# FORMULATION OF DIETS FOR *LABEO ROHITA* AND *OREOCHROMIS NILOTICUS* USING PLANT INGREDIENTS AND THEIR *IN VITRO* DIGESTIBILITY STUDY USING pH-STAT METHOD

*A Major project dissertation submitted for the partial fulfillment of the requirement for the degree of*

**Master of Technology in Industrial Biotechnology**


Submitted by

Brijesh Kumar

(2k13/IBT/07)

**Delhi Technological University, Delhi, India**


*Under the supervision of*


Dr. Rina Chakrbarty, FNASS
Professor
Aqua Research Lab
Department of Zoology
University of Delhi, Delhi

Dr. Jai Gopal Sharma
Associate Professor
Delhi Technological University

# DECLARATION

I hereby declare that the M. Tech major project dissertation entitled, **"Formulation of diets for *labeo rohita* and *Oreochromis niloticus* using plant ingredients and their *in vitro* digestibility study using ph-stat method"** submitted by me to Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of M. Tech Industrial Biotechnology is a record of bonafide work carried out by me under the guidance of **Dr. Rina Chakrabarti**, FNAAS Professor at university of Delhi , Delhi.

I, further declare that the work reported in this report has not been submitted, and will not be submitted, either in part or in full, for the award of any other degree or diploma of this University or of any other institute or university.

**Date-29/06/2015**

**Brijesh Kumar**

**Roll No. 2K13/IBT/07**

# UNIVERSITY OF DELHI

## DEPARTMENT OF ZOOLOGY

DELHI-110007


## CERTIFICATE

This is to certify that the M. Tech dissertation entitled **"Formulation of diets for *labeo rohita* and *oreochromis niloticus* using plant ingredients and their *in vitro* digestibility study using ph-stat method"** submitted by **Brijesh Kumar (2K13/IBT/07)** in partial fulfilment of the requirement for the award of the degree of Master of Technology, in Industrial Biotechnology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance at University Of Delhi, Delhi.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree/diploma in India or abroad.

Date: 29/06/2015


**Dr. Rina Chakrabarti, FNAAS**
**Professor**
**Aqua Research Lab**
**Department of Zoology**
**University of Delhi, Delhi**

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*Place: Delhi*

*Date:* *Brijesh Kumar*

# CONTENTS

# LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| FAO | Food And Agriculture Organization |
| NSPs | Non-starch polysaccharides |
| EAA | Essential amino acids |
| NEAA | Non-essential amino acids |
| TI | Trypsin inhibitor |
| ANF | Antinutritional factors |
| BTPL | Basic Technology Private Limited |
| RPM | Revolution per minute |
| DH | Degree of hydrolysis |

## 1. Abstract

Fish the aquatic animal grows good if they are provided enough nutritious food. The phytoplankton, zooplankton etc are natural food of the fish. Due to less abundance of natural food supplementry diets have to be developed. In the absence of natural food, these supplementary diets having complete nutrition can be given to fishes. So in present study the diet was formulated from plant ingredients such as black gram (*Vigna mungo)* (Black gram ), banana leaves (*Musa paradascica*), almond oil-cake (*Phaseolus limensis*), and soyabean chunk (*Glycine max*). These plants feed that contain some inhibiting factors (antinutritional factors). The most occuring inhibiting factors are tannin, saonin, trypsin inhibitors and NSP. These inhibiting factors may be heat liable or heat stable. The estimation of these antinutrients is very important for decreasing their effect on feed by the help of feed extruder. The most common antinutrient is tannin. The estimation of tannin was done in diet ingredients and prepared diets to evaluate the efficiency of extruder. Feed extruder is a machine operating at high temperature to inactivate these heat liable antinutrient. The *in vitro* digestibility of the different feed materials made from the plant ingredients was evaluated. The results of these diets showed that the ingredients used may be promising alternate feed sources in aquaculture feed industry.

## 2. Introduction

Aquaculture can be defined as the farming of the aquatic organisms like fishes, crustaceans, mollusks and aquatic plants for the purpose of research, food and maintenance of these organisms. The major aim of the aquaculture is to increase the production of the protein source derived from the fish. As fish is very good source of proteins so the fish consumption is increasing very rapidly. It was estimated that there are billions of people which eats fish, shellfish, crustaceans, molluscas and aquatic plants provides requisite nutrition. Aquaculture when compared with the terrestrial agriculture system the former one required less time for culture and also the aquaculture can resides on agriculture waste hence less dependence on chemical fertilizers.

The share of aquaculture in the total world food fish production is increasing very rapidly as it is increases 10% since 1984 as compared to 3% increase for livestock meat and 1.6% increase for capture fisheries (FAO, 1997.) To sustain of increase in aquaculture production supplementary diets or alternative diets production need to increase. A large amount of fishmeal is used in marine fishes, trout, salmon etc in western countries. Fishmeal production as done in only some regions of the world and for every country fish meal abundance is very difficult so there is need for alternative protein sources diets as replacement of fishmeal in aqua-feed was an important agenda of the International Symposium for Sustainable Aquaculture held in 1998 in Oslo, Norway. The alternate protein sources that can be used are plants byproducts, agricultural wastes, plant leaves, etc. The main problems with these plant sources are the presence of the inhibiting factors like tannins, saponis, phytates etc. Anti-nutrients have been defined as substances produced as in many metabolic reactions reacts with many physiological reaction and affect animal health (Makkar, 1993.). These anti-nutrients like protease inhibitors, tannins and lectins which affect protein digestion while others like phytates, oxalates, which affects protein

utilizations. Antinutrients may also be divided accourding to heat stability. Heat labile factors like PI , phytates and antivitamins, whereas others as heat stable factors which are saponins, NSP , antigenic proteins and some phenolic compounds (Peol, 1989; Rumsey *et al*., 1993). A large number of different processing techniques that increase the protein and starch digestibility of different plant ingredients.(Alonso and Marzo, 1998). The feed extruder which operates at high temperature decreased the content of inhibiting factors.

Proteins are the largest component of fish diets which are the very essential for the growth, reproduction, and for different physiological process. Their amino acids chains play very significant role in different modifications. these protein sources are very costly (Garcia *et al*., 2000). Protein requirements for different growing fish generally lies between 30-55% of total input diets (Rahnema *et al*., 2005; Trushenski *et al*., 2006; Kaushik and Seiliez 2010). Fish usually needs the balanced amount of essential amino acids (EAA) and nonessential amino acids. The fish tissue mostly formed from available 20 major amino acid in which there are 10 amino acids that cannot be synthesized and therefore these should present in the artificial diets. The ten EAA are:, tryptophan histidine, phenylalanine, methionine, isoleucine threonine lysine, leucine, arginine , and valine (NRC, 2011).

The digestibility of the prepared artificial diets is very important because the success of the feed depends on the digestibility. Digestion is the process of breakdown of the food into smaller molecules by the help of the enzyme, gastric juice, teeth etc. The digestion most frequently depends on the pH of the digestive enzymes, gastric juice, emulsification of the lipids and the nature of feed. During formulation of diet it should be keep in mind that the diets should be balanced so that proper digestion of food can take place.

Indian major carp *Labeo rohita* rohu and Nile tilapia *Oreochromis niloticus* (family: Cichlidae) are two commercially important fish species. Rohu is herbivore mid-column feeding species. Tilapias are most commonly produced fish all over the world for the food purpose (Stickney, 1986). Tilapia is a voracious feeder of plant materials. *Tilapia aurea, Tilapia niloticaare, Tilapia mossambica* are very common species of the tilapia and different hybrids have been studied (Strickeny, 1986).Tilapia is the fish which have high growth rate, good tolerant to large stocking densities and

also can tolerate some level of poor water quality, high reproductive rates, and resistance to many diseases. So tilapias are very excellent species for aquaculture production (Stickney, 1986; Chamberlain, 1993).

Considering the above knowledge present study has been designed to formulate diets using plant ingredients and to evaluate the effect of plant incorporate diets in rohu and tilapia. The objectives of the present investigation are:

- ❖ Formulation of artificial diet for the fishes using plant ingredients.
- ❖ Feed preparation with the help of the feed extruder from the plant ingredients.
- ❖ To check the effectiveness of the extruder in removal of the anti-nutritional factor (tannin) from the formulated feed to make it more digestible.
- ❖ To check the degree of hydrolysis (DH%) of formed feed from the plant ingredients.

# Review of literature

## 3.1. Plant ingredients diets

Aquaculture contributes to the household animal protein and income supply. The success and expansion of aquaculture depends on reducing the operational cost of diets. The major feed which is available in the market, fish meal, is too costly and there also a lot of competition with other different feeding animal industries for the available demand of fish meal. So, the utilization of plants and its derived materials in fish feeds is getting very important role in the world. Mukhopadhyay *et al*. (2006) studied the feed or nutritive potential of four different aquatic weeds namely, *Trapa natans Salvinia cuculata,, Ipomoea reptans* and *Lemna minor* for determining their importance in utilization as alternate fish diet which aim was to bring down the cost of available commercial feeds. These different prepared diets having protein content in the range of 11-33%. While the ratio of protein to energy (P/E) of these different weeds were in the range of 31-96 mg/Kcal and the highest value was displayed by *Ipomoea reptans*. These aquatic weeds also contained high amounts of vitamins E and C and mineral elements which are very essential for normal growth and development of fishes. Study analysed that the of trypsin inhibitor (TI) was in the range 1-1.5%,in gram while the concentration of the calcium oxalate was found in the range of 0.6% to 3%, while the concentration of the tannin was in the range of 0.25-0.93% and phytate concentration varied from the 0.003% -0.006%. So this study showed the, cost effective and commercial utilization of the aquatic weeds particularly *Lemna minor* and *Ipomoea reptans* and, for the preparation of artificial diets which may be easily available also for the farmers.

In another study the food processing waste and partially post-consumption waste classified as in different categories: vegetables and fruits, cereals, meat products, and bones were used for making diets. The prepared diets were checked for these traditional fish: mud carp (*Cirrhina molitorella*), bighead carp (*Aristi chtysnobili),* and grass carp (*Ctenopharyngodon idellus*) (Zhang *et al*., 2013).

The effect of different protein sources for salmonid in which the commercially available fish meal was reolaced with the plant ingredients was studied (Katheline and

Dominique, 2012). They have compared and interpreted the results of the control diets with various other feeds given in very little amount or at the moderate or little higher and these prepared diets showed no effect for the fish. However when the fish meal was replaced at very high amount the result showed the depression in the growth of the fish while the diet prepared from these ingredients were balanced in composition of the nutrients.

A large number of studied showed the various effect of using plant ingredients in place of fish meal like cottonseed meal (Rinchard *et al.*, 2002) gluten meal (Wu *et al*., 1995), soyabean meal (Wee and Shu, 1989, Shiau *et al*., 1989, Webster *et al*., 1992), lupins, (Fontainhas- Fernandes *et al*., 1999) rapeseed (Davies *et al*., 1990), maize and distillers dried grains with soluble (Coyle *et al*., 2004) for different fishes like tilapia, rohu, etc. However when there was complete replacement of fish meal by the individual plant proteins, then the result showed large decrease in fish growth performance (Sklan *et al*., 2004; Mbahinzirek *et al*., 2001). This decrease was because of the presence of different antinutritional factors in these plant proteins, like soyabean meal (Bureau *et al*., 1998), cottonseed meal and rapeseed meal.

