Ghazaghi et al. 2014). Traditional medicinal herbs rich in phytochemicals were the most acceptable as possible alternatives to antibiotic growth promoters because of their natural property, growth-promoting, and anti-oxidative effects (Liu et al. 2006). Phytochemicals, in the form of herbal biomedicine, has a long history mainly in oriental countries (Ji et al. 2009). However, the intensification of animal production and high productivity due to use of antibiotics led to a decline in their usage and less interest in providing scientific bases to their effects but the adverse effects of using antibiotics and other synthetic compounds on product quality and safety have rejuvenated interest in the fields of phytochemistry, phytopharmacology, phytomedicine and phytotherapy during the last decade (Makkar 2007).

Generally, phytochemicals have been classified into six major categories i.e., carbohydrate, lipids, phenolics, terpenoids and alkaloids, and other nitrogen-containing compounds based on their chemical structures and characteristics (Harborne and Baxter

1993). Phytogenic or phytochemical feed additives are a relatively young class of feed additives that are gaining interest within the aquaculture industry although they are quite popular in swine, poultry, cosmetic, and pharmaceutical industries (Encarnação 2016). Plants consist of several biological compounds especially alkaloids, flavonoids, phenolics, terpenoids, steroids and essential oils that have made them attractive for use as growth promoters in fish production with proliferation of gut flora that stimulates appetite and muscle conversion rate maintaining health status (Chakraborty et al. 2013) through antimicrobial, antioxidant, anti-stress and nutrigenomic effects on the development of immunity (Citarasu 2010). Plants can be used as feed additives in several forms either as crude or extract or its isolated active component but using crude plants has the advantage of little effort being made to obtain and apply it and develop practical diet especially for fish farmers (Awad and Awaad 2017). Therefore, Supplementation of natural plant products is considered as a promising preventive practice which assists in maintaining fish welfare, and a healthy environment (Pohlenz and Gatlin 2014).

1.1.6 *Urtica parviflora* as phytochemical feed additives in aquaculture

Urtica parviflora commonly known as Sishnu (Nepali) and Himalayan stinging nettle (English) which belongs to the family Urticaceae is a herb consisting of long stoloniferous rhizomes found in forests and among taller herbaceous vegetation, mainly found in the Nepal, Bhutan, Western China and India (Kumar et al. 2017). *U. parviflora* is used as a leaf vegetable, primarily in soups, vegetable pies, and salads and also traditional veterinary medicine for livestock (Barman et al. 2015). Despite the use of nettle in food and folk. veterinary medicine is well documented, it is today an underestimated and frequently neglected plant considered as a weed to be eliminated in agriculture (Vico et al. 2018).

Nettle is a good source of polyunsaturated fatty acids (60%) whose 50% corresponds to linoleic acid (C18:2), an omega-6 and it can strengthen the immune system, body resistance against bacterial or viral infections and antioxidant activity for fish (Rutto et al. 2013). *Urtica parviflora* also contains bioactive components such as histamine, serotonin (5-hydroxytryptamine), malic acid, aspartic acid, serine, tyrosine and tryptophan and acetylcholine (kumar et al. 2017) and most of these bioactive components can act as smooth muscle stimulants (Oliver 1991). Nettle plants also

possess high concentrations of minerals such as Magnesium (Mg), Calcium (Ca), Copper (Cu), Manganese (Mn) and Cobalt (Co) (Rafazlovska et al. 2013) along with vitamins A, B Complexes, C, D, E, F, K and P (Rutto et al. 2013). The phytochemical screening of *U. parviflora* revealed the presence of biochemical constituents' alkaloids, polysaccharides, saponins, flavonoids, glycosides, phenolic compounds, carotenoids and tannins in *U. parviflora* (Pandey et al. 2010). These phytochemicals can enhance various activities such as growth, feed consumption and act as a tonic in immunostimulation, antistress and to promote antimicrobial properties of fish and are redox active molecules with antioxidant characteristics that may help to improve the general physiological condition of fish. (Chakraborty and Hancz 2011).

1.1.7 Use of exogenous enzyme in rainbow trout feed

Increasing cost and limited global production of fish meal and fish oil has led to increased use of plant ingredients in aqua-feeds (Tacon and Metian 2015). Compared to other plant ingredients, soybean products are considered to be the most cost-effective alternative for partial replacement of high-quality fish meal and fish oil in feeds of salmonids, because of its high content of available protein with a relatively well-balanced amino acid profile, high digestibility, reasonable price, steady supply and low phosphorus content relative to fish meal (Biswas et al. 2007). Although soybean meal can fulfill nutritional requirements of fish, it consists of several antinutritional factors such as protease inhibitors, lectins, phytic acid, phytoestrogens, antivitamins, and allergens which limit feed performances and digestibility in compounded aquaculture feed (Francis 2001). Therefore, Enzymes are used to improve the availability of certain nutrients such proteases, amylases or to eliminate the presence of certain antinutrients (Escarnao 2016)

Rainbow trout is a predatory fish, although it consumes and assimilates plant protein, its intensity of growth on such feed is much lower in comparison to feed containing complete fish products (Singh et al. 2019). However, addition of exogenous enzymes to the feed can damage the hydration film membrane around chyme, increase the interaction between chyme and enzymes, and thus improve the utilization rate of feed and enhance growth of fish (Meale et al. 2014). Phosphorus combined with phytic acid present in soybean cannot be absorbed and utilized by fish in the feed which results in a pollution of phosphorus discharged into water as fish lack the enzyme to hydrolyze

phytate phosphorus in the digestive system (Zheng et al. 2019). Rainbow trout feed is super complex mixture composed of crude protein, crude lipid, carbohydrates, inorganic salt and so on (Ghomi et al. 2012). Thus, the use of exogenous enzyme mixture (multienzyme) is better choice rather than individual enzyme as multienzymes can synergistically degrade the target substrates of feed (Zheng et al. 2019).

1.2 Objective of study

The general objective of the study was to investigate effect of *U. parviflora* with multienzyme on growth and feed utilization of rainbow trout fingerlings.

The specific objectives were: -

1. To investigate Survival Rate (SR), Specific Growth Rate (SGR), Conditioning Factor (CF), daily growth rate, average body weight, total harvesting and Extrapolated Net yield ton per ha per cycle of rainbow trout fingerlings fed with different percentage concentration of U. parviflora with multienzyme.
2. To determine Feed Conversion Ratio (FCR) and Protein Efficiency Ratio (PER) of rainbow trout fingerlings fed with different percentage doses of *U. parviflora* with multienzyme.

1.3 Rationale of study

Urtica parviflora can be easily available whenever required without marketing and transportation expenses as they are found in wastelands of Nepal (Wagle et al. 2013). So, expensive chemotherapeutics can be replaced by herbal alternatives such as *U. parviflora* with high nutritional value to produce organic fishes and promote aquaculture in low cost.

Many researches were conducted by formulating diets by supplementing phytochemical products (Reverter et al. 2017). But, investigations on interactions between plants and commercial multienzyme with aim of assessing the potential of their combined effects on growth performance of fish is not enough (Zheng et al. 2019). So, this study is designed to assess the possibility of supplementation of *U. parviflora* and commercial multienzyme as growth promoters in diet of rainbow trout fingerlings.

