

MICROBIOLOGICAL AND NUTRITIONAL ANALYSIS OF HERBAL FORTIFIED BISCUITS AND IN VIVO GROWTH ANALYSIS USING THE ZEBRAFISH

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Technology degree in Biotechnology

by

ERIC JOHNSON (38230011)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600119

MAY – 2022



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited with "A" grade by NAAC | 12B Status by UGC | Approved by AICTE
Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai – 600 119
www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of ERIC JOHNSON (38230011) who carried out the project entitled "**Microbiological and Nutritional Analysis of Herbal Fortified Biscuits and In Vivo Growth Analysis using the Zebrafish**" under our supervision from January 2022 to April 2022.

Dr. Gracy Jenifer

Internal Guide

Dr. Jerrine Joseph

External Guide

Head of the Department
Dr. V Ramesh Kumar

Submitted for Viva voce Examination held on _____.

Internal Examiner

External Examiner

DECLARATION

I, **Eric Johnson (38230011)**, hereby declare that the Project Report entitled **“Microbiological and Nutritional Analysis of Herbal Fortified Biscuits and In Vivo Growth Analysis using the Zebrafish”** done by me under the guidance of **Dr. Gracy Jenifer and Dr. Jerrine Joseph** at **Sathyabama Institute of Science and Technology, Chennai** is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE:

A handwritten signature in black ink, appearing to read 'Eric Johnson', with a horizontal line drawn through the middle of the signature.

PLACE: CHENNAI

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V Ramesh Kumar**, Head of the Department, Dept. of Biotechnology for providing me the necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guides **Dr. Gracy Jenifer** and **Dr. Jerrine Joseph** for their valuable guidance, suggestions and constant encouragement paved the way for the successful completion of my project work.

I wish to express my thanks to **Dr. P Prakash**, Year Coordinator, all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project

ABSTRACT

Urbanization has led to an increase in the consumption of industrially produced food, including by those in developing countries. Many countries have now reached a larger segment of their populations by using fortified foods to combat micronutrient malnutrition. Poverty has its effects on a population that largely lacks adequate micronutrients. Therefore, it is common practice to include micronutrients in multiple forms. The purpose of the study was to formulate plant-based fiber-enhanced biscuits (Spinach, Holy Basil, and Moringa) that contain sufficient nutrients (protein, vitamins, and fiber). Research objectives were to determine the microbiological, physical, and organoleptic properties of herbal biscuits, as well as their nutritional value. Fiber enriched herbal biscuits were developed by incorporating Spinach (*Spinacia oleracea*) leaves, Holy Basil (*Ocimum sanctum*) leaves, Moringa (*Moringa oleifera*) leaves, finger millet and whole wheat flour, coconut oil, jaggery powder and other necessary ingredients. This recipe should be standardized and evaluated for organoleptic acceptability by a five point's hedonic scale. The nutritive values of the normal standard biscuits were protein 14.7g, fat 19.5g, carbohydrate 56.6g, fiber 2.3g, ash 2.1% and 458Kcal. We should make sure the herbal biscuits contain more moisture content, crude fiber, energy and protein, fat, carbohydrate, and ash. It should also be noted that the quality and microbiological characteristics should also be improved to acceptable levels. The plant-based fiber-enhanced biscuits are then subjected to Zebrafish (*Danio rerio*) at different concentrations (10mg, 20mg, 30mg / per fish) to analyze the physical, teratological characteristics and growth of the fish.