Vegetables which are very common contain different nutrients which include phytochemicals, minerals and vitamins. The photochemical or ANF includes saponins, alkaloids, triterpenoid and glucosides. Phytochemicals or ANF is the type of the molecules which are non-nutritive plant chemicals. These are also called as the secondary metabolites which have resistance to fight against the different diseases like metabolic syndrome, cancer and stroke Most of these chemicals are produced by plants itself to protect against different diseases and pests and some of these chemicals showed that they can protect human again some diseases. So the green plants are the great reservoirs for the chemotherapy and provide a lot of sources of natural pesticides. Saponin is inhibitor which is the causative factor of the rupture of the blood cells in the fish and some animal also is of different types and the type also depends that whether the plant was cultivated or it was not cultivated like tripenoids saponin and the cultivated herbal plants used as medicines contain steroidal saponin. Vegetable of different types could be wild or cultivated for food purpose need to be

generally processed before consumption (Olusola *et al*., 2013). Saponins are also known to causative agents of hemolysis (lysis of erythrocytes with that leads to release of hemoglobin), have a bitter taste, and it is very toxic to cold-blooded animals. Even though these attributes are not common to all known saponins, they are sometimes used to characterize this class of compounds. Many plant drugs and folk medicines, especially those that have origins in Asia, contain saponins. The non-sugar or the aglycone unit of the saponin molecule is called as genin or sapogenin. The saponins molecules are generally divided into the three major classes on the basis of the structure of genin: steroid glycosides, Triterpeneglycosides and steroid alkaloid glycosides (Madland, 2013). Various studies showed that tannin is very common antinutritional factor present in different plant ingredients tannin estimation in feed is very important.

### 3.2. Tannins

Tannins are mainly the secondary compounds of different chemical structures which is major inhibitor in the plants and vegetables and the tannin is generally divided into condensed tannins and hydrolysable. Tannin is the compound which affects the digestive process by binding to different component available in the feed like minerals, vitamins, carbohydrates and amino acids (Liener, 1989). Tannins also affect the absorption of vitamin B12. In a study it was found that Common carp (*Cyprinus carpio*) was able to survive or with no effect of 2% condensed tannin in the feed. So at this level the fish was growing and fish health was also good but the same amount of the hydrolysable tannin showed that the similar fish rejected the feed after 28 days (Becker and Makkar, 1999).The hydrolysable tannin is easily degraded I the water in comparison to the condensed tannin, So this hydrolysable tannin easily bind to the feed complexes and affecting the physiological process forming smaller compounds that can enter the blood stream and after a certain period of time this will cause serious problems to different organs e.g. kidney and liver . The naturally occurring in plant products shoul be investigated before considering for diets preparation. In a study it was found that Condensed tannins which is present in the copra at concentration of the 3.4% could have been the causative agent for the growth depression in commonly available tilapia (*Oreochromis niloticus***)** and rohu

(*Labeo rohita*.) fingerlings at a such low level of concentration(Jackson *et al*. 1982; Mukhopadhyay and Ray, 1999). Other feeds which contains tannin like peaseed meal (*Cajanus cajan*) and rapeseed (*Brassica napus*) showed that there is difference in tolerance limit of tannin between different fishes. Some fishes showed very good tolerance to tannin while some at very low level could not survive. Broad bean (*Vicia faba)* meal, which have very high amount of the condensed tannin content, showed lower protein digestibility than soybean in an *in vitro* experiments (Grabner and Hofer, 1985). Tannins are the type of the inhibiting factors which interact with other antinutrients. For example, the interaction between tannins and lectins reduced the inhibitory action of tannins on amylase (Thompson, 1991). Different plant ingredients, their antinutritional factors and protein percentage were given in table 1.

**Table1:** Different plant ingredients – antinutritional factors and protein percentage

| **Plants** | **Protein Contents(%)** | **Major Inhibitors** | **References** |
|---|---|---|---|
| *Coffea arabica* (Coffee) pulp | 10.0 | Tannins and polyphenols | Didanna, 2014 |
| *Trapa natans (*Water chestnut) | 11.4 | Trypsin inhibitor (TI) tannin and phytic acid | Kalita *et al*., 2007 |
| *Salvinia cuculata* (water fern) | 11.0 | Trypsin inhibitor (TIA tannin and phytic acid | Kalita *et al*., 2007 |
| *Pennisetum glaucum* (Pearl millet) | 12.0 | Phytate and tannin | Lestienne *et al*.,2005 |
| Oryza sativa (Rice) | 13.0 | Phytate | Hou *et al*., 2013. |
| *Musa acuminate* (Banana) leaves | 16.1 | Saponin, tannin and phytate | Dongmeza and Steribronn, 2009 |
| *Manihot esculenta* (Cassava) Leaves | 23.1 | Saponin, tannin and phytate | Dongmeza and Steribronn, 2009 |

| | | | |
|---|---|---|---|
| *Musa paradascica* (Bamboo)(leaves) | 15.3 | Saponin, tannin and phytate | Dongmeza and Steribronn, 2009 |
| *Zea mays* (Maize) | 9.4 | Trypsin and phytate | Wikipedia |
| *Cicer arietinum* (Chickpea) | 24.0 | Protease, amylase inhibitors and lectins | Bampidisa and Christodoulou ,2011 |
| *Vigna mungo* (Black Gram) | 26.7 | Tannin and protease inhiitors | Sreerangarajua and Krishnamoorthy , (2000) |
| *Phaseolus vulgaris (*Kidney beans) | 24.0 | Protease, tannin and phytate | Mallikarjunan and Marathe, 2014 |
| *Arachis hypogaea* (Groundnut) cake | 42.1 | Protease, phytate | Ghadge et al., 2009 |
| *Glycine max* (Soyabean) | 46.2 | Protease, phytate and saponins | Ghadge et al., 2009 |
| *Prunus dulcis* (Almond) fruit mesocarp | 0.1 | Tannin | Nwosu *et al*., 2008 |
| *Hyphaene thebaica* (Dulm palm) | 0.01 | Tannin | Nwosu *et al*., 2008 |
| *Lemna minor* (Duckweed) | 32-34 | No inhibitor | Yılmaz *et al*, 2004 |
| *Vigna mungo* (Black lentil) | 29-30 | Protease inhibitor | Keembiyehetty and Silva,1992 |
| *Echinochloa crus-galli* (Baryard grass) | 14.1 | Tannin, phytic and saponin | Dongmeza and Steribronn, 2009 |
| *Pennisetum purpureum* (Napier grass) | 16.3 | Tannin, phytic and saponin | Dongmeza and Steribronn, 2009 |
| *Morus alba* (Mulberry) (leaves) | 25.2 | Tannin, phytic and saponin | Dongmeza and Steribronn, 2009 |

| | | | |
|---|---|---|---|
| *Arachis hypogaea* (Peanut leaves) | 17.6 | Tannin,phytic,andsaponin | Dongmeza and Steribronn, 2009 |
| *Ipomoea batatas*(Sweet Potato)(leaves) | 17.4 | Tannin, phytic and saponin | Dongmeza and Steribronn, 2009 |
| *Manihot esculenta* (Cassava)(Peels) | 41.2 | Tannin and phytic | Dongmeza and Steribronn, 2009 |
| *Manihot esculenta*(Cassava)(Tubercule) | 14.1 | Tannin and phytic | Dongmeza and Steribronn, 2009 |
| *Mangifera indica* (Mango) leaves | 8.7 | Trypsin inhibitor | Abarike *et al.*, 2014 |
| *Vitellariaparadoxa* (Shea) Leaves | 35. | Tannin and phytate | Abarike *et al*., 2014 |
| *Allium cepa*(onion) | 1-3 | Tannin and phytate | Olusola *et al*., 2013 |
| *Allium sativum*(garlic bulb) | 2-3 | Tannin and phytate | Olusola *et al*., 2013 |
| *Tetracarpidium conophorum* (Walnut) Leaves | 15-16 | Phytic acid | Olusola *et al*., 2013 |
| *Tetra selmischuii* (Microalgae) whole | 39.0 | Trypsin inhibitors | Olusola *et al*., 2013 |
| *Phaeodactylum tricornutum*(Microalgae) Whole | 35.0 | Trypsin Inhibitors | Olusola *et al*., 2013 |
| *Euglena viridis* (Microalgae) Whole | 32-35 | Trypsin Inhibitors | Olusola *et al*., 2013 |
| *Andrographis paniculata*(Nees) Leaves and shoots | 32.14 | Phytate ,tannin | Olusola *et al*., 2013 |
| *Lonicera japonica* (Honey suckle) Leaves | 8-12 | Phytate ,tannin and saponin | Olusola *et al*., 2013 |

| | | | |
|---|---|---|---|
| *Ganoder malucidium*(lacquered)(Leaves) | 25-35 | Phytate, tannin and saponin | Olusola *et al*.,2013 |
| *Calotropis gigantea* + *Azadirachta Indica* (Akanda + neem leaf) | 30-35 | Trypsin inhibitors and phytate | Olusola *et al*., 2013 |

### 3.3. Diet extruder

Extruders are generally the screw pumps in which the different diet mixture is forced and in this process is subjected to heat, pressure and shear forces. Extrusion is a process, which includes several unit operations including cooking, kneading, mixing, forming ,shaping and shearing, There are the some factors that most affects the nature of the different extruded product are the operating parameters of the extruder and the different rheological properties of the food. The most important operating parameters which mostly affects are the pressure, temperature, diameter of the die apertures and shear rate. The diet extruder is of mainly two types as mentioned below (Vijaygopal, 2014).

#### 3.3.1: Twin screw extruders

Twin screw extruder is a better design because in this one screw wipes out the cavity of the other screw thus ensuring positive displacement of diet materials through the barrel thereby preventing burning out of products which was found in single screw extruders. Moreover, a single screw extruder requires elaborate drying, utilizing higher energy. In a twin screw extruder, lower moisture content in feed ensures that it takes less time for drying. In addition the feed nutritional value is more in case of twin screw extruder (Vijaygopal, 2014).

#### 3.3.2: Effects of extrusion on proteins

Extrusion effects denatured protein and undenaturded protein in different ways. Denatured proteins are the types of the proteins which have been pre-cooked up

to some extent in which some of the available amino acid chains have been broken up into single amino acid units or into shorter chains (fish meal, meat meal, and extracted soyabean meal). So these proteins are not able to form gel during extrusion. So they they do not contribute in the binding of the different raw material mix. Undenatured proteins are the type of the proteins that have been not previously heat-treated. So their amino acid chains are not damaged and under some extrusion conditions these are capable for gel formation in much the same way as starch do, so that these materials can act as a binder.

### 3.3.3: Effects of extrusion on carbohydrates

The capability of the starch molecules to absorb water serves as the test for the degree of gelatinization. However, there is need to take care when applying this test as some extrusion processing conditions further changes starch structure and this process is known as dextrinization. A stage ahead of the gelatinization at which the starch molecules start to break down in various smaller parts known as dextrin, and the process is known as dextrinization. Dextrins are polysaccharides of intermediate chain length. These smaller molecules get dissolves in the water as it should have absorbs water. Extrusion process occurring at temperature and moisture of medium level results in good amount of gelatinization and a low amount of dextrinization. When the temperature level gradually increases and the addition of moisture decreases. As a result gelatinization and dextrinization will start to increase. And if this process remains continue a point is reached at which all the starch present in diets is gelatinized and some of it is dextrinized. After that point dextrinization increases and the amount of gelatinized starch will decrease and the gelatinized molecules continue to be broken down into dextrins and some other small molecules. The effect of all the above process is that water adsorptive capacity of the starch molecules and solubility increases in line with extrusion having condition of the increasing temperature and decreasing moisture until a saturation point is arrived at which solubility still continues to rise but the adsorptive capacity of the starch molecules starts to fall. The properties of extruded starch can be manipulated by varying different parameters of the processing conditions.

### 3.3.4. Effects of extrusion on fats

Fat is the bio molecule which is made up of fatty acids. These fatty acids may be of essential or non-essential type of fatty acids Fat is molecule which acts as lubricant. So fat molecules generally increase the density of the feed and also form complexes with the starch and inhibiting the gel forming characteristics of this material. In general practice, the fat levels in the extruded mix should be less than 6% if floating feed have to be prepared and higher for the sinking feed up to 15%. To some nutrition we need higher value of fat so that can be added after extrusion. The expansion produced by extrusion is mostly created by moisture that was trapped within the mixture as vaporizing into steam when the material exits the die.