2. LITERATURE REVIEW

2.1 Sustainable and responsible aquaculture

Despite several benefits of aquaculture such as the provision of good quality and accessible food for population and the generation of millions of jobs and economic development for the developing countries, aquaculture has been considered as responsible for cause of many environmental, social, economic including esthetic problem (Martinez-Porchas and Martinez-Cordova 2012). However, the environmental impacts of aquaculture are largely determined by species, system, production methods, location and quality of management. The major issues associated with aquaculture are increasing the feed efficiency, minimize water exchange, shifting of fishmeal with crop-based ingredients (World Bank 2013). The major target to achieve sustainable aquaculture are 10% improved feed efficiency in input use, sustainable intensification, shifting energy supply, shifting species mix (higher share of freshwater species, lower share of marine species) and replacement of fishmeal and fish oil with crop-based ingredients (Waite et al. 2014).

In order to control mortality, promote growth and avoid huge economic losses, fish farmers frequently adopt inappropriate practices such as excessive use of chemical products which violates the concept of sustainable and responsible aquaculture (Valladão et al. 2015). Wide range of pharmaceuticals including hormones, steroids, antibiotics, and parasiticides used in aquaculture have caused imbalances in both aquatic and terrestrial ecosystems (Boxall 2004). The chemical use to improve water quality and antibiotic to treat disease and promote growth with massive water exchange can harm wildlife (Mugg et al. 2007). The feed formulation with improper composition of fish requirement cause nutrient-loaded effluent can lead to eutrophication (Hasan 2001). Along with environmental degradation, chemically treated fish also cause negative health impacts on human by accumulations of chemical residues in muscle and production of antibiotic resistant pathogens on human body (Makkar et al. 2007).

However, phytochemicals contained in plants can enhance the innate immune system, possess antimicrobial capabilities, stimulate appetite and promote growth without affecting human health and environment (Chakraborty et al. 2013). As a result, scientist communities are more focused in exploiting Plants in crude form or its extract or its

isolated phytobiotic compounds as potential natural alternatives for fish productivity as sustainable and environmentally friendly approach to aquaculture with use of Plants would be a win–win situation for farmers, consumers and environment (Makkar et al. 2007

2.2 Leaves of plants as growth promoter in aquaculture

Plants can be administered as a whole plant or their parts such as leaf, root, seed, stem and fruit and can either be used fresh or as prepared herbal extracts with different solvents (Altemimi et al. 2017). Although interest in the use of Plants and plant extracts in aquaculture has exploded recently, they have long been used by rural fish farmers (Reverter et al. 2017). To improve water quality, reduce fish stress, increase fish resistance to pathogens, treat fish diseases and promote growth, fish farmers directly introduce fresh leaves of Plants in crude form into the rearing water as leaves are aerial part and easy to harvest without damaging plants (Reverter et al. 2017). The leaves of plants possess significantly higher quantity of bioactive compounds and micronutrients in comparison to other parts (Kregiel et al. 2018). Therefore, investigation based on the utilization of plants as feed additives in aquaculture used leaves (37%) while 22% used the whole plant as powder, plant essential oil or extract as well as root (18%) was also often used followed by seeds (8%), barks (6%), fruits (6%) and finally flowers (4%) (Reverter et al. 2017).

2.2.1 Effects of leaves of Plants in survivality of fish

Various factors such as stocking density, water quality, feed quality and improper handling of fish results stress and rapid mortality of fish in commercial aquaculture which decrease fish and its products in market and cause economic loss to fish farmers (Shakya 2017). So, many researches were conducted by addition of leaves of plants or its extracts to assess the survivability of cultured fish. Most of previous studies reveal that inclusion of leaves of plants as feed additives don’t cause any significant impact on survivality of cultured fish demonstrating that these feed additives don’t have any toxic effects to the fish (Abdel-Tawwab and Hamed, 2020, Bisht et al. 2020, Salomón et al. 2020) as well as positive results were observed in survival rate and relative percent survival (RPS) while performing challenge test with fish pathogens.

Dietary administration of different concentration (2 and 3%) of stinging nettle (*U. dioica)* powder in diets of rainbow trout (*O. mykiss*) significantly enhanced survival rate when compared with fish fed control diet (Saedi et al. 2017). In same species, addition of various levels (1, 1.5 and 3 g/Kg) of extract of Dill *Anethum Graveolens* in diets significantly increased the survival rate of fish in comparison to that of fish fed control diet (Sendijani et al. 2020). In an experiment conducted by Omitoyin (2018), dietary supplementation of different concentration (0.5, 0.25, 0.5 and 0.75%) of aqueous extract of guava (*Psidium guajava*) significantly enhanced the survival rate of Nile Tilapia (*Oreochromis niloticus*) in comparison to control diet. Similarly, Sogbesan (2019) observed that addition of different doses (0.5, 1 and 1.5%) of *Chromolaena odorata* leaves paste to *Clarias gariepinus* significantly increased the survival rate of fish. Hence, leaves of Plants even used in small quantity (< 5%) has potential to control mortality, avoid economic loss and decrease management cost.

2.2.2 Effects of leaves of plants on growth performance of fish

Investigations have been carried out to evaluate growth performance of fish by dietary administration of leaves of Plants in aquafeed. The results of most of the previous study demonstrated that bioactive compounds present on leaves of plants can improve the growth of fish as a result of enhanced activities of digestive enzymes and high feed to muscle conversion rate (Reverter et al. 2017). In this connection, the growth promoting effect of nettle (*Urtica* sp.) have been well demonstrated in either species of economic interest, such as rainbow trout (*O. mykiss*) (Awad et al. 2012, Saedi et al. 2017, Mehrabi and Firouzbakhsh, 2019) or endangered species Victoria labeo (*Labeo victorianus*) (Ngugi et al. 2015). Awad et al (2012) observed that leaves of *U. dioica* in dried powder form (1 and 2 %) was effective as growth promoter for Rainbow trout (*O. mykiss*) where significant increase in final weight, weight gain and specific growth rate and length Also, Saedi et al (2017) found using 3% of *U. dioica* powder as feed additives in diets of same species significantly enhanced weight gain and specific growth rate after 8 weeks of feeding trial. More recently, Mehrabi and Firouzbakhsh, (2019) found that feeding rainbow trout (*O. mykiss*) with lower levels (0, 1 and 1.5%) of *U. dioica* leaves powder significantly enhanced final weight, specific growth rate and weight gain when compared with fish fed control diet. Ngugi et al (2015) observed that leaves of *U.*

dioica in dried powder form (1, 2 and 5%) can promote growth of Victoria Labeo (*Labeo victorianus*) with significant rise in weight gain and specific growth rate.