LIST OF CONTENTS

CHAPTER NO	TITLE	PAGE NO
	ABSTRACT	
	LIST OF FIGURES	
	LIST OF TABLES	
1	INTRODUCTION	1
2	LITERATURE SURVEY	5
3	AIM AND SCOPE	7
4	MATERIALS AND METHODS	8
5	RESULTS AND DISCUSSION	11
6	SUMMARY AND CONCLUSION	22
7	REFERENCES	23

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO
5.1	Day 05 in control	12
5.2	Day 10 in control	12
5.3	Day 15 in control	12
5.4	Length of zebrafish in control (cm) VS. Day	13
5.5	Weight of zebrafish in control (mg) VS. Day	13
5.6	Day 05 in 10mg HFB	14
5.7	Day 10 in 10mg HFB	14
5.8	Day 15 in 10mg HFB	14
5.9	Length of zebrafish in 10mg of HFB (cm) VS. Day	15
5.10	Weight of zebrafish in 10mg of HFB (mg) VS. Day	15
5.11	Day 05 in 20mg HFB	16
5.12	Day 10 in 20mg HFB	16
5.13	Day 15 in 20mg HFB	16
5.14	Length of zebrafish in 20mg of HFB (cm) VS. Day	17
5.15	Weight of zebrafish in 20mg of HFB (mg) VS. Day	17

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO
5.16	Day 05 in 30mg	18
5.17	Day 10 in 30mg	18
5.18	Day 15 in 30mg	18
5.19	Length of zebrafish in 30mg of HFB (cm) VS. Day	19
5.20	Weight of zebrafish in 30mg of HFB (mg) VS. Day	19

LIST OF TABLES

TABLE NO	TITLE	PAGE NO
5.1	Proximate analysis	11
5.2	Microbial analysis	11
5.3	Overall Growth rate of zebrafish	20

1. INTRODUCTION

Human malnutrition is characterized by a low or high supply of macronutrients, micronutrients, or both in the body (Ortiz-Andrellucchi *et al.*, 2016). In the meantime, undernutrition and micronutrient deficiencies are persistent and negatively impact public health (Murray *et al.*, 2006). In fact, poor food access and low income are significant causes of micronutrient deficiencies in rural areas (Shetty, 2009). The world is estimated to have two billion micronutrient deficient people (Allen *et al.*, 2006). Unfortunately, in developing countries most people cannot afford these foods in their daily diet. As a result, they suffer from micronutrient deficiencies (Bain *et al.*, 2013; Müller & Krawinkel, 2005). Many children worldwide suffer from nutritional deficiencies, which can negatively affect their physical and mental development and increase susceptibility to infections. Moreover, undernutrition amplifies the effect of every disease, including measles and malaria Black *et al.* (2005). In addition, according to Black *et al.* (2008), women and children are the major targets suffering from consequences of micronutrient deficiency such as poor pregnancy outcomes, children's impaired mental, and physical development. Up to 3.1–3.5 million of children under 5 years old die every year and women of reproductive age living in low and middle income countries because of undernutrition (fetal growth restriction, suboptimal breastfeeding, stunting, wasting, and deficiencies of vitamin A, iodine, zinc, iron, vitamin D deficiency, rickets, osteomalacia, and thyroid deficiency) (Black *et al.*, 2008, 2003; Mandelbaum, 2004). Therefore, elimination of these deficiencies is essential, not only to improve health, but also for sustained economic growth and national development (Mishra, 2011). Fortification of basic foods, seasonings, or condiments can be an effective way to increase their nutritional value by adding micronutrients such as vitamins and minerals (Mannar & Gallego, 2002). By incorporating synthetic micronutrients into foods, foods can be fortified (Zimmermann *et al.*, 2006). Most commonly fortified foods in developing countries are foods that are commonly consumed. These include oil, fat, salt, rice,