### 3.3.5. Potential of extrusion in aquaculture feed manufacture

The production of diets for aquaculture is providing a promising and a sector with the fastest growth in the global feed industry. Although today alot amount of aquatic feed and different animal feed is still made by old and convenient pelletizer technology, extrusion is used in various food industries because it offers several advantages over the palletizing which include the following:

- Buoyancy can be controlled so it is easy to produce floating and slow sinking feeds.
- Extruded feeds are capable of carrying higher fat levels than pelleted diets.
- For many species extrusion also increases carbohydrate availability.
- Extrusion increases the rate of the destruction of micro-organisms and some other contaminants.
- Extruded pellets are generally having greater physical integrity and produce fewer fines.
- Under some conditions the extruded products shows greater water solubility than normal feeds,
- Wet materials such as the fresh minced fish can be readily incorporated into extruded products.

**Table 2:** Type of extruders

| Extruder | Temperature(°C) | Pressure(bar) | Max. Fat (%) | Moisture (%) |
|---|---|---|---|---|
| Single screw | 80-140 | 15-30 | 22 | 15-35 |
| Twin screw | 80- 160 | 15-40 | 27 | 10-40 |

### 3.4. Estimation of antinutritional factors:

Estimation of these factors can be done by the various methods. Depending upon the amount of these factors the different methods can be chosen. The different methods that are commonly used for the estimation of these Factors are:

#### 3.4.1. HPLC method:

This method is very sensitive and costly for the estimation. HPLC which is called as High Performance Liquid Chromatography and also known as High Pressure Liquid Chromatography. HPLC method that has been used from around 35 years and also can be said as one of the largest separations technique. History of HPLC: it was from the years of 1960 where it has been called as High Pressure Liquid Chromatography. This is most widely used as separation technique as it has very broad applicability for organic and inorganic molecules as it is sensitive, accurate and precise. This is very suitable for separation of the molecules which are nonvolatile in nature and are used in numerous uses in industry, environmental areas, clinical settings, pharmaceuticals, etc. HPLC is very common in pharmaceuticals for separation technique because the purity level need to be very high. It involves the injection of the small volume of liquid sample into a tube which is packed with very tiny particles of diameter 3 -5 micron (µm) in known as the stationary phase in which different components of the test sample moves down along the column which is also packet liquid column called as mobile phase. In this mobile phase the molecules is being forced through the column by pressure which is delivered by a pump. An output from the detector which called as “liquid chromatogram”. These plant molecules are also used in various drugs. So when these secondary molecules need to be separated

from the plants for drugs the HPLC method should be used but as for estimation less costly less time taking spectrophotometric method can be used which is discussed below.

### 3.4.2. Spectrophotometric method

Spectrophotometry is a method in which the different chemical substances chemical substance absorbs light. The absorption of the light varies according to the amount of the chemical substances present in the samples. By measuring the intensity of light as the beam of light passes through sample solution. As different compounds possess different colors. The intensity of these a color is proportional to the concentration of the compound. This spectroscopy is off many types depending upon the wavelength used like visible, UV, infrared spectroscopy. Normally the spectrophotometer are designed to measure the diffusivity on any of awider range of the light that usually that covers mostly 200 nm to 2500 nm, Depending upon the materials and the standards to prepare the different range can be selected.

## 3.5. Diet formulation

For diet formulation there are several methods which are used for making the different animal's diets. For making of animal diet it is very important that the different ingredients should be mixed in the ratio that the nutritional value of the diet should be desired.

Some of the method which has been listed here are:

- computer method
- simultaneous method
- 2 × 2 matrix method
- square method
- trial-and-error method
- Pearson Square

### 3.5.1. Trial and error method

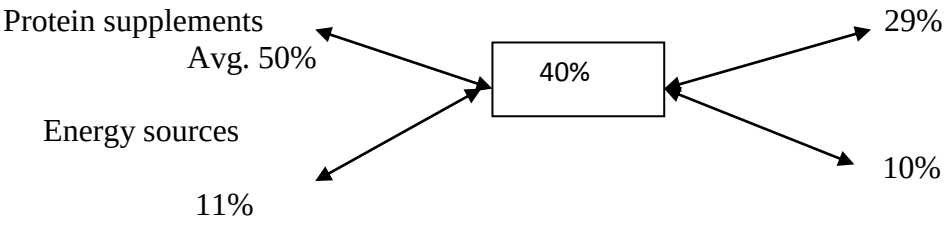
Table 3**:** Choose a combination of the ingredients shown in Table 3 that will provide a feed containing 30 to 40% crude protein.

| Ingredients | Amount of ingredient (kg) | Crude protein in ingredient (%) | Crude protein in feed (%) |
|---|---|---|---|
| Rice bran | 22 | 10 | 2.2 |
| soybean oil meal | 30 | 45 | 13.5 |
| Fish Meal | 28 | 60 | 16.8 |
| Wheat floor | 20 | 12 | 2.4 |
| **Totals** | **100** | | **34.9** |

### 3.5.2. Pearson square method

The method that was used for the diet formulation was Pearson square. This method is simple and easy to use. It gives quick substitution of diet ingredients without disturbing protein content. This is very important to note that in this method balances crude protein requirements only. There is no consideration given to the energy, vitamin, mineral or other nutritional requirements. So only one consideration of protein is taken for the diet formulation considered the protein content of the diet should be 40%. For this one input was protein source and other was energy source. The solution is to make two groups into the ingredients, the first ingredients should be taken whose crude protein percentage is higher than the requested and the second one should be of ingredients whose crude protein is lower than the request. In this method the first group will be consisted of fish meal and alternate meal and then the second group which will consist of wheat meal. So first calculate the average crude protein percentage for first group and then it should do the same for second group (Bolorunduro, 1995).

Two or more than two ingredients: To find the proportions of wheat flour (12%), rice bran (10%) and fish meal (60%), and Soya source (40%) required to make a feed containing 40 % crude protein. First draw a square. Placed the desired protein level at the center of the square. As,40%. Now group the ingredients into energy sources) and protein supplements sources. Calculate average of protein supplements and energy sources and place them at left input corner. If protein supplements are to be mixed in 1:1 ratio then divide the obtained ratio equally or if 2:1 then mix the obtained amount in 2:1 ratio .Similar for energy source also. Place the average value of protein supplements (Avg. of fish meal and soya source 50) and energy source (Avg. of wheat flour and Rice bran 11) on the two left corners of the square. Calculate the difference in crude protein content of these two (50 and 11) and place this number (39) near the lower left corner of the square. Subtract the decided protein level (40 %) of the feed from the protein content of each Ingredients and record the answer at the corner diagonally opposite of each ingredients. .The difference between percentages of protein in energy sources and in the feed (29%) denotes the amount of protein supplements needed. The difference between protein supplements source and the feed (10%) denotes the amount energy sources of needed. Add the difference. So the calculated difference should be equal to 39 (quantity at left corner).




Difference=39 Add = 39

### 3.6. Protein estimation

The protein estimation is a very important study for almost every research. The protein estimation can be done with various methods. Total protein estimation is used to analyze compounds in several areas like industrial, agricultural, aquaculture and biotechnology products. They are very basic for research purposes, especially for calculating the specific activity (i.e., total activity/total protein) of enzymes, antibodies, and lectins. It is very important to note that, accuracy and precision for the specific activity measurements depend as much on how accurate measurement was of total protein as on determination of total activity. The different methods which are discussed here in short are – Some are copper-based assays for quantitate estimation of the total protein: the biuret method having some variation with the Lowry method, and the bicinchinonic acid (BCA) assay. Acid hydrolysis of the proteins can be coupled with ninhydrin test to determine quantitate amino acid content of a sample. The Ultraviolet spectrophotometry which is a spectrometric method used to measure total protein and used to evaluate samples for the presence of contaminants in disease, food and toxic etc. The Coomassie dye which is a binding dye used in Bradford assay. It is a quite simple, easy and that's why used frequently, although sometimes it gives variable response depending on the extent of protein binds the dye in acid pH.

### 3.7. Bradford assay

Bradford assay is a common method for the estimation of the total protein to determine the enzyme activity of any protein in different samples like food, toxic etc. Coomassie dye which at the acidic pH binds with the protein molecules. The triphenylmethane group of the dye which binds with the different nonpolar structures present in the proteins and the sulfonate anion groups of the dye reacts with cationic side chains (like arginine and lysine side chains) present in the protein molecules at acid pH (Lovrien *et al*., 1995). As this methos is very simple and very little time consuming and also less costly this method is mostly used for protein estimation. Bovine serum albumin is used to calibrate the Bradford assay.

### 3.8. Micro-Kjeldahl method to measure total protein

This method is used for the determination of nitrogen occurring in the trinegative state in different samples of food and raw materials.This method is generally used for the determination of total protein in plant samples. This method mainly consists of three steps: 1) Digestion is the step in which the sample or plant ingredients are boiled with the sulphuric acid in the presence of the catalyst (PotaassiumSulphate and Sodium Sulphate). The nitrogen present in the sample is converted to ammonia. 2) Distillation is the second step in which ammonia which was trapped by the sulphuric acid solution is released from the ammonium sulphate after the addition of the excess amount of sodium hydroxide 3) Titration is the last step to analyze the end point or to measure the amount of released ammonia.

### 3.9. Enzyme Activity

Enzyme activity is measured to rate of the reaction. Simply it can be said as the speed of changing the substrates into the products. The enzyme activity estimation is generally done *in vitro*. If the parameters affecting the condition are set correctly the n the activity measured will show close resemblances with the in vivo activity, (Sedmak and Grossberg, 1977).The factors which mostly affect the activity of the enzyme are), temperature, ionic strength, substrate concentrations, pH and nature of salts present .Enzyme inhibition is another very important parameter should keep in mind while measuring the enzyme activity. A lot of inhibiting substrates may present in the solution when measuring the activity. The enzyme inhibitors may be off different type like competitive or noncompetitive or mixed one. Generally the enzyme when reacts with the substrates possess hyperbolic curve and that curve may be studied using the formula called as Michaelis– Menten equation. The formula is as, V= (VmaxS/Km+S) and in this equation Vmax is the maximum speed of the reaction, whereas {S} denotes the concentration of substrate. The substrate concentration that is used for the measurement of the activity should be chosen keeping minds other factors like solubility, stability etc. (Van Kley and Hale, 1977). The rate of the

reaction decreases more rapidly when the concentration of the substrates decreases which is shown in fig, 1.

The enzyme which does not obey the above equation are called as allosteric enzymes. (Fig.1). these allosteric enzymes are the enzyme which required an activator. These enzymes possess very specific properties like some may be active over a large range of pH while some in very short range (Robert, 2000). Total protease activity is the activity of the total different enzymes present in the test which breaks the protein molecules into smaller molecules.