Guava (*Psidium guajava*) leaves are considered safe for cultured fish species and also widely used as phytogenic feed additives in different forms in aquaculture. In case of rohu (*L. rohita*), dietary supplementation of (0.5 and 1 %) guava (*P. guajava*) leaves significantly enhanced the final weight gain, percent weight gain and specific growth rate in comparison to that of control diet (Giri et al. 2015). In an experiment conducted by Gobi et al. (2016), increasing dietary levels of aqueous and ethanol extract (1, 5 and 10 mg/g) of guava (*P. guajava*) leaves in the diets of mozambique tilapia *mossambicus* significantly increased the final weight and specific growth rate. Feeding aqueous (0.5 and 0.75%) or ethanolic (5gm/kg) guava (*P. guajava*) leaves extract based diets to Nile tilapia (*O. niloticus*) also improved the final weight, mean weight gain and specific growth rate (Omitoyin et al. 2019, Abdel-Tawwab and Hamed 2020). Also, using 0.025% of guava (*P. guajava*) leaves in shrimp culture significantly elevated final weight, mean weight gain, percent weight gain and specific growth rate (Yin et al. 2014)

Leaves of plants native to Asian countries are widely used and more extensively studied to assess the growth performance of cultured fish species. Lower-level inclusion of *Houttuynia cordata* leaves extract (0.5 and 1%) or leaves powder (1%) significantly improve final weight, weight gain and specific growth rate of Rohu (*Labeo rohita*) (Garg et al. 2019). Also, feeding diet supplemented with dill (*Anethum Graveolens*) extract (1 and 1.5 gm/kg) to rainbow trout (*O. mykiss*) elevated the weight gain and specific growth rate than that of control diet (Sendijani et al 2019). Rahman et al. (2019) conducted an experiment in which increasing dietary concentration (0.1, 0.2 and 0.4%) of Indian Lotus (*Nelumbo nucifera)* leaf powder significantly improved final body weight, weight gain and specific growth rate Nile Tilapia (*O. niloticus*). More recently, Srichaiyo et al. (2020) also found that dietary administration of another native Asian plant gotu kola (*Centella asiatica*) powder (5gm/kg and 10 gm/kg) in diets of Nile Tilapia (*O. niloticus*) also significantly enhanced the final weight, weight gain and specific growth rate.

However, leaves from banana (*Musa nana*) and maize (*Zea mays*) found in Southeast Asia don't have any effects on the weight gain and specific growth rate of grass carp (*C. idella*) (Mayrhofer et al. 2017). The use of Leaves extracts of Chinese medicinal

plant gingko (*Ginkgo biloba*) suppressed on weight gain rate and specific growth rate of hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀) (Tan et al. 2017). Also, reduction on Final fish weight, Weight gain and Specific growth rate was reported with dietary administration of dried leaves' powder (1, 2 and 3%) of purslane (*Portulaca oleracea* L.) native to Indian subcontinent (Abdel-Razek et al. 2019).

The aromatic plants containing essential oil rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis),* peppermint (*Mentha piperita*), olive (*Olea europea),* oregano (*Origanum vulgare), thyme (Thymus vulgaris)* and lemon verbena *(Aloysia citrodora* and *Lippia citriodora*) are also used as phytogenic feed additives in various commercially important fish species. Adel et al. (2015) observed that with increasing dietary concentrations of peppermint (*Mentha piperita*) on diet of Caspian white fish (*Rutilus frisii kutum*) resulted enhancement of weight gain and specific growth rate. Also, increasing dietary levels (1, 2 and 3%) of rosemary (*R. officinalis*) leaves powder significantly improved the weight gain, final weight and specific growth rate of common carp (*Cyprinus carpio*) (Yousefi et al. 2019). Similar results were observed when various levels (0.4, 0.7, 1 and 3 g/kg) of rosemary (*R. officinalis*) leaves extract were supplemented in diets of rainbow trout (*O. mykiss*) (Karataş et al. 2020) but no effects on growth performance was recorded with dietary supplementation of olive (*Olea europea*) leaf extract, thyme (*Thymus vulgaris*) extract and lemon verbena (*Aloysia citrodora*) leaves powder in same species (Baba et al. 2018, Hoseini and Yousefi 2018, Hoseinifar et al. 2020). Also, dry oregano (*Origanum vulgare*) leaves supplementation does not affect final weight. average daily gain and percentage weight gain and specific growth rate in Nile Tilapia (*O. niloticus*) (Santo et al. 2019). However, 0.1% dietary supplementation of leaves extract from mixture of sage (*Salvia officinalis)* and Lemon verbena (*Lippia citriodora)* significantly increased final body weight and specific growth rate of gilthead seabream (*Sparus aurata*) (Salomón et al. 2020).

Indeed, the effects of leaves of plants in fish feed used for enhancement of fish growth is variable among fish species. In an experiment conducted by Puycha et al. (2017), increasing the dietary concentration (100, 150 and 250 gm/kg) of moringa (*Moringa oleifera)* leaves in diets of Bocourti's catfish (*Pangasius bocourti*) decreased the average daily gain and specific growth rate. Similarly, reduction on weight gain and thermal growth factor was reported in Gilthead seabream (*Sparus aurata*) fed diet

containing 15 % *M. oleifera* leaves (Mansour et al. 2018). Guppy *(Poecilia reticulata)* fed with diets containing different concentrations of moringa leaf powder did not show any significant changes in final mean weight, net weight gain, weight gain and specific growth rate (Bisht et al, 2020). In contrast, 1.5% supplementation of *M. oleifera* in diets of Nile tilapia (*O. niloticus*) significantly enhanced final weight, body mass gain, specific growth rate and length gain rate (Elabd et al. 2019).

The effects of plants on growth promotion of fish also depend on various physical factors such as (dosage and trial period) and chemical factors (methods used in diet preparation). For example: Dietary inclusion (5, 10, 15 gm/kg) of leaves extract of clove basil (*Ocimum gratissimum*) in diets of African catfish (*Clarias gariepenus)* fingerlings for 12 weeks significantly increased the final weight, weight gain and specific growth rate (Abdel-Tawwab et al. 2017) whereas the use of fermented *O. gratissimum* (1, 2 and 3%) in diets of African catfish (*C. gariepenus*) fingerlings significantly decreased the same growth parameters when compared with fish fed control diet (Sogbesan et al. 2017).

2.2.3 Effects of leaves of plants on feed utilization of fish

Superior feed efficiency ratio and lower feed conversion ratio suggest the decreased amount of feed necessary for fish growth which could result in reduction of production cost (Omitoyin et al. 2019). There have also been some researches which demonstrates the feed efficiency of fish increases with dietary supplementation of leaves of Plants in fish feed. Adel et al. (2015) observed that increasing dietary concentrations of peppermint (*M. piperita*) extract (1, 2 and 3%) decreased the feed conversion ratio of Caspian white fish (*Rutilus frisii kutum*). Rahman et al. (2019) reported that increasing the concentrations (0.1, 0.2 and 0.4%) of Indian Lotus (*N. nucifera*) powder in diets of Nile Tilapia (*O. niloticus*) increased feed intake and decreased the feed conversion ratio. Feeding Rosemary (*R. officinalis*) leaf powder (1, 2 and 3 %) supplemented diets to common carp (*C. carpio*) significantly improved feed efficiency ratio when compared to fish fed control diet (Yousefi et al. 2019). In an experiment conducted by Garg et al. (2019), dietary administration of *H. cordata* leaf meal (0.25, 0.5 and 1 gm/kg) and leaf extract (1 and 2 gm/Kg) significantly decreased feed conversion ratio in comparison to control diet. Also, in case of rainbow trout, dietary administration of Rosemary (*R. officinalis*) leaf extract (0.4, 0.7, 1 and 3 gm/kg) significantly increased

Feed Efficiency ratio and decreased feed conversion ratio (Karataş et al. 2020). Salomón et al. (2020) 0.1 % supplementation of mixture of sage (*S. officinalis*) and lemon bee brush (*L. citriodora*) in diets of gilthead seabream (*S. aurata*) also decreased feed conversion ratio.