wheat, or maize flour. Fortification of foods has a significant impact on health in terms of level of fortification; bioavailability of fortificants; and the quantity of fortified food consumed (Verma, 2015; Bhutta *et al.*, 2013). Traditionally, zinc has been added to foods in high amounts (100 mg zinc/kg zinc for wheat flour; (Brown *et al.*, 2010), where a lower level of zinc fortification does not likely impact the nutritional benefits. (Brown *et al.*, 2009). A very efficient food fortification program, especially one that takes advantage of existing technology and native distribution, will greatly improve the health level of a population. Rice, therefore, is thought of as a food fortifier in developing countries that may be enforced at the population level (Moench-Pfanner *et al.*, 2012). Rice flour fortified with iron, zinc, and vitamin M has been well-tried to boost the expansion and matter standing of youngsters below five years recently, with speedy iron and Zinc absorption (Hettiarachchi *et al.*, 2004). invigorating flour is far easier as a result of the nutrients that are unit on the market in fine-grained kind will with success be mixed into the flour. As such, rice flour was counseled as an appropriate vehicle for fortification. Long-term measures are enforced to combat vitamin A and iron deficiencies, above all, fortification of cotton oil with vitamin A and of flour with iron, zinc, folic acid, and B vitamin. Multiple matter fortification seems comparatively additional useful and will be thought of as a result of multiple matter deficiencies existing in several cases. This thought justifies why several fortification programs are unit minded toward multi-micronutrients and vehicles chosen adequately for an honest acceptableness of fortified foods by the target cluster. The fortification of food can take many forms and employ many different techniques (Liyanage & Hettiarachchi, 2011).

Biscuits generally consist of wheat flour, starch, sugar, sugary syrup, and flavorings., and they are generally small, flat, and crisp. Biscuit, originally written bisket, is derived from the Old French bescuit and Medieval Latin biscoctum, both meaning "baked twice." Because biscuits are often filled with sugar and fat, they are often considered unhealthy food. The biscuit, on the other hand, can transport

many nutritionally beneficial ingredients. Biscuits can be fortified in several ways, according to Manley (2003b). The consumption of cookies fortified with fresh corn pericarp improved the fecal parameters in individuals suffering from constipation (Sevá-Pereira *et al.*, 1991). Van Stuijvenberg *et al.* (2001) propose that red palm oil can be used as an alternative to dietary vitamin A fortification. Sharif *et al.* (2005) showed that shortening could be replaced with rice bran oil in a recipe to produce superior-quality cookies. In Rababah *et al.* (2006) study, chickpea flour, broad bean flour, and soy protein were used to fortify biscuits. These ingredients can be combined to produce high-protein biscuits, according to their study. With maltodextrin and glycerol monostearate or guar gum, biscuit textures can be significantly improved (Sudha *et al.*, 2007). According to studies conducted in India, 30% soy flour can provide increased nutritional value without deteriorating the physical properties of biscuits (Singh and Sabapathy, 2006). Savitha *et al.* (2008) explored how 0.5% sucralose and different levels of maltodextrin affected dough properties and biscuit quality in the replacement of 30% sugar. The results show that sucralose and maltodextrin can replace sugar in biscuits. *Amorphophallus konjac* plant produces glucomannan, a polysaccharide derived from its tuberous roots. Glucomannan was extracted and incorporated into biscuit dough by Jenkins *et al.* (2008). Researchers conclude that biscuits enriched with other viscous and starchy foods can replace snack foods with a high glycemic index. Using a blend of navy bean, pinto bean, green lentil, and yellow pea flour, Zucco *et al.* (2011) were able to produce cookies with acceptable physical characteristics and an improved nutritional profile. Sharma and Zhou (2011) prepared biscuits fortified with green tea extract. These biscuits were found to be fairly stable in the presence of tea catechins.

By consuming fortified foods regularly and frequently, dietary supplements will maintain nutrient stores more efficiently and effectively than by consuming intermittent supplements. Additions of nutrients to the foods help reduce the incidence of deficiency problems as well as those caused by seasonal fluctuations