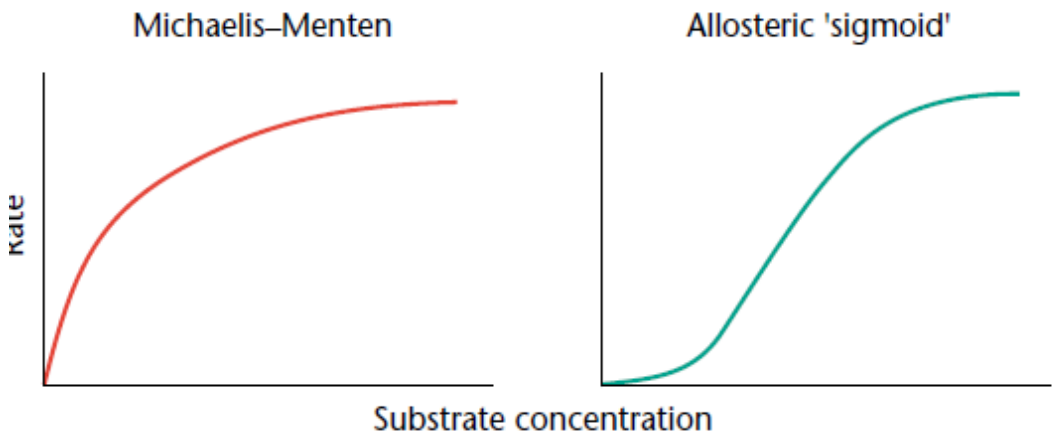



Fig 1: Enzyme – Substrate graph

### 3.10. *In vitro* digestibility with pH Static Method

*In vitro* digestibility study has been reported as one of the most useful method to understand the digestibility of diet in fish (Lee and Lawrence, 1997) and many authors have used it to study digestibility in different fishes (Ezquerra *et al*., 1997; and Tibbetts *et al*., 2011). *In vitro* techniques has became very important method for the evaluation of ruminant feeds (Alarcon et al., 2002). However, this methods presently available and still time consuming (Lemos *et al*., 2009 ) . *In vitro* digestion can that can match with the in vivo digestion if all the parameters are set very natural to the in vivo. The pH-Stat assay method is a method which based on the measurement of the broken bonds by the enzymes or total protease. (El-Mowafi *et al*.,

2000) The aims of the present investigation were to evaluate the protein content of easily available plant ingredients and estimate their in vitro digestibility in the two economically important fish Tilapia and rohu. The results from this study will be useful for evaluation on digestibility of plant ingredients may help in replacing costly fish meal for the production of cost-effective and easily digestible diet for these important carps. *In vitro* digestion method was developed which simulates the conditions in the digestive tract of a fish as closely as possible. The physiological pH, water content in the stomach, optimum temperature value, gut, proteolytic activity and passage time through the stomach and from the gut are taken into consideration. As the name suggests that this is a method in which pH will remain the static.

## 4. Materials and methods

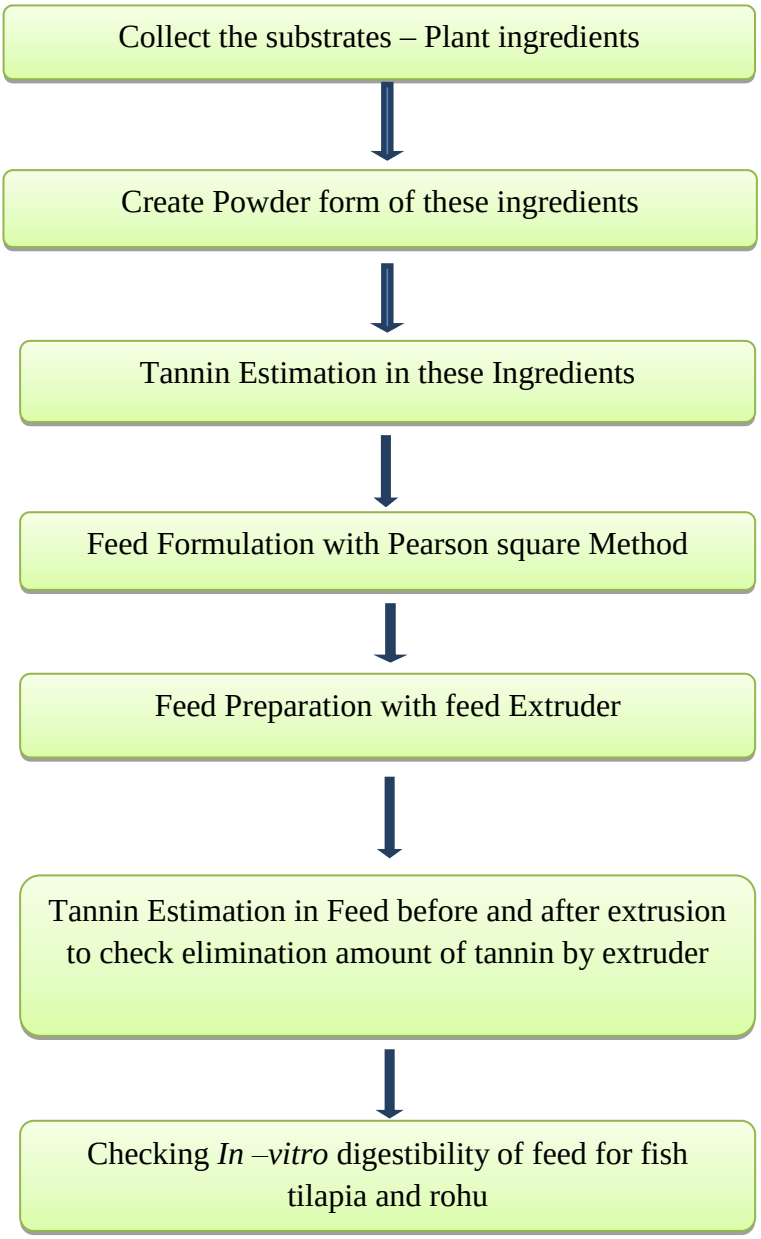



Fig. 2. Basic scheme of experiment.

### 4.1. Plant ingredients

The leaves of banana (*Musa paradascica*), almond oil-cake (*Phaseolus limensis*), black gram (*Vigna mungo*), and soyabean chunk (*Glycine max)* were collected from the local markets and from the DTU campus and was kept to dry in an oven at 65°C. Then these ingredients were ground using mixer and stored in an airtight container to protect from moisture and contamination. These powdered materials (Fig. 3a-d) were used for further study of biochemical estimation (tannin, protein) and *in vitro* digestibility).


Fig. 3(a) Powder of banana leaves, (b) Powder of almond oil-cake (c) Powder of black gram (d) powder of soyabean chunks.

### 4.2. Experimental Fish Diets

#### 4.2.1. Formulation by Pearson square method

Pearson square is mathematical method which can be used for more than one ingredient. The four different diets (containing 40% protein) with black gram (diet 1, D1), soyabean chunk (diet 2, D2), banana leaves (diet 3, D3) and almond oil cakes (diet 4, D4) were formulated using Pearson square method. In D1, D2 and D4, the protein source fish powder was substituted with respective plant ingredient in 1: 1 ratio, in case of D3, fish powder and banana leaves powder were in 2:1 ratio. In all diets wheat flour was used as energy source. Apart from these some more ingredients were used for the preparation of diets (Table 4) were cod liver oil, vitamin and mineral premixes.

**Table 4.** Composition of various plant incorporated diets.

| | Diets | | | |
|---|---|---|---|---|
| **Ingredients(g/Kg)** | **D1** | **D2** | **D3** | **D4** |
| Fish powder | 445 | 325 | 285 | 360 |
| *Vigna mungo* (Black gram) | 445 | _ | _ | _ |
| *Glycine max* (Soyabean chunk) | _ | 325 | _ | _ |
| *Musa paradascica(*Banana leaves) | _ | _ | 145 | _ |
| *Phaseolus limensis (*Almond oil-cake) | _ | _ | _ | 360 |
| Wheat flour | 96 | 316 | 53 | 246 |
| Cod liver oil | 20 | 30 | 15 | 30 |
| Vitamin and minereal complex | 4 | 4 | 2 | 4 |
| **Total** | **1000** | **1000** | **500** | **1000** |

### 4.3. Preparation of diet using Twin screw extruder

After diet formulation the fine powders of all ingredients were mixed thoroughly, 10-15% water was added and mixed properly using a blender. After mixing it was sieved (500 µm). The amount of water varied according to the nature of the diet (floating or

sinking) to be prepared. For floating type of diet less water was added and for sinking more water was added. According to the desired diet size different dies (2.5 and 3 mm) were used. Colouring substances were used for the development of colour of the experimental diet to attract the fish. All test diets were prepared using Twin screw extruder (BTPL, Kolkata, lab model) (Fig. 4a) with control panel (Fig. 4b). The following conditions were used for the preparation of diets: extruder RPM 180-250, dieter RPM 5-7, cutter speed 950-975 RPM, torque 5-10 N.m. The temperature was 60-75°C. After setting all these parameters the prepared mixture was added continuously in the dieter and the prepared diet was collected in a clean tray (Fig. 5a). The prepared diet (Fig. 5b) was dried in oven at 60-65°C for 10-15 min. After diet preparation, jamming or barrel cleaning inching was performed. The prepared diets were of two types sinking (Fig.6a-c) and floating (Fig.6d) whose amount shown in table 5.

**Floating diet**: This is the type of the diet which has fewer densities and it floats on the surface of water. This diet is suitable for the fish which are surface feeder. For black gram 670 g of floating diets was prepared.

**Sinking diet**: This is the type of the diets which has higher densities and it sinks and goes to the bottom of water. As this diets is suitable for the fish which are bottom feeder. As in diets mixture proper binder or wheat flour and oil was present so it was stable in the water.

Fig. 4. The feed making unit, (a) Twin screw extruder (b) Panel board


Fig. 5 (a) Collection of diet, (b) Prepared diet.

**Table 5.** Amount of diets.

| **Diets** | **Floating diet (g)** | **Sinking diet (g)** | **Total diet (g)** |
|---|---|---|---|
| *Vigna mungo (Black* gram) (D1) | 670 | 220 | 880 |
| *Glycine max (Soyabean chunk)* (D2) | _ | 845 | 845 |
| *Musa paradascica* (Banana leaves)(D3) | _ | 435 | 435 |
| *Phaseolus limensis* (Almond oil cake) (D4) | _ | 880 | 880 |


Fig.6.(a) Soyabean diet (D2), (b) Almond oil cake (D4), (c) Banana leaves diet (D3), (d) Black gram diet (D1)

### 4.4. Fish species

Two different species *Labeo rohita*, rohu and *Oreochromis niloticus*, tilapia were used for *in vitro* digestibility study. Rohu (length: 34.5±2.3 cm, weight: 330.51±30 g) and tilapia (length: 22.4±2.5 cm, weight: 150.5±22 g). These fishes were taken from Jahangirpuri, Delhi and were brought carefully to the wet laboratory facility. The scientific classification of these two fishes is as follows (Table 6).

**Table 6**. Classification of fishes.

| | **Rohu** | **Tilapia** |
|---|---|---|
| **Kingdom** | Animalia | Animalia |
| **Phylum** | Chordata | Chordata |
| **Class** | Actinopterygii | Actinopterygii |
| **Order** | Cypriniformes | Perciformes |
| **Family** | Cyprinidae | Cichlidae |
| **Genus** | *Labeo* | *Oreochromis* |
| **Species** | *rohita* | *niloticus* |

### 4.5. Maintenance of fish

Fish were separately maintained in the plastic tanks (40x30x30 cm) for 5-7 days for acclimation (Fig. 7.a, b). Fish were fed twice daily with diets containing 40% protein. Excess food and excreta were removed regularly by siphoning. Fishes were kept in fasting condition for 72 h before sampling for complete evacuation of the digestive tract for *in vitro* digestibility study.

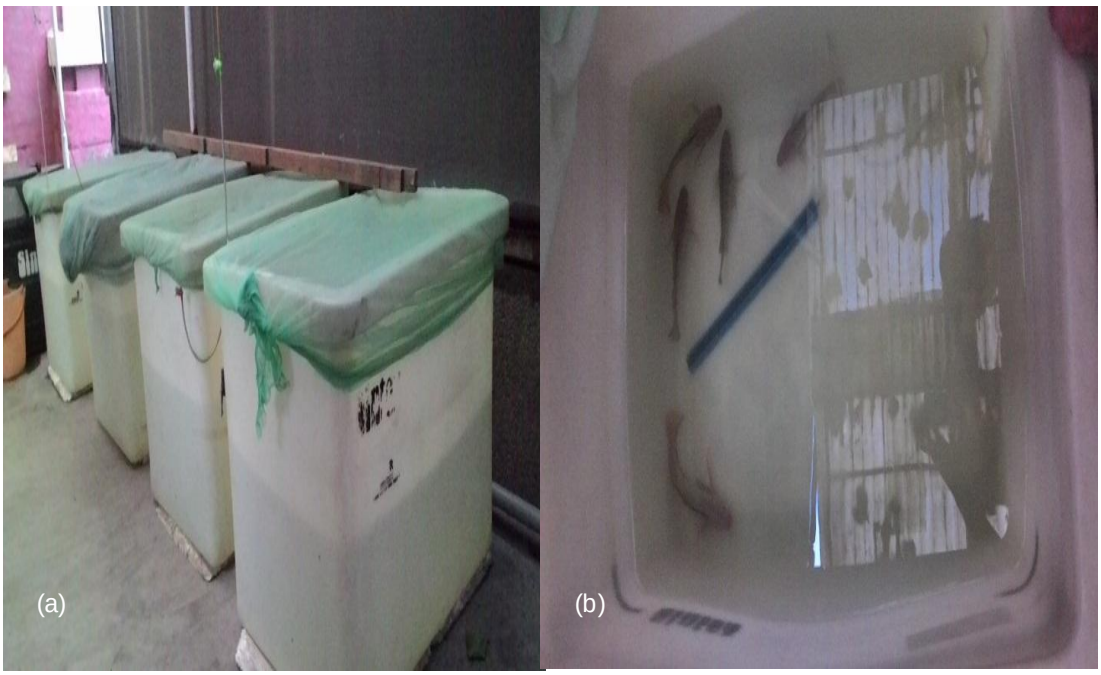



Fig.7 (a) Fish culture tanks, (b) Fish in tanks.