The effects of plants are also dependent upon life stages of experimental fish. Dietary administration of stinging nettle (*U. dioica*) powder (0.5-3%) significantly decreased feed conversion ratio and enhanced feed efficiency ratio in juvenile rainbow trout (*O. mykiss*) (Saedi et al. 2017, Mehrabi and Firouzbakhsh 2019) but feeding *U. dioica* supplemented diets (1 and 2 %) on fingerlings of rainbow trout (*O. mykiss*) does not cause any effects on feed conversion ratio (Awad et al. 2015). Similarly, no effects were recorded with dietary supplementation of stinging nettle (*U. dioica*) powder (1, 2 and 5%) in case of Victoria Labeo (*L. victorianus*) (Ngugi et al. 2015) which reveals that the effects of Plants on feed utilization also varies among the experimental fish. Dietary supplementation of Moringa (*M. oleifera*) leaves (100, 150 and 200 gm/Kg) in diets of Bocourti's catfish (*P. bocourti*) significantly increased the feed conversion ratio (Puycha et al. 2017). Also, increased feed conversion ratio and feed intake was recorded in gilthead seabream (*S. aurata*) with dietary supplementation of Moringa (*M. oleifera*) leaves (5, 10 and 15%) (Mansour at al. 2018), whereas no effects in feed conversion ratio was reported with inclusion of Moringa (*M. oleifera*) leaves (5, 10 and 15%) in diets of guppy (*P. reticulata*) (Bisht et al. 2020). In contrast, 1.5% dietary administration of Moringa (*M. oleifera*) leaves significantly decreased feed conversion ratio in Nile Tilapia (*O. niloticus*) (Elabd et al. 2019).

It is essential to find out optimal dose to lower feed conversion ratio, enhance feed intake and simultaneously improve feed utilization. In an experiment conducted by Omitoyin et al. (2019), 0.50 and 0.75% inclusion of guava (*P. guajava*) aqueous extract significantly decreased the feed conversion ratio whereas 1 % inclusion increased feed conversion ratio. In an experiment conducted by Abdel-Tawwab et al. (2017), 10 and 15 gm/kg dietary administration of clove basil (*O. gratissimum*) leaf extract in diets of African catfish (*C. gariepinus*) significantly enhanced the feed intake no significant effects were seen in case of feed conversion ratio whereas decreased feed conversion ratio and feed intake was reported with dietary inclusion (1, 2, 3 and 4%) of clove basil (*O. gratissimum*) in same species (Sogbesan et al. 2017).

Mayrhofer et al. (2017) reported that dietary inclusions of leaves of banana (*M. nana*) or maize (*Z. mays*) in diets of grass carp (*C. idella*) do not cause any significant effects in Feed Conversion Ratio. Dietary supplementation of thyme *(T. vulgaris)* extract, olive leaf (*Olea europea*) extract, Dill (*A. Graveolens*) extract, lemon verbena (*A. citrodora*) leaves powder doesn't cause any effects in feed intake and Feed Conversion Ratio in rainbow trout (*O. mykiss*) (Hoseini and Yousefi 2018, Baba et al. 2018., Sendijani et al. 2020, Hoseinifar et al. 2020). Dietary inclusion of dry oregano (*O. vulgare*) leaves powder, gotu kola (*C. asiatica*) powder purslane (*P. oleracea*) doesn't affect feed intake and feed conversion ratio of Nile Tilapia (*O. niloticus*) (Santo et al. 2018, Abdel-Razek et al. 2019, Srichayo et al. 2020). Similar results were observed when Mozambique tilapia (*O. mossambicus*) was fed aqueous or ethanol extract of guava (*P. guajava*) leaves (Gobi et al. 2016).

Dietary supplementation of leaves of Plants also has positive effects in protein efficacy. In an experiment conducted by Garg et al. (2019), dietary administration of *H. cordata* leaf meal (0.25, 0.5 and 1 gm/kg) and leaf extract (1 and 2 gm/Kg) significantly improved protein efficiency ratio. Guava (*P. guajava)* leaves extract-based diet 0.25, 0.50, 0.75 and 1.00 % also significantly increased protein efficiency ratio in Nile Tilapia (*O. niloticus*) as compared to control diet (Omitoyin et al. 2019). Similar results were observed in an experiment performed by Sendijani et al. (2020) when Dill (*A. Graveolen*) extract (1, 1.5 and 3 gm/kg) were added in the diets of rainbow trout (*O. mykiss*). But no significant impact is recorded with dietary supplementations of Moringa (*M. oleifera*) leaf powder in diets of Bocourti's catfish (*P. bocourti*) (Puycha et al. 2017)

2.2.3 Effects of multienzymes on growth of fish

Supplementation with exogenous enzyme into feed could not only decrease the burden of the environment through recycling of the feed input (Verdegem 2013) but also promotes digestion of animals by transforming feed into small molecules (Bedford 2000). many studies have also demonstrated that exogenous multienzyme mixture with reasonable adjustment could significantly enhance fish growth and reduce feed efficiency (Ghomi et al. 2012, Zhamini et al. 2012, Hlophe-Ginindza et al. 2016) Ghomi et al. (2012) found that great sturgeon (Huso huso) fingerlings fed with 250 mg/kg exogenous multienzyme complex containing phytase, lipase, xylanase, protease, β-

glucanase, α-amylase, pentosanase, hemicellulase, cellulase and pectinase, exhibited higher weight gain and specific growth rate, and also significantly improved feed conversion ratio. Similarly, it was also reported that Caspian salmon (*Salmo trutta caspius*) fed diets containing multienzyme complex i.e., mixture of Natuzyme50® with protease, lipase, fitase, α amilase, cellulase, amiloglucosidase, β-glucanase, pentosonase, hemicellulase, xylanase, pectinase, acid phosphatase and acid phytase and Hemicell® with Endo-β-mannanase, amylase, xylanase, cellulose and α-galactosydase could significantly promote its growth performance (Specific growth rate, final weight and percentage body weight gain) (Zamini et al. 2012). Hlophe-Ginindza et al. 2016 demonstrated that *Oreochromis mossambicus* fed diets with Natuzyme50® (0.05 gram/kg) in kikiyu based diets significantly increased weight gain and specific growth rate of fish.

However, the addition of multienzyme containing bacterial protease and α galactosidase for hydrolysis of raffinose and stachyose (Alpha galactosidase) to the lupin-based diet had no significant effect on the growth parameters of rainbow trout (Farhangi and carter 2007). A multienzyme with xylanase, amylase, cellulase, protease and β-glucanase) introduced into diet of rainbow trout showed no significant effect on the growth performance (Ogunkoya et al. 2006). Thus, to maximize the combination efficiency of enzymes, it is crucial to understand how enzymes synergistically hydrolyze their respective substrate

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental setup apparatus

a) bucket
b) scoop nets
c) scrubber
d) metallic raceway covers
e) metallic screens

3.1.2 Instruments used in growth experiment

a) ordinary scale (± 1mm) attached with wooden board

b) electronic weight balance (± 0.1-gram SN-014739), Phoenix instrument model

c) heavy duty electronic weighing machine

3.1.3 Equipment used in feed preparation

a) Electric dryer
b) Electric grinder
c) pellet feed machine
d) mixture machine
e) soybean roaster