in food supply. This will benefit children and pregnant women who need adequate nutrient stores during pregnancy and lactation as well as pregnant women who need a steady supply of micronutrients for growth and development. Fortification can be beneficial to women who are postpartum and to their infants because it boosts vitamin content in breast milk and reduces their nutritional requirements. The fortification of widely consumed and widely distributed foods would not only improve the nutritional status of a large portion of the population, but also serve other purposes. The fabricated food is delivered in the form of a biscuit to enhance immunity and satisfy the nutritional value needed for the day and it is completely plant-based which makes it bio-compatible. It relates to fabricating vegan friendly biscuits with multifaceted benefits including improved nutrition and immunity. The predominant benefits include the improvement of the nutritional value and the enhancement of immunity. It is plant-based which is a bio-available and environment-friendly alternative to generic packed snacks which are full of harmful additives and ingredients with low nutritional value. In accordance to this invention, several plant based ingredients were recognized based on their contribution to enhancing immunity and nutritional value , amidst which a few ingredients namely , Fiber enriched herbal biscuits were developed by incorporating Spinach (*Spinacia oleracea*) leaves, Holy Basil (*Ocimum sanctum*) leaves, Moringa (*Moringa oleifera*) leaves, finger millet and whole wheat flour ,coconut oil, jaggery powder and other necessary ingredients were identified suitable for the fabrication of the biscuit. The acquisition of the ingredients were done by comparing the cost from multiple sources and the most efficient source was finalized. Then on a trial and error basis, the biscuit was fabricated.

2. LITERATURE SURVEY

- Md. Ariful Alam *et al.*, (2014) study was designed to formulate herbal biscuits with sufficient fiber content (Holy Basil and Moringa) and sufficient nutrients (protein and fiber). The study aimed to assess the biochemical and organoleptic characteristics of herbal biscuits by substituting them with four different percentages of Tulsi and Moringa leaves, namely Normal, Tulsi -1 (1%), Tulsi -2 (2%) and Tulsi -3 (3%) leaf extracts. In order to make fiber-enriched herbal biscuits, Tulshi leaves (*Ocimum sanctum*) and Moringa leaves (*Moringa oleifera*) were incorporated, as well as whole wheat flour, egg whites, vegetable oil, margarine (dalda) and other necessary ingredients. On a five-point hedonic scale, this recipe was evaluated for organoleptic acceptability. According to the acceptance scores for the normal biscuit, sample biscuit 1, 2 and 3 the scores were 4.46, 4.43, 4.03, and 4.14 respectively. A normal standard biscuit has the following nutritional values: protein 14.7g, fat 19.5g, carbohydrates 56.6g, fiber 2.3g, ash 2.1% and 458Kcal. As of sample-1, the prepared herbal biscuits contain 4.6% moisture, 2.9% crude fiber, 460 Kcal energy, 14.8% protein, 20.1% fat, 55.4% carbohydrates, 2.2% ash. Nevertheless, Samples-2 and 3 biscuits contain 4.5 percent moisture, 3.5 percent crude fiber, 450 kcal energy, 14.6% protein, 19.9% fat, 55.2% carbohydrate, 2.3% ash, and 4.7 percent moisture, 4.1% crude fiber, 449 kcal energy, 14.9% protein, 19.3% fat, 54.6% carbohydrate, 2.4% ash, respectively. A certain proportion of Tulsi and Moringa leaves at an acceptable level improved the quality characteristics of the product. Comparatively with all other quality characteristics, the addition of Tulsi and Moringa leaves at 1% was more acceptable.
- Arshad. U *et al.*, (2006) replaced wheat flour with defatted wheat germ (DFWG) at levels of 0–25% and was investigated for its effect on functional and nutritional properties of cookies. The crude protein content of DFWG was as high as 27.8% with a highly valuable amino acid profile, rich in essential amino acids, especially lysine (2.32 g/100 g). The physicochemical and sensory evaluation of cookies

revealed that up to 15% substitution of wheat flour with DFWG produced acceptable cookies similar to the control (100% wheat flour) cookies. The protein quality of the cookies was assessed through weanling albino rats by feeding a diet of cookies for 10 days, which was formulated to supply 10% protein, with a casein diet as a control. The cookies containing 15% DFWG, were best regarding protein bioavailability in rats. The protein efficiency ratio (PER), net protein utilization (NPU), biological value (BV) and true digestibility (TD) differed significantly among diets containing cookies with 0–10% DFWG, and casein diet when fed to rats. Diets containing 15% DFWG have values, of these parameters,