### 4.6. Sampling and preparation of crude enzyme extract

Fish were anesthetized by using MS 222 (a product of Sigma, USA), and was dissected (Fig. 8a) the digestive tract (Fig. 8b and associated glands were collected from each fish and was properly cleaned. The digestive tissue of each species was pooled (4 rohu and 7 for tilapia). Pooled sample of both fishes were collected separately in petri plates (Fig.8b). The tissues were weighed and homogenized using distilled water (1:3, w/v) which was chilled. The homogenate (Fig. 9) was filtered through cheese cloth which was pre-treated in EDTA (ethylenediaminetetraacetic acid, 0.5%). The obtained filtrate was centrifuged at a speed of 10,000 *x* g for a time 30 min at 4°C and supernatant was collected. This supernatant was called as crude enzyme extract. The crude extract of both fishes were stored separately at -20 °C for further use.

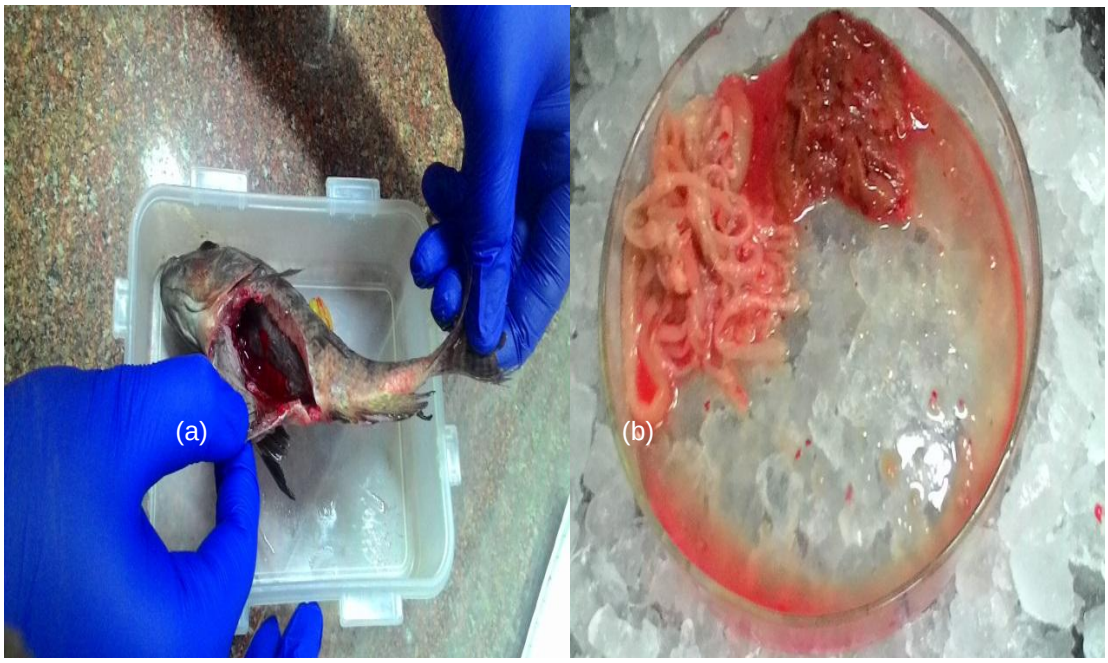



Fig. 8 (a) Dissected fish, (b) Digestive tract of fish.

Fig. 9 Digestive tract collected in bottle after homogenization.

### 4.7. Biochemical assays

#### 4.7.1. Tannin estimation

Tannin estimation is based on the principle that it reduces phosphotungstomolybdic acid in a solution which is alkaline in nature produce a blue colored solution, and the intensity of which is directly proportional to the amount of tannins (Anonymous, 1980). For estimation of tannin, 0.25 g of the test sample in powdered form was taken in a conical flask of 100 ml**,** 37.5 ml distilled water was added and after that it was boiled for 30 min. Then the sample was allowed to cool at room temperature; then it was centrifuged at 2,000 rpm for 20 min at a temperature of 25°C. Supernatant was collected. 0.1 ml of the supernatant was taken and 7.5 ml of distilled water was added to this. Folin-Denis reagent (0.5 ml) was added and 1 ml of sodium carbonate solution was added. The volume was made 10 ml with distilled water and was mix properly using vortex and then it was allowed to stand for 10 min and absorbance was taken at 725 nm in Spectrophotometer (Spectronic 21D). The standard solution was prepared adding 100 mg tannic acid in 100 ml of distilled water. The tannin content of the samples as tannic acid equivalents was calculated from the standard graph. Distilled water was used as blank. The tannin equivalent was calculated as:

Tannin in µg /0.25 g = (absorbance – intercept)/ Slope

Tannin in µg/ g = value obtained x 4

#### 4.7.2. Estimation of protein

The protein content of the samples was determined by micro-Kjeldahl method using an automated nitrogen estimating system (Pelican Instruments, Chennai, India, Fig. 10a). In this system there were three consecutive steps: digestion, distillation and titration. The first step is digestion which was performed taking 250 - 300 mg of sample in the digestion tube. After that concentrated sulfuric acid (10ml) was added in presence of catalyst mixture (potassium sulfate: copper sulfate, 5:1). Digestion started as preheated at 350°C for 30 min and then the temperature was raised to 420°C for 2 h.

After 2 h, the samples were allowed to cool on a stand and then 10 ml of distilled water was added to the digested sample. The sample was loaded in the distillation. Excess base (10 ml) NaOH was added to the digestion product to convert ammonium ($NH^+_4$) to ammonia ($NH_3$) and it was collected in boric acid (4%). Distillation process was conducted on an automated digestion unit (Fig. 10b) (Kelplus Classic DX VA, Pelican Instruments, Chennai, India) and this whole process took 10 min.

Titration is based on the principle that it quantifies the amount of ammonia in the receiving solution (2.5% Boric acid solution with mixed indictor methyl red and bromocresol green, 2:1). The amount of nitrogen in a sample was calculated from the quantified amount of ammonia ion in the received solution. Titration was carried out in an Autotitrator (pH-STAT 902 Titrando, Metrohm, Switzerland; Fig. 10c). The distillate was collected and then titrated against 0.1 N HCl. The dosing unit of the autotitrator which accurately detected the sharp shift in pH as the end point of the titration. In this instrument the end point detection is based on change in voltage. As manual titration end point is based on colour change so there chance of error is more. The percent of nitrogen and protein for each sample were analyzed using pre-installed programme software (TIAMO 2.2-81, Metrohm, Switzerland). The calculation was as follows:

$$\text{N}(\%) = \frac{14 \text{ x normality of acid x titrant value x } 100}{\text{Sample weight x } 1000}$$

Crude protein (%) was determined by multiplying nitrogen (%) with a conversion factor of 6.25.

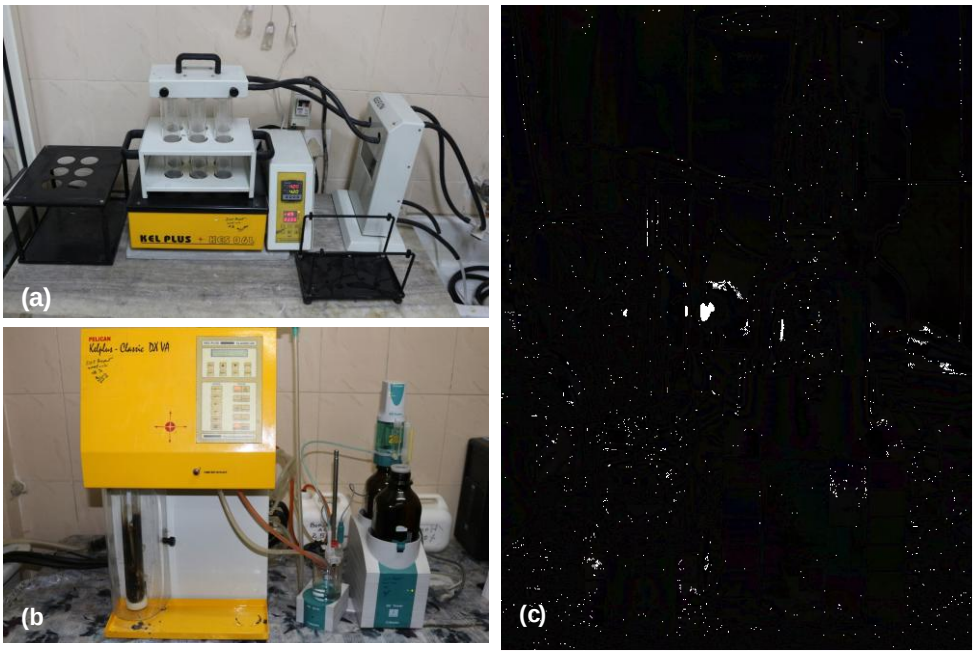



Fig. 10 (a) Kjeldahl digestion unit, (b) Distillation unit and auto titration unit, (c) pH-STAT Autotitrator.

### 4.7.3. Estimation of protein in crude enzyme extract

The soluble protein in enzyme extract was estimated by the method of Bradford (1976). In this method absorbance was measured at 595 nm using UV-visible Spectrophotometer (Shimadzu, Japan). Protein content was calculated using bovine serum albumin (1 mg/ ml) as standard. In this method the crude enzyme extract (5 µl) was taken in eppendorf and distilled water was added to make it to up to volume of 100 µl. After that 1 ml of Bradford reagent was added and was mixed properly with the help of vortex. The following calculation was done to obtain the amount of protein in crude enzyme extract.

Protein in µg /µl = (absorbance – intercept)/ slope.

As the aliquot taken was 5 µl so the obtained value was dived by 5.

### 4.7.4. Total protease activity

Total protease activity which includes endopeptidase and exopeptidase of the crude extract was estimated using azocasein as substrate (Garcia-Carreno, 1992). Crude extract was taken (10 µl) and was incubated with 500 µl of 1% azocasein in presence of Tris-HCl 50 mM (pH 7.5) at 25°C for 10 min. The reaction was terminated after adding 500 µl of 20% trichloroacetic acid. The assay mixture was centrifuged at 10,000 x g for 10 min. The absorbance was measured at 366 nm. Distilled water was used against crude extract for the blank. In test sample the TCA was added after 10 min of substrate addition while in blank sample the TCA was added just after substrate addition to stop reaction. Total protease activity was expressed as

Activity Units = $Abs_{366}$ / min mg protein in the reaction mixture; where, $Abs_{366}$ = Assay Abs. - Blank Abs.