3.3.4 Instruments used for determination of water quality

a) digital thermometer (Orion 5-star S.N. 005840)

b) DO meter (Orion 5 star, thermo electric cooperation U.S.A)

c) P^H meter (Thermo-electron Cooperation Russell 060p)

d) eXact Ecocheck Kit #48698K

3.3.5 Instruments used for determination of feed quality

a) Physical balance
b) Soxhlet extraction apparatus
c) Berzelius beakers (600 ml)
h) Pipette (10 ml)
i) Sintered glass crucibles
j) Washing bottles (1 liter)
o) Thimble
p) Burette (10 ml)
q) Calorimeter

d) Analytical Balance k) Hot air oven r) Fuse wire (10 cm)

e) Kjeldahl flasks (300 ml) l) Aluminum tray s) Sample capsule

f) Macro Kjeldahl digester and distiller m) Refluxing apparatus

t) Electronic balance g) Measuring cylinder (25 ml) n) Filtering apparatus

3.3.6 Chemical reagents used for determination of feed quality

a) 40 % Sodium hydroxide (NaOH) f) Catalyst, Na_2SO_4: $CuSO_4$

b) Methyl red indicator g) Petroleum benzene ether

c) Concentrated sulphuric acid (H_2SO_4) h) Acetone

d) 2 % Boric acid (H_3BO_3).

e) Benzoic acid

3.2 Methods

3.2.1 Experimental set up

Fingerlings of rainbow trout was obtained from Fisheries Research Centre, Nuwakot. Fish was acclimatized to the experimental rearing conditions for one month. Fingerlings were fed with control diet during acclimation. Advanced fish with average weight of 38.89 ± 5.55 gram was be selected from acclimatized fish and salt treatment was done to avoid fungal infection. The stocking density was 125 fingerlings per 2.5m^2. Eight raceways of National Fisheries Research Centre, Godawari with length 5m, breadth 1m and height 0.6m was subdivided into equal portion with 2.5 m^2 area for fish stocking on the basis of treatments. Each treatment was replicated thrice in completely randomized design (CRD). The raceways were supplied with the water from nearby water resource with the help of cemented canal. The depth of the water was maintained 50 cm and flow of water was maintained 6 liters per second. The raceways were covered with metallic raceway covers to protect fish from predatory birds and metallic screens were added in outlet of raceways to prevent escaping from outlet of water. The floors and bottom of raceways were scrubbed to clean at the interval of four days.

3.2.2 Feed preparation

Fresh flag leaves were collected from Rainbow Trout Fishery Research Station in Dhunche, Nepal. Then, they were cleaned, cut into small pieces and shade air dried shade to prevent the loss of vitamins and other volatile nutrients. Other major feed ingredients were bought from nearby market of Godawori, cleaned and sundried whereas fish meal was dried in electric dryer. Soybean was roasted in soybean roaster. 50 grams of each feed ingredients were collected to conduct proximate analysis of feed ingredients in order to prepare diet with similar protein levels.

Table 1. Biochemical composition of different feed ingredients used in preparing experimental diets

Feed ingredients	Crude Protein (CP)	Crude Fat (CF)	Crude Ash	Moisture
Wheat flour (%)	14.78	5.26	5.41	11.33
Fish meal (%)	60.76	1.95	18.66	9.56
Soybean (%)	36.33	2.15	5.75	5.36
Nettle powder (%)	24.45	2.13	18.86	9.56

All the major ingredients were pulverized into fine powder in a grinder, and stored at 4°C until use. In the mixture machine, nettle powder was mixed with other feed ingredients in different percentage compositions. (Table 1). The compositions of different feed ingredients required to prepare feed pellet was determined by using solver tool on MS-Excel. Pellets was prepared by help of pellet feed machine. The multi-enzyme was supplied from Alembic pharmaceuticals Ltd, Vadodara was used in research and each 500 grams of it contained Amylase 250000 units, Protease 350000 units, Lipase 20000 units, Cellulase 30000 units, Phytase 45000 units, Alpha Galactosidae 45000 units, Glucanase 70000 units, Petinase 120000 units and Xlanase 35000 units. Feed formulations include Treatment 1 (T1): Control diet with 0.05gm/kg multienzyme, Treatment 2 (T2): Control diet without 0.05gm/kg multienzyme, Treatment (T3): Control diet with 0.05gm/kg multienzyme and 1.5% nettle powder, Treatment 4 (T4): Control diet with 0.05gm/kg multienzyme 3% nettle powder and Treatment 5 (T5): Control diet with 0.05gm/kg multienzyme 3% nettle powder.

Table 2: Formulation of Experimental diets

Experimental diets	T1	T2	T3	T4	T5
Full fat Soybean (%)	35	31.55	35	35	35
Soybean oil (%)	6	6	6	6	6
Wheat flour (%)	11.22	12.45	10.05	8.88	7.31
Fish meal (%)	47.78	50	47.45	47.12	46.69
Slack (%)	5.3	5.25	5.3	5.3	5.3
Nettle powder (%)	0	0	1.5	3	5

After the preparation of experimental diets, 50 grams of each experimental diets were collected to proximate analysis of experimental feed (Table 3)

Table 3: Biochemical composition of experimental diets

Experimental diets	T1	T2	T3	T4	T5
moisture (%)	5.06	5.14	5.05	5.12	5.73
Crude protein (%)	33.09	35.5	37.79	41.49	44.06
Crude Fiber (%)	2.15	1.55	1.52	2.41	3.78
Crude fat (%)	10.72	15.78	15.32	15.08	15.46
Digestible energy (Cal/g)	4287.06	2635.74	3633.68	3452.66	2947.06

3.2.3 Proximate analysis of feed ingredients and experimental feed

Proximate analysis of macro ingredients and experimental diets was conducted at animal nutrition division by following standard protocol of Analysis of Office Association of Chemists (AOAC, 1995)

3.2.3.1 Determination of moisture

Representative clean dry sample of approximately 100-gram was pre-weighed in aluminum tray. The weighed sample was dried in hot air oven at 100^0 C for 24 hours to a constant weight. The weighed sample was quickly weighed after cooling at the room temperature without undue exposure to atmosphere for prolonged period using the desecrator to avoid absorption of moisture. The difference in weight is expressed as moisture percent the oven dried sample was stored after grinding in laboratory grinder (1mm size), in air tight containers for further estimation of various proximate principles.

$$\text{Moisture \%} = \frac{\text{Initial sample weight - final sample weight}}{\text{Sample wt.}} \times 100$$

3.2.3.2 Determination of protein

Weighed 0.5-1.0g of air-dry sample was kept into Kjeldahl flasks tube. about 5g (2 spatula) of Digestion mixture (Na_2SO_4 and $CuSO_4$ in ratio of 5:1) was added to it. 25ml of H_2SO_4 was also added carefully. the flasks were kept on digestion racks and heat. the flasks were swirled gently with continuous heating for about 2hour and cooled. 100 ml of volume (sample + distilled water) in Volumetric flask was prepared and left for overnight and then if the level was lower at marked point flask. Then, distilled water water was added to reach up to the marked level. 10 ml of 2% boric acid was placed into Erlenmeyer flask (triangle flask). the water tap was opened to the cooling system and the heaters of distillation apparatus was switched on. 10ml of Sample and 10 ml NaOH was added into Kjeldahl flask. The flask was immediately to distillation apparatus, mixed completely and distilled for about 3 minutes boiling or until you collect about 30 ml distillate. The distillate turned from red to green. Distillate was titrated with 0.03N H_2SO_4 until turns from green to pink

$$\text{Crude protein (\%)} = \frac{14 \times \text{Normality} \times (\text{Reading point - blank point}) \times 6.25}{\text{Dry Matter} \times \text{Sample Weight}}$$

3.2.3.3 Determination of fat

Clean numbered flasks were kept in an oven at 135^0C for 2 hours. The flasks were cool in desiccators and the initial weight was recorded. About 2 grams of sample was weighed and placed it in a folded filter paper and the sample was dried at 100^0 C at 2

hours in Oven. The paper-containing sample was put in to the extractor. About 150ml Petroleum benzene ether into the flask (half the flask) was put and placed it on the heating system while connecting to the extractor and the condenser. The condenser was covered with cotton wool, and temperature of water was set at 70^0C. Extract was removed from the flask and left overnight. The flask was placed in an oven set at 135^0C for 2 hours and cooled in a desiccator and the final weight was recorded.