- Amadi, Joy A.C (2019) recently evaluated the nutrient, phytochemical and sensory evaluation of biscuits made from composite flours of wheat and okra pod for enrichment. Wheat flour, fresh okra pods and all ingredients used in biscuit making were purchased from Relief market Owerri, Imo State. The okra pod was washed, sliced into pieces, oven dried at 60°C for 8 hours and milled into flour. The biscuits were formulated as 100% wheat flour (WF), 80% wheat flour : 20% okra pod flour (WOF1), 70% wheat flour : 30% okra pod flour (WOF2) and 60% wheat flour: 40% okra pod flour (WOF3). Chemical and sensory analysis was carried using standard methods. Results were expressed using means and standard deviation using SPSS. Biscuit WOF3 had the highest moisture ($13.56 \pm 1.16\%$), ash ($3.49 \pm 0.01\%$) and protein ($14.99 \pm 1.38\%$) contents, while biscuit WF recorded the highest fat ($15.68 \pm 0.22\%$), crude fiber ($2.73 \pm 0.05\%$), carbohydrate ($63.03 \pm 0.32\%$) and energy ($439.08 \pm 2.93\%$) contents. The mineral composition shows that biscuit WOF3 recorded the highest calcium ($212.50 \pm 0.010 \text{ mg/100g}$), magnesium ($83.50 \pm 0.009 \text{ mg/100g}$), potassium ($594.00 \pm 0.00 \text{ mg/100g}$) and manganese ($1457.00 \pm 0.24 \text{ mg/100g}$), while biscuit WOF1 had highest iron ($109.12 \pm 0.88 \text{ mg/100g}$) and copper ($23.00 \pm 0.42 \text{ mg/100g}$).

3. AIM AND SCOPE

3.1. AIM

To fabricate vegan friendly biscuits with multifaceted benefits including improved nutrition and immunity.

3.2. SCOPE

It relates to fabricating vegan friendly biscuits with multifaceted benefits including improved nutrition and immunity. Enhancement of immunity and improvements in nutritional value are the two most significant benefits. It is plant-based which is a bio-available and environment-friendly alternative to generic packed snacks which are full of harmful additives and ingredients with low nutritional value.

3.3. PLAN OF WORK

- Identification and procurement of raw materials from cost-effective and efficient sources.
- Fabrication of the biscuit by appropriate addition of the ingredients and baking at 150 degrees for 10 minutes.
- Performing nutritional analysis to justify nutritional value. Microbial and physical analysis was also performed to determine the quality of the product developed.
- The fabricated product is converted to suit fish consumption and subjected to zebrafish (*Danio rerio*) in different ratios to understand the most suitable dosage.
- Performing an in vivo toxicity test using zebrafish embryos to detect whether the biscuits are toxic.
- Observation and justification of the objectives.

4.MATERIALS AND METHODS

4.1 MATERIALS

4.1.1. Spinach

Spinach contains Calcium, Folate, Iron, Magnesium, Phosphorus, Potassium, Vitamin A, Vitamin B6, Vitamin C, Vitamin E, Vitamin K

Several important plant compounds, including : Lutein, Kaempferol, Nitrates, Quercetin and Zeaxanthin

4.1.2. Moringa Leaves

Moringa leaves are rich in Vitamin A, Vitamin C, Vitamin B1 (thiamin), Vitamin B2 (riboflavin), Vitamin B3 (niacin), Vitamin B6 and Folate, Magnesium, Iron, Calcium, Phosphorus, Protein and Zinc.

They are rich in amino acids, 18 types of amino acids are found which makes it an important contribution towards our wellbeing.

4.1.3. Holy Basil

Holy basil leaf contains various beneficial constituents, such as Eugenol, Ursolic acid, Rosmarinic acid.

Other active compounds include Beta-Caryophyllene and Oleanolic acid, Carotenoids, Vitamin C, Calcium, Iron, Zinc.

4.1.4. Finger Millet flour

Finger millet is also an excellent source of Vitamin A and B Niacin Calcium Iron Phosphorus Potassium Antioxidants

4.1.5. Refined wheat flour

Refined Wheat Flour is usually used in low amounts for the proper fabrication of biscuits.