**Activity Unit** = Assay Abs. - Blank Abs. /mg protein in the reaction mixture

### 4.8. *In vitro* digestibility: pH-Stat method

The degree of hydrolysis (DH%) or digestibility of the protein sources was evaluated using pH-Stat titration method using the crude enzyme extract collected from the two species (Garcia-Carreno *et al.,* 1997, Kumar *et al.,* 2007). The finely grounded powdered substrates were homogenized with distilled water using a blender. Substrate suspension (8 mg/ml) for each plant ingredient was prepared and the pH was adjusted to 8.0 using appropriate amount of 0.1 N sodium hydroxide (NaOH). If the obtained activity was more than of 0.250 U mg/protein then the crude enzyme was diluted appropriately to obtain an activity of 0.250 U mg/protein. The pH of enzyme sample was also adjusted at 8.0 using 0.1N NaOH before the assay.

The *in vitro* digestibility assay was performed in the pH-Stat Autotitrator interfaced to desktop computer with a preinstalled Stat-titration programme (Tiamo Version 2.2). It was set on the following parameters: mode - Stat titration, control pH at 8.0; dynamics, 0.2; maximum rate - 0.1 ml /min ; minimum rate - 10.0 µl /min ; dosing

rate - 0.1 ml /min ; stop time - 3600 s and report output. Now 10 g of the substrate suspension (pH 8.0) was taken in a jacketed vessel and 200 µl of crude enzyme extract was added to start the reaction at 25°C. Sodium hydroxide (0.1 N) was added to maintain the pH of the reaction mixture at 8.0. All experiments were performed in triplicate for each ingredient. The volume of NaOH consumed to keep the pH constant (8.0) was recorded and used to calculate the degree of hydrolysis (DH%) for the plant ingredient using the formula (derived from algorithm developed by Adler-Nissen, 1986):

$$\text{Degree of hydrolysis}(\%) = \frac{B \text{ x } N_B \text{ x } 1.4 \text{ x } S\% \text{ x } 100}{8 \text{ x } 100}$$

Where, B = ml of 0.1 N NaOH consumed to maintain the reaction mixture at pH 8.0, $N_B$ = normality of the titrant, S% = protein content in the reaction mixture expressed as %.

# 5. Results

## 5.1. Estimation of tannin

### 5.1.1. Black gram powder and black gram supplemented diet

The concentration of tannin was estimated in black gram powder, which was 419.6 µg/g. In the diet, the ratio of black gram powder and fish powder was 1:1. The concentration of tannin was estimated in diet mixture before and after extrusion. The concentration of tannin in the diet mixture before extrusion was 380.3 µg/g, while after extrusion the level of tannin in processed diet was 334.2 µg/g (Fig.11). The tannin level reduced 12.1% in processed diet compared to the raw mixture before extrusion. Probably the exposure of raw ingredient at 65°C helped to reduce the anti-nutritional factor tannin.

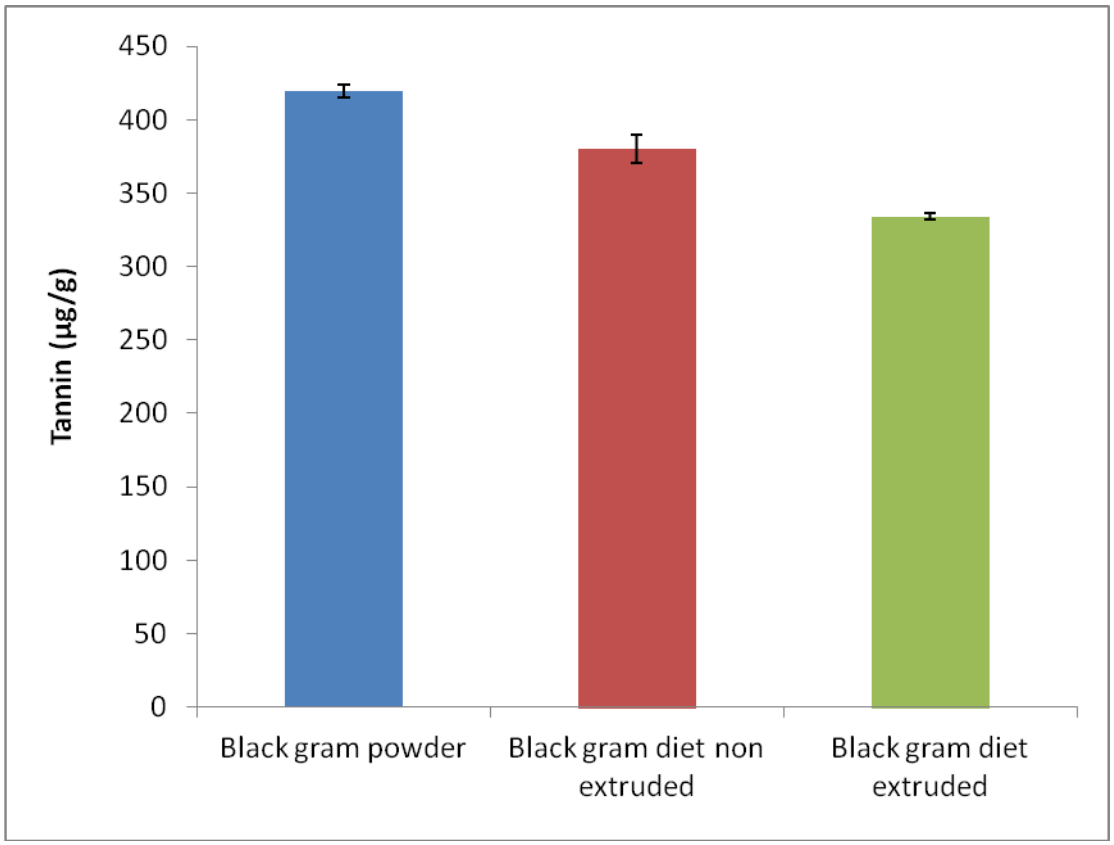



Fig. 11. Tannin concentration in black gram powder, black gram non extruded and extruded diet (D1). Results are expressed as mean ± SE (n=4).

### 5.1.2. Soyabean chunk powder and soyabean chunk supplemented diet

The concentration of tannin was estimated in soyabean chunk powder, which was 459.11 µg/g. In the diet, the ratio of soyabean chunk powder and fish powder was 1:1. The concentration of tannin was estimated in diet mixture before and after extrusion. The concentration of tannin in the diet mixture before extrusion was 385.3 µg/g, while after extrusion the level of tannin in processed diet was 363.06 µg/g (Fig.12). The tannin level reduced 6% in processed diet compared to the raw mixture before extrusion. Probably the exposure of raw ingredient at 65°C helped to reduce the anti-nutritional factor tannin.

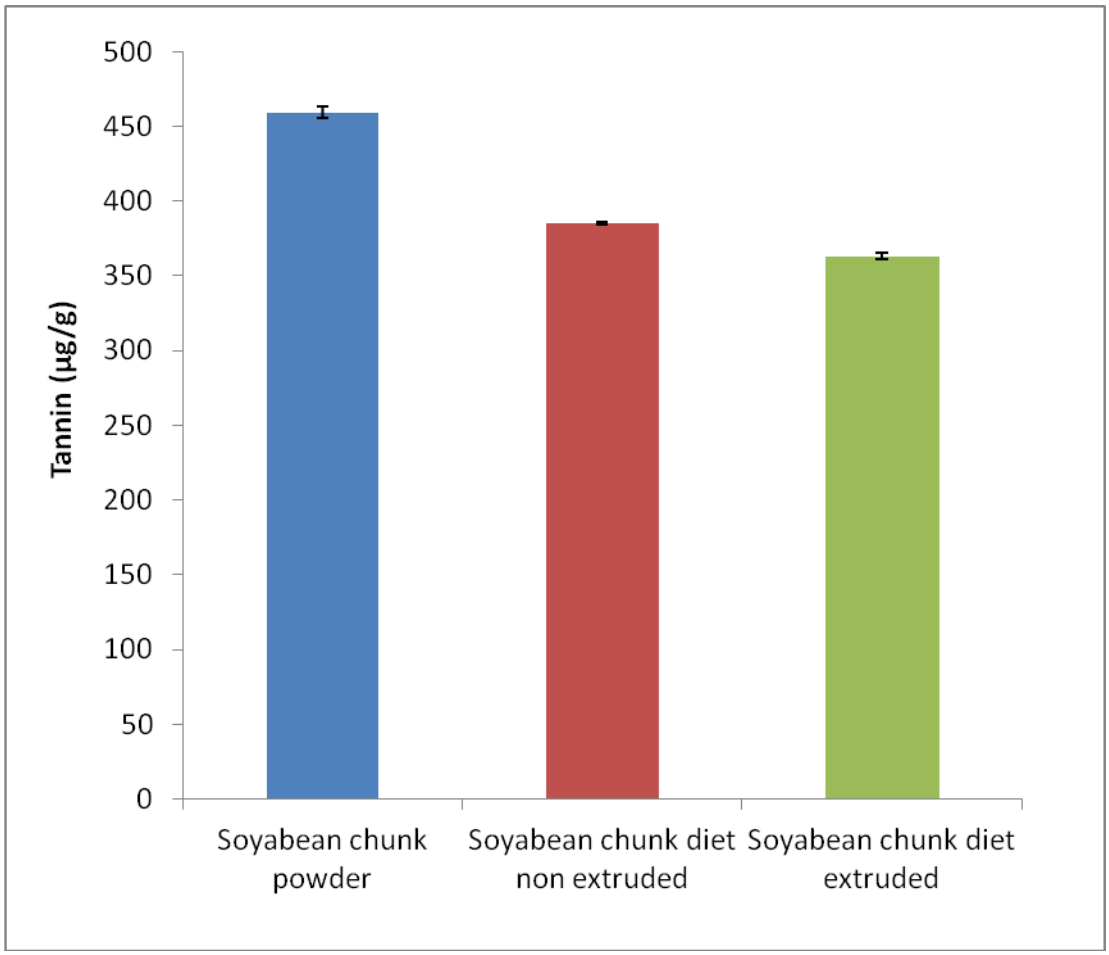



Fig.12.Tannin concentration in soyabean chunk powder, soaybean chunk non extruded and extruded diet (D2). Results are expressed as mean ± SE (n=4).

### 5.1.3. Banana leaves powder and banana leaves supplemented diet

The concentration of tannin was estimated in banana leaves powder, which was 1035.6 µg/g. In the diet, the ratio of banana leaves powder and fish powder was 1:2. The concentration of tannin was estimated in diet mixture before and after extrusion. The concentration of tannin in the diet mixture before extrusion was 626.1 µg/g, while after extrusion the level of tannin in processed diet was 490.7 µg/g (Fig.13). The tannin level reduced 21% in processed diet compared to the raw mixture before extrusion. Probably the exposure of raw ingredient at 70 °C helped to reduce the anti-nutritional factor tannin.