$$\text{Crude fat (\%)} = \frac{\text{initial weight of flask - initial weight of flask}}{\text{Sample weight} \times \text{Dry Matter}} \times 100$$

3.2.3.4 Determination of Crude fiber

About 1 to 2gram of grinded air-dry sample was weighed and passed through a 1mm meshes into a 600ml refluxing beaker. 200 ml 1.25 % H_2SO_4 solution was added and placed the beakers on hot refluxing apparatus and the condenser was placed in place and heated to boiling for 5-10 minutes. Onset of boiling was adjusted to about 60^oC and refluxed for 30 minutes from onset of boiling. Tare crucibles was weighed (initial weight) and placed on the filtering apparatus. Beakers were swirled to suspend the solids. The crucibles were filled and filtered by using low vacuums initially and increased vacuum when necessary and wash with hot water 2 to 3 times. 200 ml of 1.25 % of NaOH solution was added and boiled as a same procedure in 1^{st} boil. Sample in the beaker was rinsed into the crucible with minimum hot water (100^0C), and the crucibles were filled twice with hot water and filtered with Petroleum benzene ether and acetone. the crucible was dried at 135^0C for 2 hours and cooled in Desiccators and weighed (Final weight). Crucible was dried at 135^0C for 2 hours for ash and cooled in Desiccators and weigh and weighted (Ash weight).

$$\text{CF\%} = \frac{\{(\text{Final weight - initial weight}) - (\text{Ash weight})\}}{\text{Sample weight} \times \text{Dry Matter \%}} \times 100$$

3.2.3.5 Determination of digestible energy

About 0.5 to 1 gram of sample of experimental diets were weighed and kept in capsule. The capsule was kept on the bomb head and attached with 10 cm fuse wire. Sample needs to be touched by the fuse wire. The prepared sample were kept inside the bomb and the screw cap was well tightened up and pressure valve was closed. O_2 gases was filled in bomb at 30 atom pressure. The pressure should not exceed more than 40 atoms. the gas from lever was removed if excess gasses is present in the lever, gasses was again

refilled again but the previous gasses from the bomb was removed. the water bucket was cleaned and filled with the accurate 2000-gram water. the bucket that contains 2000 gm of water was kept in to bigger bucket and bomb head was set into the water bucket. The ignition cord was inserted inside the bomb head. the cover was set and content was stirred with the stirrer by hand to check whether the stirrer has touched the bomb or bucket. If it was touched, stirrer was readjusted to make it untouched. the motor was started if the stirrer was free and kept running till five minutes. Then, the temperature of the motor (first Reading) was recorded. The ignition switch was pressed on for 5 second. After 6 minutes, the second reading of temperature was taken.

$$\text{Energy (cal/g)} = \frac{(\text{First reading - second reading}) \times (2000 + \text{standard})}{\text{Sample weight} \times \text{Dry Matter \%}} \times 100$$

3.2.3.6 Determination of Ash content

Ash content of feed ingredients was determined by weighing the crucible containing the dried sample and transferring to a muffle furnace at 580°C for 3 hours. The crucible containing the sample was reweighed and the difference between sample weights indicated the ash content.

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

3.2.4 Physicochemical parameters of water

Rainbow trout can only survive at certain range of water quality. So, water parameters are to be maintained within acceptable range of fish. Dissolved oxygen (DO) in ppm, pH value and temperature in degree centigrade (°C) were measured during growth checkup at out flow of each raceway. Digital pen thermometer, Portable Hanna p^H and Orion 5-star S.N. 005840, thermo-electron corporation, U.S.A was used to monitor temperature, p^H and dissolve oxygen respectively. Water samples were collected from each race at the same time and stored in refrigerator. Alkalinity, hardness, ammonia, Nitrite (NO_2) and Nitrate (NO_3) was determined in laboratory from collected water sample with the help of eXact Ecocheck Kit #48698K.

Total hardness was found < 5 ppm, Nitrite (NO2) was found < 0.01 ppm, Nitrate (NO3) was found > 0.12 ppm and Ammonia (NH3) was found < 0.01 ppm. No significant differences were observed in water quality parameters among treatments (Table 5).

Table 4: Mean and standard error (SE) of water quality parameters

Water quality	T1	T2	T3	T4	T5
Temperature(˚C)	14.0714±0.87	14.61±0.79	14±0.96	14.03±0.92	14±0.95
DO (ppm)	8.06 ± 0.18	8.07± 0.21	7.94±0.18	7.98±0.18	7.93±0.18
P^H (ppm)	7.89± 0.12	7.9±0.12	7.93±0.11	7.94±0.14	7.88±0.11
Alkalinity (ppm)	103.14± 5.12	107± 7.99	101.29±3.74	101.71±4.68	99.28±1.92

3.2.5 Fish sampling and growth performance

Fish sampling was done for determine the growth performance of the fishes. Ten percent of the fish was taken out randomly by help of scoop net at the interval of 15 days. Length and weight of sampled fish was measured by ordinary scale (± 1mm) attached with wooden board and electronic weight balance (± 0.1-gram SN-014739, Phoenix instrument model respectively. During final growth checkup, all fish stocks in each treatment were weighed by using heavy duty electronic weighing machine to determine the harvesting weight of fish as well as total number of survived fishes in each treatment were also counted one by one simultaneously to determine the survival rate of fish.

The fish production and related parameters were analyzed using following formulae:

$$\text{Daily growth rate (g/fish/day)} = \frac{\text{Average harvest weight (g)} - \text{Average stock weight (g)}}{\text{Culture days}}$$

$$\text{Specific growth rate (\% per day)} = \frac{\text{(Log. harvest weight - Log. stock weight)}}{\text{Culture days}} \times 100$$

$$\text{Extrapolated Net fish yield (ton/ha/cycle)} = \frac{\text{(HW in kg - Stocked weight in kg)/1000}}{10000 \times \text{Culture days}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Quantity of feed supplied (kg)}}{\text{Net fish yield (kg)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{gain in weight (g)}}{\text{Protein intake in feed (g)}}$$

$$\text{Condition Factor (gm/cm}^3\text{)} = \frac{\text{Weight of fish in gram}}{(\text{Length of fish in cm})^{\wedge 3}}$$

$$\text{Survival Rate (SR)} = \frac{\text{Number of fish that survived}}{\text{Total number of fish}} \times 100\%$$

3.2.6 Statistical analysis

The differences between the group means of total harvesting weight was tested by analysis of covariance (ANCOVA) with average stocking weight. Daily growth rate, Specific Growth Rate, Feed Conversion Ratio, Condition Factor, Survival Rate and Average body weight was tested by analysis of variance (ANOVA). Duncan's Multiple Range Tests (DMRT) was applied to determine the significance of differences between any two means after ANOVA. All statistical tests were performed using statistical package SPSS 16.0 Software (SPSS, Chicago II). Comparisons was made at 5% level of significance

4. Results

4.1 Survival rate

Fishes in all the treatments were in healthy condition during experimental period because symptoms of fish disease and infections were not observed. No significant differences were observed in survival among the treatments since fish survival rate ranges from 98.4–100% (Table 2).