4.1.6. Jaggery Powder

Jaggery may also contain small amounts of B vitamins and minerals, including calcium, zinc, phosphorus and copper .

4.1.7. Coconut oil

Coconut Oil contains Vitamin E.

4.1.8. Baking powder

Baking powder is usually used in low amounts for the proper fabrication of biscuits.

4.1.9. Cocoa powder

Baking powder is used in low amounts for the proper taste of biscuits.

4.2 METHODOLOGY

4.2.1. Preparation of Herbal Fortified Biscuit

The fabrication process begins with leaves being extracted and dried and grinded in the form of powder. Sieve the finger millet flour(170g), refined wheat flour(30g), moringa leaf extract (5g) spinach leaf extract (5g) and holy basil (5g) leaves in powdered form and other dry ingredients. Mix the Coconut oil (250ml) and Jaggery powder (200g) together to obtain sweetened shortening cream. Mixed dry flour is added to shortening cream with addition of water to make dough. The dough was prepared by manual kneading of all the dry and liquid ingredients to attain uniformity with desirable visco-elastic characteristics. Dough is kept for 10-15 minutes as it is and then used for sheeting, sheets were made by rolling balls of dough on wooden platforms. Sheets are cut by hand operated metal dye, arranged on olive oil coated tray and were kept for baking. While baking, structural changes take place due to heating of dough. The Color of the biscuit changes to the typical light brown color of the finished biscuit. Each lot requires 10 minutes at 150 o C for baking.

4.2.2. Analysis to justify quality and nutritional value

The fabricated nutrition bar should be analyzed for various physical, nutritional and microbial parameters. The physical parameters to be analyzed are texture and color. The nutritional parameters to be tested include Protein, Fiber, Carbohydrate, Sugar, Energy, Ash and Fat. Iron is also tested as it is a prevalent deficiency. Microbial analysis is performed for *Bacillus*, *Salmonella* and Coliforms.

4.2.2.1. Proximate Analysis

The total ash is determined by : $\text{Ash content} = \frac{\text{weight of the ash}}{\text{weight of the original sample}} \times 100\%$.

The total protein is determined by : $\text{Protein} = \text{N} \times 6.25$ (where N is the total nitrogen content present in food)

The total fat is determined by : Using GLC

The total fiber is determined by : $\text{Fibre} = \text{Weight of the Residue} - \text{Weight of Protein and Ash}$

The total energy content is determined by : $\text{Food energy} = (\% \text{ Crude Protein} \times 4) + (\% \text{ Fat Content} \times 9) + (\% \text{ Carbohydrate} \times 4)$

4.2.2.2. Microbiological Analysis

Two grams of the sample is weighed out for analysis and evaluated.

Quarter strength of peptone water solution is prepared by dissolving 3 g in 200 mL of distilled water.

Nutrient Agar (NA) is prepared by dissolving 4.6 g in 200 mL of distilled water; serial dilution was carried out on the biscuit samples and plating of the sample is performed.

4.2.3. Suspension of food as flakes for confirmatory studies

A zebra fish (*Danio Rerio*) facility is maintained and the fish feed on commercially available fish feed for the adaptation period. A total of 15 fishes are to be obtained from which 6 will be used as control. 3 Fishes will be added into 3 separate tanks and the feed will be subjected to different ratios. The fabricated fortified biscuits are added with a negligible amount of dry fish to curate it to suit fish consumption and are pelleted and suspended to the fish at different ratios for 30 days.

5.RESULTS AND DISCUSSION

5.1. PROXIMATE ANALYSIS RESULT

Proximate analysis was compiled with the data of sensory and nutritional analysis for 100g of sample.