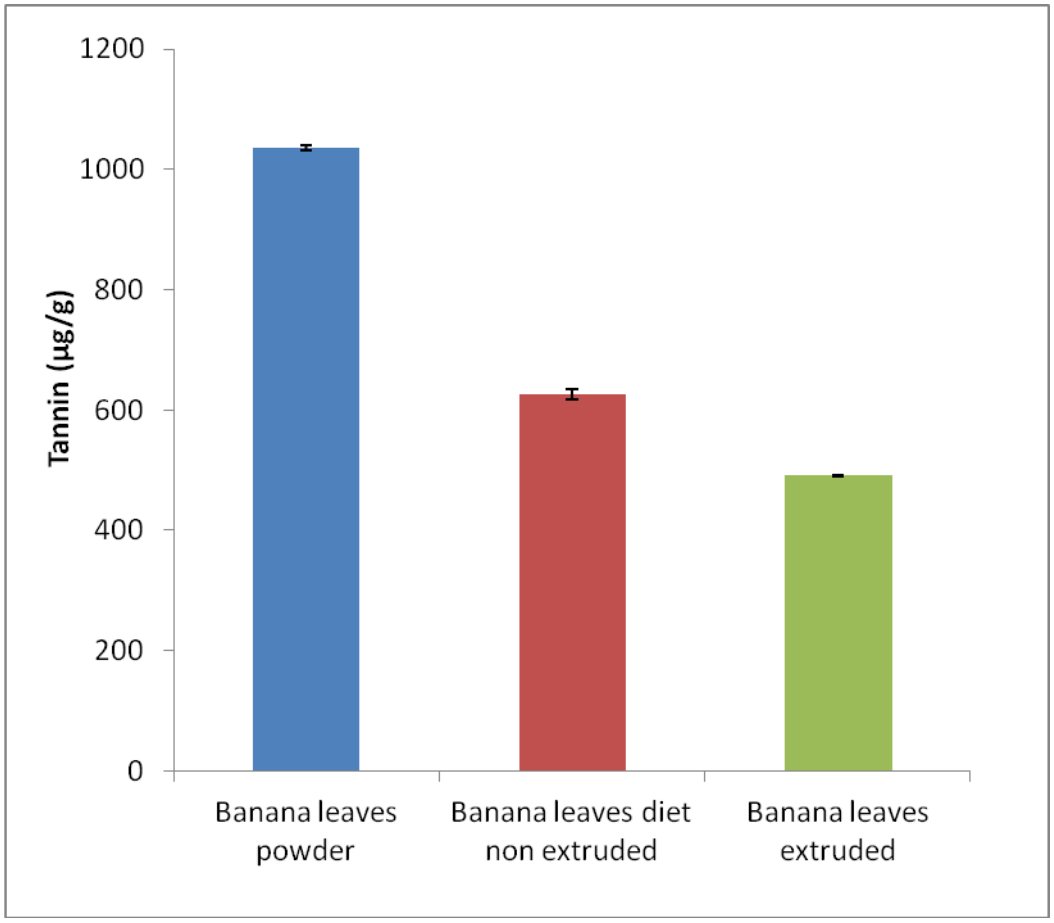



Fig.13. Tannin concentration in banana leaves powder, banaana leaves non extruded and extruded diet (D3). Results are expressed as mean ± SE (n=4).

### 5.1.4. Almond oil cake powder and almond oil cake supplemented diet

The concentration of tannin was estimated in almond oil cake powder, which was 575.9 µg/g. In the diet, the ratio of almond oil cake and fish powder was 1:1. The concentration of tannin was estimated in diet mixture before and after extrusion. The concentration of tannin in the diet mixture before extrusion was 411.9 µg/g, while after extrusion the level of tannin in processed diet was 350 µg/g (Fig.14). The tannin level reduced 15% in processed diet compared to the raw mixture before extrusion. Probably the exposure of raw ingredient at 66°C helped to reduce the anti-nutritional factor tannin.

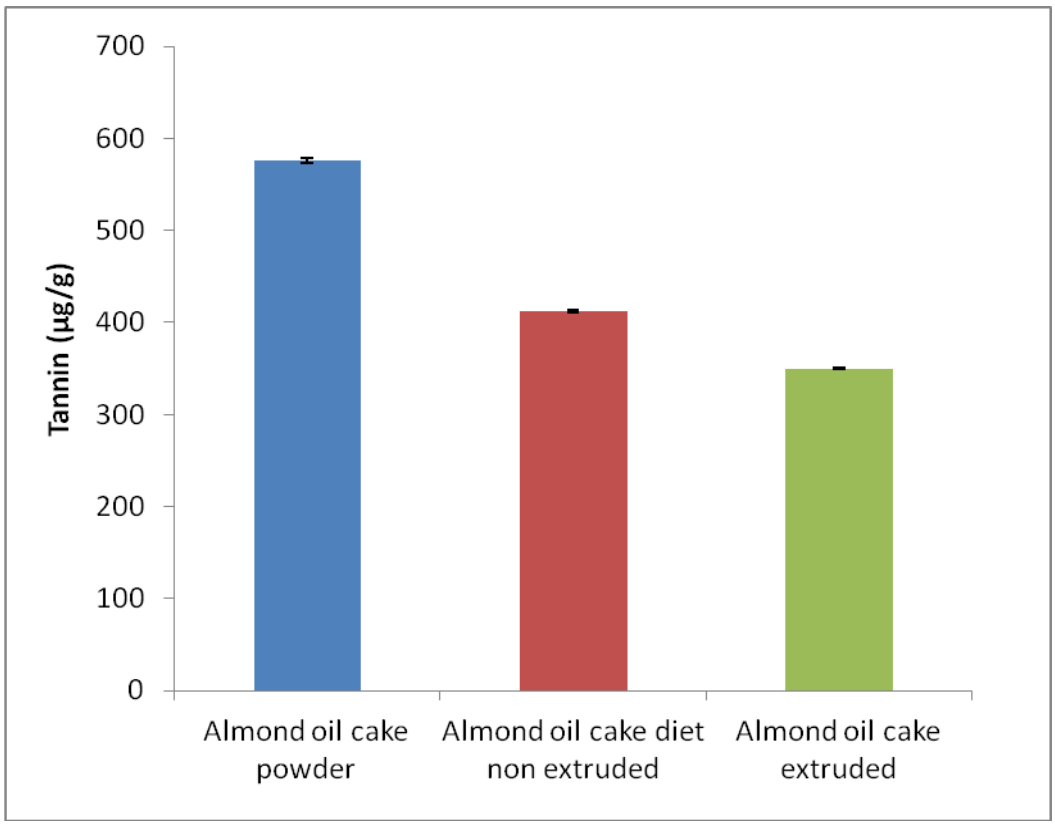



Fig.14. Tannin concentration in almond oil cake powder, almond oil cake non extruded and extruded diet (D4). Results are expressed as mean ± SE (n=4).

## 5.2. Protein estimation in ingredients and diets by micro-Kjeldahl method

The protein content of four different ingredients that were used for the preparation of the diets was estimated by the micro-Kjeldahl method. Maximum protein content was in soyabean chunk followed by almond oil cake (Fig.15). The protein content of the diets prepared from these ingredients was also estimated. To check whether the extrusion temperature affected protein content of the diets. The diets before extrusion and after extrusion were compared. The result showed in that there is very little effect of temperature at 65-70°C ( Fig.16). The protein content in diets after extrusion was reduced very little 0.5-1%.The protein content in the diets were in the expected range from 40-45% in different diets prepared from different plant ingredients.

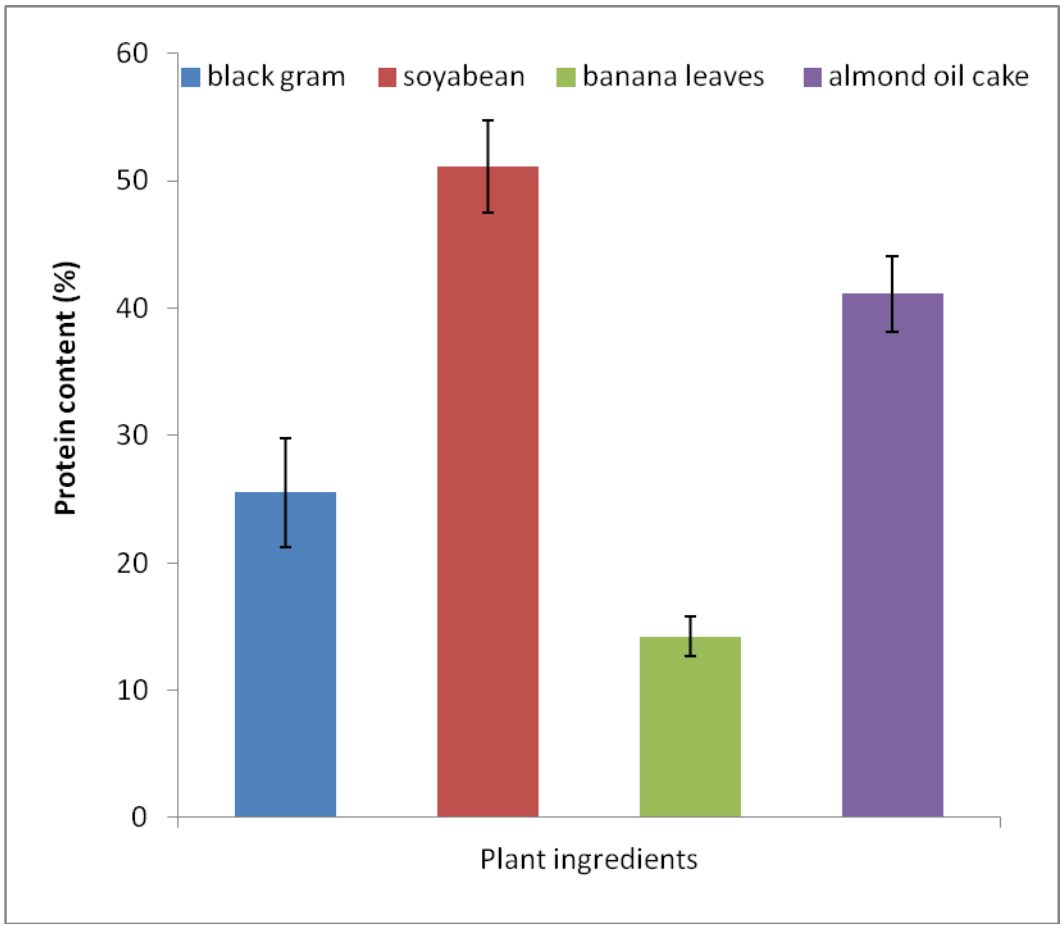



Fig. 15. Protein content of different ingredients. Results are expressed as mean ± SE (n=3)

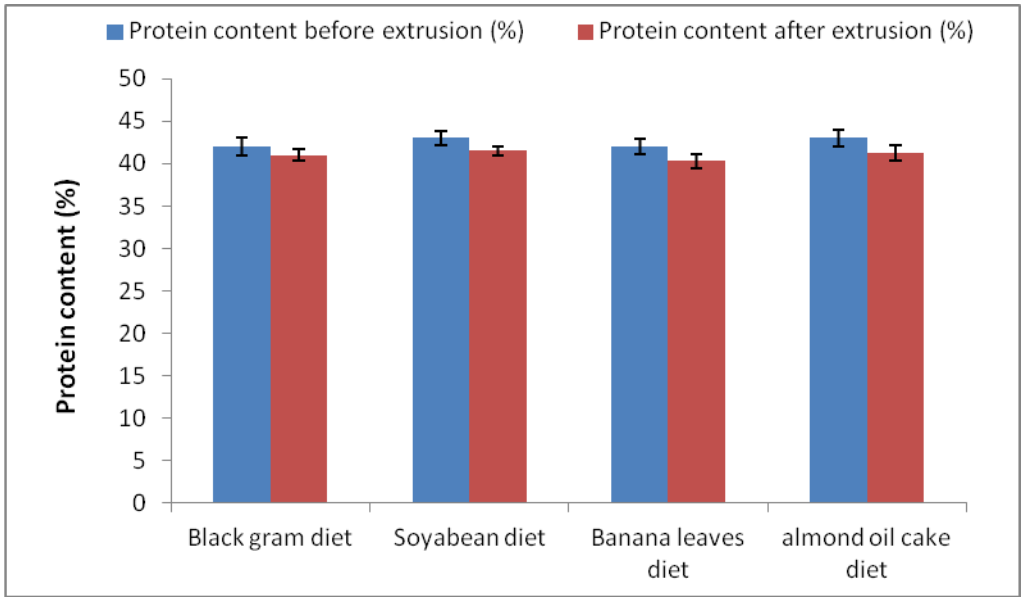



Fig 16: Protein content of different Plant ingredient supplemented non extruded and extruded diets. Results are expressed as mean ± SE (n=3)

### 5.3. Protein estimation in the crude enzyme extract of fish

The protein content of the digestive extract of the two fishes was estimated. The protein content of the digestive extract of the rohu (*Labeo rohita)* was 3.009 mg/ml while for the tilapia (*Oreochromis niloticus)* protein content was 4.31 mg/ml. ( Fig. 17).