Table 5: Mean and standard error (SE) of survival rate

Treatments	**Survival rate (gm)**
T1	98.41±0.79
T2	100.00±0.00
T3	99.21±0.79
T4	100.00±0.00
T5	99.21±0.79

4.3 Growth performance

4.3.1 Daily growth rate

Highest daily growth rate (g/fish/day) was observed ($P<0.05$) in diet with 1.5% nettle and 0.05-gram multienzyme (T3) followed by diet with 3% nettle and 0.05-gram multienzyme (T4), diet with 0.05-gram multienzyme (T1), diet with 5% nettle and 0.05-gram multienzyme (T5) and diet without nettle and multienzyme (T2) (table 3).

Table 6: Mean and standard error (SE) of Daily growth rate

Treatments	**Daily growth rate (gm)**
T1	1.12 ± 0.02^{c}
T2	0.95 ± 01^{a}
T3	1.19 ± 0.01^{d}
T4	1.16 ± 0.01^{cd}
T5	1.03 ± 0.02^{b}

Mean value with different superscript letters is significantly different.

4.3.2 Specific growth rate

Specific growth rate was found significantly higher (P<0.05) in 1.5% nettle and 0.05-gram multienzyme (T3) than other diets. Diet with 0.05-gram multienzyme (T1) also showed significantly better (P<0.05) specific growth rate than diet with 3% nettle and 0.05-gram multienzyme (T5), diet with 5% nettle and 0.05-gram multienzyme (T5) and diet without nettle and multienzyme (T2). Lowest specific growth rate was observed diet without nettle and multienzyme (T2)

Table 7: Mean and standard error (SE) of Specific growth rate.

Treatments	**Specific growth rate (gm)**
T1	0.61 ± 0.02^{b}
T2	0.57 ± 0.006^{a}
T3	0.65 ± 0.01^{c}
T4	0.58 ± 0.004^{a}
T5	0.59 ± 0.01^{a}

Mean value with different superscript letters is significantly different.

4.3.3 Condition factor

The condition factor (length-weight relationship) was found similar among treatments during experimental period. Condition factor did not show any significant difference among treatments (fig 1).

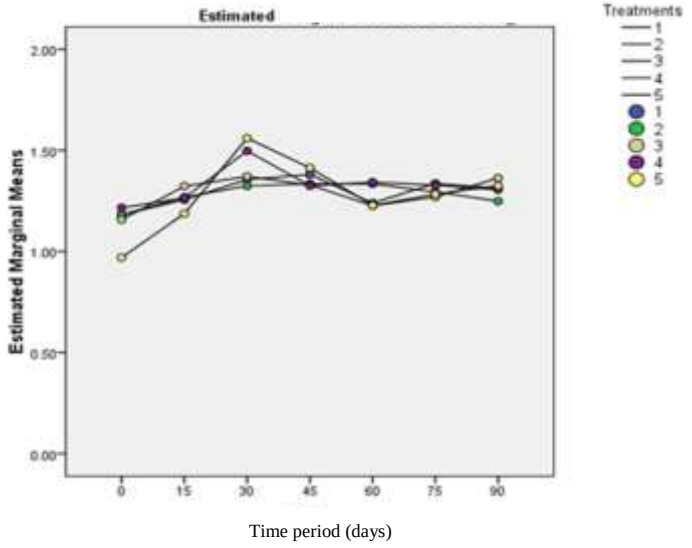

Fig 1: The relationships between dietary nettle levels supplementation and condition factor

4.3.4 Average body weight

The initial stocking weight. of fish was 38.56±2.44 gram in all the treatments. Average body weight after 15, 30, 45 and 60 days did not show any significant difference in all treatments. Average body weight after 75 days in all treatments were significantly higher than Treatment 2. Treatment 3 (170.70±11.30 gram) showed significantly ($P<0.05$) higher average body weight followed by Treatment 4 (163.02 ± 4.71gram), Treatment 1 (153.51± 0.91 gram), Treatment 5 (143.71±7.31gram) and Treatment 2 (139.15 ± 1.67 gram)

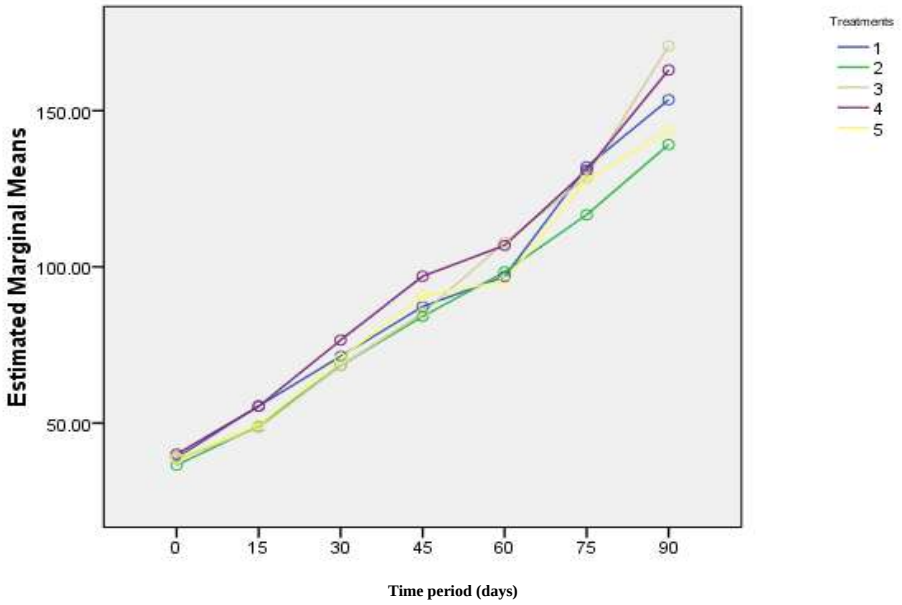

Fig 2: The relationships between dietary nettle levels supplementation and average body weight

4.3.5 Total harvesting weight

Total harvesting weight after the end of the experimental trial was found to be significantly higher ($P<0.05$) in treatment 3 followed by treatment 1, treatment 5, treatment 4 and treatment 2 (Table 5).

Table 8: Mean and standard error (SE) of Total harvesting weight

Treatments	Total harvesting weight (gm)
T1	5856.29 ± 106.63^{b}
T2	5418.78 ± 109.59^{a}
T3	6383.32 ± 111.52^{c}
T4	5565.545 ± 93.357^{a}
T5	5625.237 ± 108.75^{ab}

Mean value with different superscript letters is significantly different.

4.3.6 Extrapolated Net yield ton per ha per cycle

Treatment 3 showed significantly higher extrapolated net yield ton per ha per cycle than other treatments followed by Treatment 4, Treatment 1, Treatment 5 and Treatment 2 at the end of experiment (Table 6).

Table 9: Mean and standard error (SE) of extrapolated Net yield ton per ha per cycle

Treatments	**Extrapolated Net yield ton per ha per cycle**
T1	51.32 ± 0.94^{c}
T2	45.15 ± 0.82^{a}
T3	53.92 ± 0.09^{e}
T4	52.80 ± 1.17^{d}
T5	46.61 ± 0.06^{b}

Mean value with different superscript letters is significantly different.

4.4 Feed utilization

4.4.1 Feed Conversion Ratio

Feed conversion ratio (FCR) was found significantly lower in treatment 3 followed by treatment 4, treatment 1, treatment 2 and treatment 5 during the experimental period of 90 days.