Table : 5.1. Proximate analysis

SL.NO	TEST PARAMETER(S)	TEST METHOD	UNIT	RESULTS
1	Texture	CVR/FD/SOP/002	-	Crunchy, Oily, Chewy, Round Shaped biscuits
2	Colour	CVR/FD/SOP/002	-	Dark Brown
3	Protein	IS 7219	%	5.23
4	Crude Fiber	FSSAI manual of methods of analysis of foods (Cereal & Cereal Products)	%	1.38
5	Carbohydrate	CVR/FD/SOP/001	%	68.29
6	Total Sugar	FSSAI manual of methods of analysis of foods (Cereal & Cereal Products)	%	28.90
7	Energy	CVR/FD/SOP/001	Kcal/100g	475.16
8	Ash	FSSAI manual of methods of analysis of foods (Cereal & Cereal Products)	%	1.91
9	Fat	AOAC 21 st Edn -922.06	%	20.12
10	Iron as Fe	CVR/INS/SOP ICPMS/003	mg/100g	6.92

5.2. MICROBIOLOGICAL ANALYSIS RESULT

Microbiological analysis was done with common food fouling bacteria and therefore the quality and shelf life of the biscuit is found for 100g of sample.

Table : 5.2. Microbial analysis

SL.NO	TEST PARAMETER(S)	TEST METHOD	UNIT	RESULTS
1.	Bacillus	IS:5887 (Part-6)	Cfu/g	< 10
2.	Salmonella	IS:5887 (Part-3)	-	Absent/25g
3.	Coliform	IS:5401 (Part-1)	Cfu/g	< 10

Note:

< 10 cfu/g can be taken as absent in 1:10 Dilution.

5.3. SUSPENSION OF FOOD AS FLAKES TO ZEBRAFISH FOR CONFIRMATORY STUDIES RESULTS

5.3.1. Control - Fish Growth

The length of the zebrafish was measured using a ruler and the weight of the zebrafish was measured using a weighing balance.

Fig: 5.1 : Day 05 in control



Fig: 5.2 : Day 10 in control



Fig: 5.3 : Day 15 in control

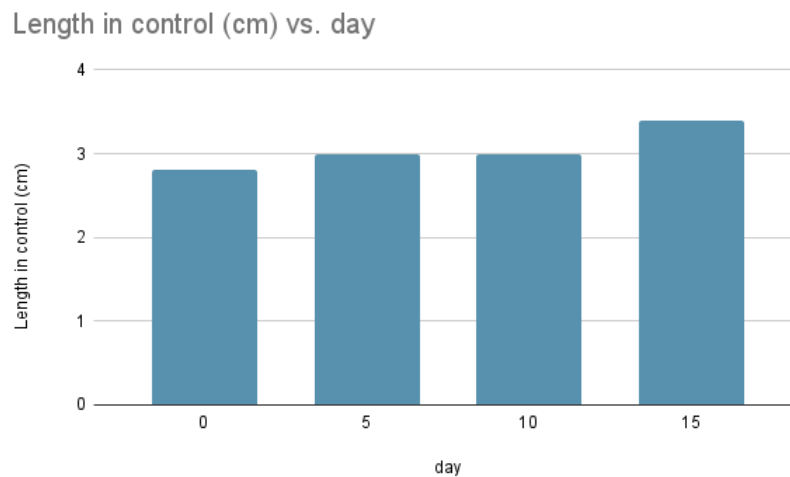


The length of the zebrafish on day 5, day 10 and day 15 are 3.0 cm, 3.0cm and 3.4 cm respectively. The weight of the zebrafish on day 5, day 10, day and 15 are 17mg, 23 mg and 25mg respectively.

5.3.1.1. Length in Zebrafish in control (cm) VS. Day

The length of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank 1 (Control) and a graph was to check the growth rate.