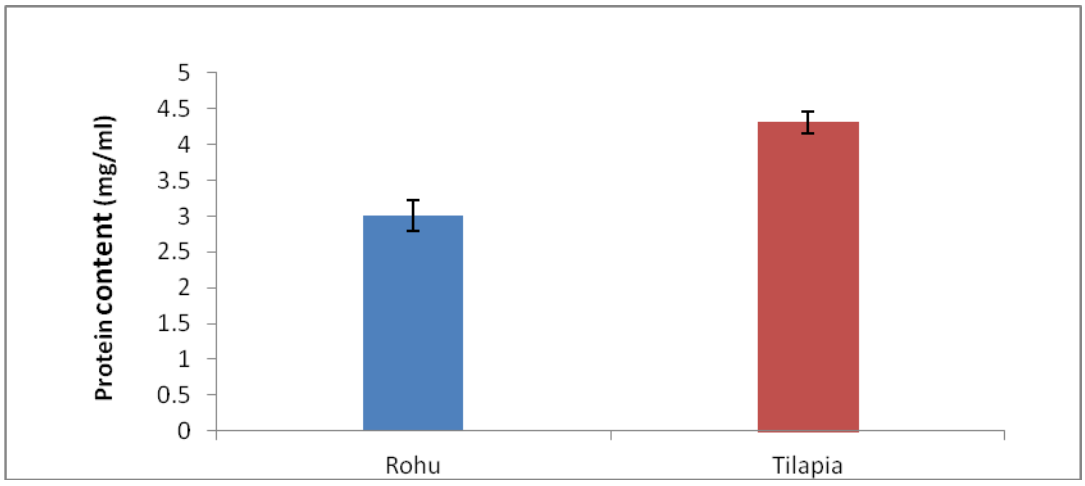



Fig 17: Protein concentration in crude enzyme extract of fish. Results are expressed as mean ± SE (n=3)

### 5.4. Total protease activity estimation

The total protease activity of the fishes used for the *in vitro* digestibility study was estimated. The total protease activity was 0.330 U/mg protein for rohu and 0.257 U/mg protein for tilapia (Table 14, Fig. 18).The total protease activity was brought to 0.250 U /mg protein for the *in vitro* digestibility study.

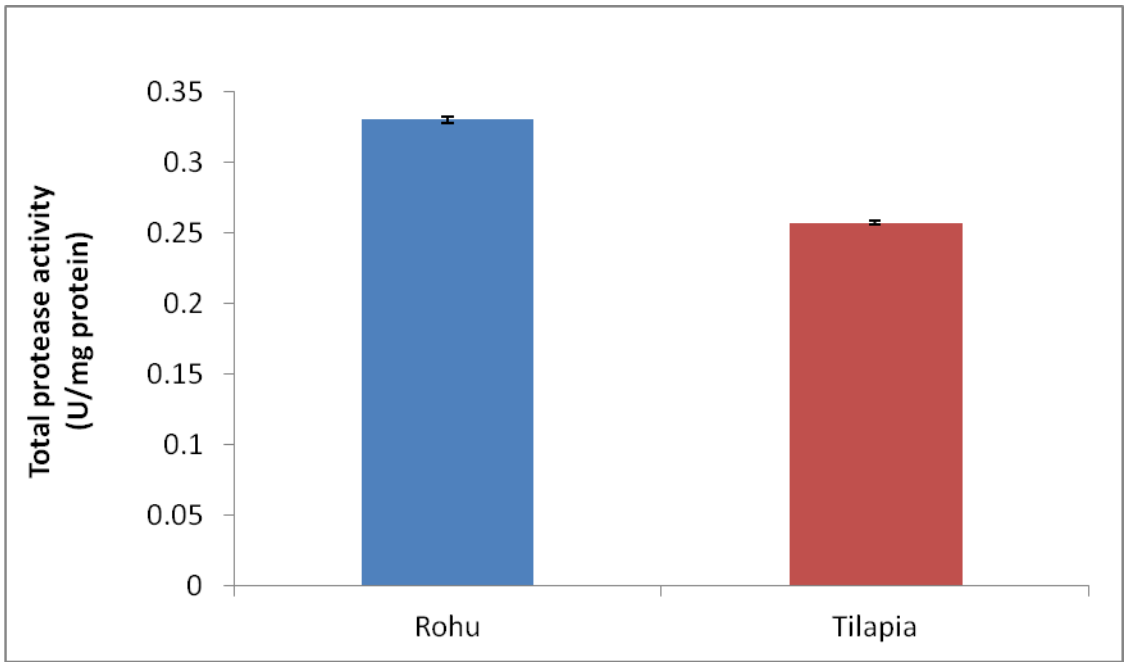



Fig. 18 Total protease activity of fishes. Results are expressed as mean ± SE (n=3)

### 5.5. *In vitro* digestibility by pH-Static method

The *In vitro* digestibility as the degree of the hydrolysis of the different diets prepared from the various plant sources with the help of the extruder showed the suitability of the plant ingredients as supplementary diets. The degree of the hydrolysis varied from species to species. The degree of hydrolysis of black gram, soybean, banana leaves and almond oil-cake incorporated diets for rohu was 7.3, 10.4, 10.5, and 11. 5 percentages respectively. and the degree of hydrolysis of black gram, soybean, banana leaves and almond oil-cake incorporated diets for tilapia was 9.5, 10, 12.5 and 12.5 percentage respectively as shown in (Fig.19) showed that this alternative protein source can be a good supplementary fish diets for the aquaproducers.

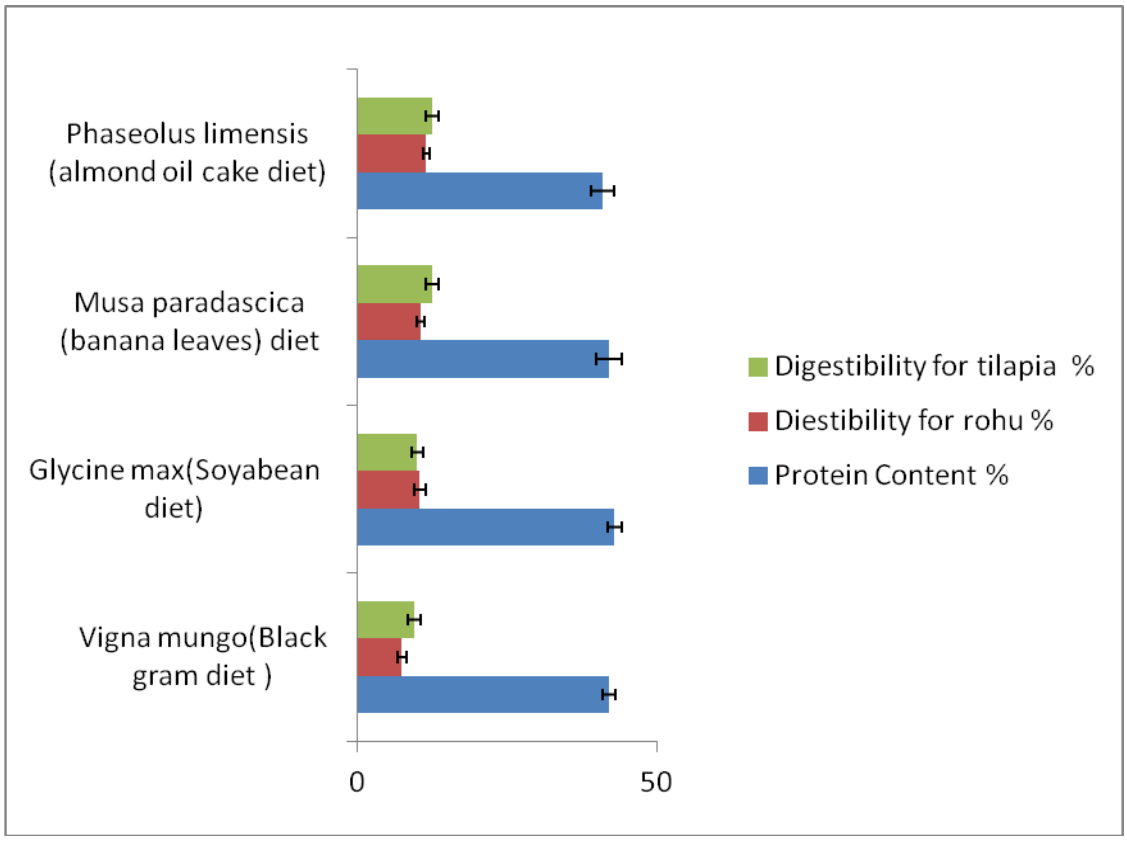



Fig. 19. Protein content of plant ingredient supplemented diet and their digestibility for rohu and tilapia. Results are expressed as mean ± SE (n=3)

## 6: Discussion

The utilization of plant sources in the formulation of aquadiet is an essential requirement for future development of aquaculture. The limitation in the usage of these products and waste is the presence of the antinutrients. Some interaction increases their effects and blocks many reaction like the interaction of tannins and polyphenols. Some interaction cause the increase in cross-linking and also decrease the protein solubility and these protein complex becomes easily degradable (Cheryan, 1980, Sathe and Salunkhe, 1985). So the estimation of these antinutrients is very important for ingredients and for different diet to check the efficiency of the extruder. Decrease in the inhibiting factors increases the digestibility of the diet. The extruder effectiveness in the present study has shown that extruder had removed antinutriton (tannin) to from 10-15% at a temperature about 65 °C. As this extruder when operating at more temperature say about 95-100 °C then the antinutrition value will be removed more. The removal of the antinutritional facors like tannin which decreased in different amount in different diets. The difference is because of operating condition of the extruder parameters and nature of the feed. So the result showed that with the help of this extruder utilization, plant ingredients as fish diet has became a good choice for aquaproducers. The other advantage of the extruder is that as in this study the twin screw extruder reduced the amount of antinutritional factors but it showed very less effect on protein. A good alternative to fish meal and other animal protein in aquadiets must have certain characteristics such as wide availability, cost effectiveness and relatively high protein content and digestibility with reasonable palatability (Gatlin III, 2007). The selection of these plant ingredients should be on the basis of some these parameters like it should be available. It should be economic and most important is that it should be non-toxic. The selection of the materials which are less available and also are very competent among humans should be avoided. For example the pulses which shows higher degree of the hydrolysis but it should be avoided because pulses are less available and also very competent among humans itself so like other sources like leaves, aquaweeds, oil cakes etc can be replaced fish meal in 1:1 with fish meal or in 2:1 with fish meal depending upon the degree of the hydrolysis. The nutritional status of the various aquatic plants for fish

diet has been reported by various researchers (Hasan and Chakrbarti, 2009). Indian major carps rohu and mrigal were reported to be able to utilize some of the aquatic weeds in their diet to some extent (Patra *et al*., 2000; Vhanalakar *et al*., 2008). Edible oil cakes as have very high nutritional value and also they have been reported to show very good degree of hydrolysis in both fishes rohu and tilapia and from the past study it has been seen that these oil cakes had used in animal diet especially for the fish and ruminants (Ramachandran *et al*., 2007). Rohu and tilapia are very common species found in India. The *in vitro* digestibility result showed that the digestibility varied between the different species. It may because of their size, feeding habbit or type and amount of digestive enzymes present in the gut. The digestibility of all the diet was good but as the plant ingredients cake and leaves are very less costly and also not used by human in large extent. So this type of the plant ingredients gives a good scope of use of these alternative resources in aquaculture.

## 7: Conclusion and future perspective

Aquaculture production may increase by use of the alternate protein sources like plant ingredients, agricultural wastes and industrial waste. Farmers can get easily available and less costly fish meal for their aquaculture. Further biological trials for different diets should be evaluated and extrusion effects on these diets. So that antinutrients amount should decrease and nutrients amount should increase and to correlate these with the subsequent nutritive values of fish meal . The *in vitro* digestibility of raw and processed feed should be assessed in vivo to compare the results . To increase the production of the aquaculture and more usage of the extrusion techniques training programs need to be conducted at regular interval. The aquaculture production should be boosted by providing skills and techniques to farmers and researchers. The interaction between researchers and farmers is very important for aquaculture.

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