Table 10: Mean and standard error (SE) of Feed Conversion Ratio

treatments	Feed Conversion Ratio
T1	1.61 ± 0.03^{bc}
T2	1.65 ± 0.02^{cd}
T3	1.53 ± 0.02^{a}
T4	1.59 ± 0.01^{b}
T5	1.68 ± 03^{d}

Mean value with different superscript letters is significantly different

4.4.2 Protein efficiency ratio

During the experimental trial of 90 days, Protein efficiency ratio was found to be significantly better in Treatment 3 and Treatment 1 in comparison to the other treatments (Table 8).

Table 11: Mean and standard error (SE) of Feed Conversion Ratio

Treatments	Protein efficiency ratio
T1	3.44 ± 0.04^{b}
T2	2.84 ± 0.06^{a}
T3	3.52 ± 0.29^{b}
T4	2.67 ± 0.11^{a}
T5	2.53 ± 1.18^{a}

Mean value with different superscript letters is significantly different.

5. DISCUSSIONS

5.1 Survival rate

Survival rate (98.4 – 100 %) was not affected with the dietary supplementations of nettle in diets of rainbow trout. The results of previous studies also demonstrated that that inclusion of leaves of Plants (>5%) don't cause any significant impact on survivality of cultured fish which reveals that these feed additives don't have any toxic effects to the fish (Yin et al. 2014, Tan et al. 2017)

5.2 Growth performance and feed utilization

Growth and improvement in fish health can provide benefits for aquaculture by decreasing production times, reducing FCR, and increasing productivity. The present study indicated that the highest fish growth was obtained at Treatment 3 i.e., 1.5% nettle and 0.05-gram multienzyme supplemented diet followed among the treatments. The reason behind better growth performance at treatment 3 could be due to growth promoting bioactive components of Nettle, combined interaction between the multienzyme and substrates present in feed which ensured the availability of nutrients, acceptable range of crude fiber, and sufficient digestible energy. Besides Treatment 3 i.e., diet with 3% nettle supplementation and 0.05-gram multienzyme showed better growth performance with decreased feed conversion ratio.

These results were consistent with Awad and Austin (2012) who found that rainbow trout fed for 2 months with a diet supplemented with 1% and 2% of stinging nettle, (*Urtica dioica*) powder recorded significant increase in growth performance, especially weight gain, SGR and digestive enzymes. Similarly, in an experiment conducted by Mehrabi and Firouzbakhsh (2019), 0.5% nettle powder supplemented diets improved Final weight, Weight Gain, Feed Efficiency Ratio and SGR. In another study, methanolic extract (0-12%) of nettle was examined on growth performance of rainbow trout within 30 days of feeding, significant rises were recorded in final weight and SGR in supplemented treatments compared with the control (Bilen et al. 2016) with decreased feed conversion ratio and supplementation of diet with various percentages (1-5) % of nettle powder fed to *Labeo victorianus* for 4 weeks revealed significant elevations in final weight and SGR values with improved and feed conversion ratio in comparison with the control (Ngugi et al. 2015). When nettle is used in extract form,

high doses are required for better performance but even low doses of nettle powder can cause positive impact on fish growth which can be more economical and practical.

Treatment 5 i.e., diet with 5% nettle showed highest feed conversion ratio among all treatments which may be because of increased fiber content, lack of adequate multienzyme for interaction with substrate and lower digestible energy in comparison to other diets supporting that the effects of Plants and feed additives as feed additives is dose specific (Awad and Awaad 2017). Similar results were observed in an experiment conducted by Omitoyin et al. (2019), in which 0.50 and 0.75% inclusion of guava (*P. guajava*) aqueous extract significantly decreased the feed conversion ratio whereas 1 % inclusion increased feed conversion ratio.

The average body weight was similar in all treatments up to 75 days of feeding trial. But significant improvement of average body weight was observed in treatment 3 than other treatments after 90 days of feeding trial which demonstrates that growth promotion of fish also depends upon time period of feeding trial. An experimental research on effects of clove basil (*Ocimum gratissimum*) in diets of African catfish (*Clarias gariepenus*) fingerlings for 12 weeks significantly increased the final weight, weight gain and specific growth rate (Abdel-Tawwab et al. 2017) but while experimental trial was conducted for 3 weeks using same species and same Plants, negative results in growth performance was observed (Sogbesan. 2017)

Similarly, supplementation of exogenous multienzyme complex in fish diets also helps to enhance the growth performance in fish by degrading the substrate present in fish feed, removal of antinutritional factors and ensuring the availability of nutrients in fish feed (Zheng et al. 2019). Therefore, diet with 0.05-gram multienzyme (treatment 1) showed better growth performance than diet without multi enzyme (treatment 2). Similar results were observed in an experiment conducted by Ghomi et al. 2012, Zhamini et al, 2012 and Hlophe-Ginindza et al. 2016 where significant improvement on Specific growth rate, final weight and body weight gain was observed with decreased feed conversion ratio in great sturgeon (*Huso huso*), Caspian salmon (*Salmo trutta caspius*) and *Oreochromis mossambicus.*

Protein efficiency ratio was also found highest in diet containing treatment 3 among all treatments. Dietary administration of *H. cordata* leaf, Guava (*P. guajava)* leaves and Dill (*A. Graveolen*) in Nile Tilapia (*O. niloticus*), rainbow trout (*O. mykiss*) also

improved protein efficiency ratio (Garg et al. 2019, Omitoyin et al. 2019, Sendijani et al. 2020). Besides treatment 3, improved protein efficiency ratio was also observed in treatment 1. This may be due to decreased plant product content (Francis, 2001), high digestible energy and effects of multienzyme which ensured the availability of nutrients for rainbow trout (Zheng et al. 2019).

6. CONCLUSIONS AND RECCOMENDATIONS

6.1 Conclusion

A dietary level *U. parviflora* of 1.5% with multienzyme provided significantly better growth performance in comparison to the fish fed other diets due to the combined effects of *U. parviflora* and 1 multienzyme. Also, control diet with multienzyme (T1) also showed better performance than control diet without multienzyme (T2) demonstrating that multienzyme supplementation in diets of *O. mykiss* can also significantly enhance growth performance and feed utilization. The feed conversion ratio was found significantly higher in diet with 5% *U. parviflora* than the other diets due to the presence of unacceptable range of crude fiber.

6.2 Recommendations

a. Further research work is needed to explore *U. parviflora* impact with or without multienzyme on nutrient digestibility, fish health and innate fish immunity.
b. Fish farmers should prioritize using plant products as feed additive to enhance the fish growth and feed utilization instead of chemotherapeutics.

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APPENDIX

PHOTOPLATES I

Photo 1: Experimental set up in raceway of National Fisheries Research Centre, Godawori

Photo 2: Drying of experimental feed in an electric dryer

Photo 3: measurement of temperature of raceway using digital thermometer

Photo 4: Salt water treatment before the stocking of fish

Photo 5: Measurement of weight using electronic weight balance (± 0.1-gm SN-014739, Phoenix instrument)

Photo 6: Measurement of length using ordinary scale (± 1mm) attached with wooden board

PHOTOPLATES II

Photo 1: Measurement of harvesting weight using heavy duty electronic weighing machine

Photo 2: counting the number of fish

Photo 3: Determination of moisture in hot air oven distiller

Photo 4: distillation of sample in kjeldalc apparatus

Photo 5: Titration of distillate with 0.03N H_2SO_4 to determine protein

Photo 6: Addition of petroleum benzene ether into Sample in soxlet apparatus