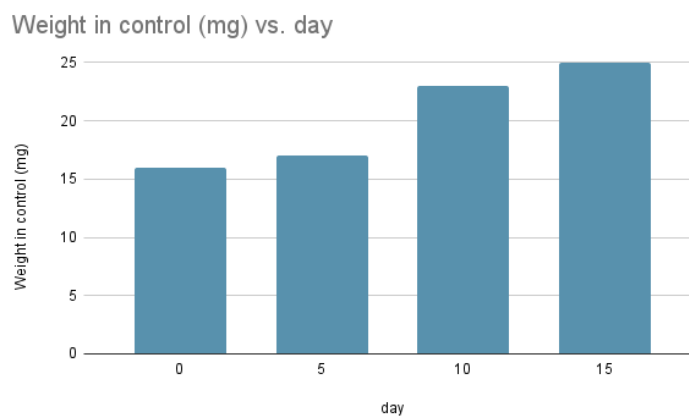
Fig: 5.4 Length of zebrafish in control (cm) VS. Day



5.3.1.2. Weight of Zebrafish in Control (mg) VS. Day

The weight of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank1 (Control) and a graph was to check the growth rate.

Fig: 5.5 Weight of zebrafish in control (mg) VS. Day



5.3.2. 10MG HFB - FISH GROWTH

The length of the zebrafish was measured using a ruler and the weight of the zebrafish was measured using a weighing balance.

Fig: 5.6 Day 05 in 10mg HFB



Fig: 5.7 Day 10 in 10mg HFB



Fig: 5.8 Day 15 in 10mg HFB

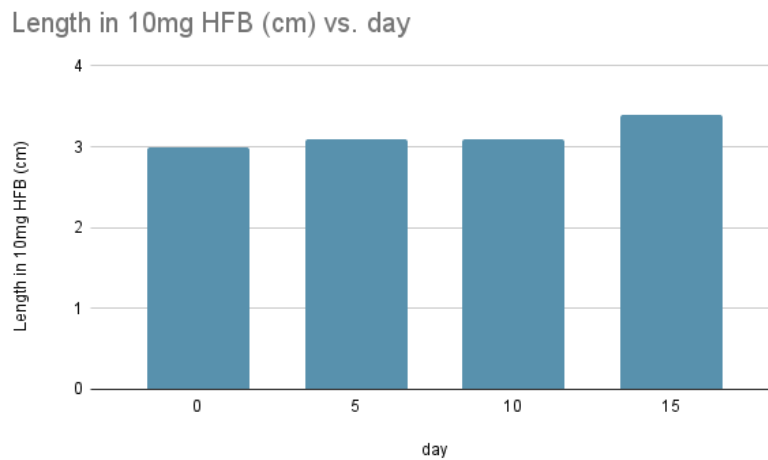


The length of the zebrafish on day 5, day 10 and day 15 are 3.1 cm, 3.1cm and 3.4 cm respectively. The weight of the zebrafish on day 5, day 10, day and 15 are 19mg, 29 mg and 30mg respectively. The length and weight of the zebrafish was slightly increased compared to the control.

5.3.2.1 Length in 10mg of HFB (cm) VS. Day

The length of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank 2 (10mg Herbal Fortified Biscuit) and a graph was to check the growth rate.

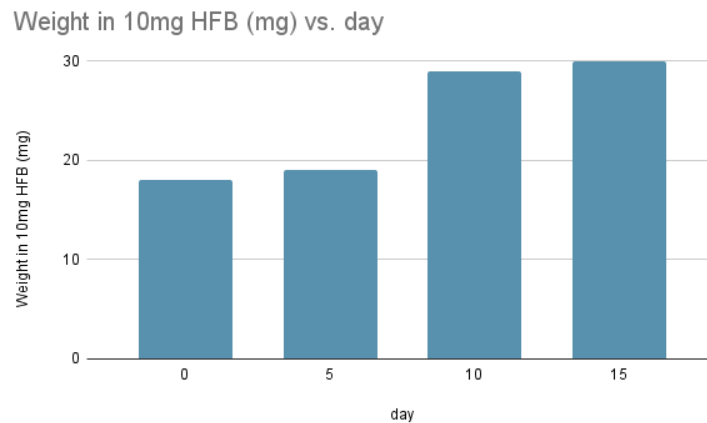
Fig: 5.9 : Length of zebrafish in 10mg of HFB (cm) VS. Day



5.3.2.2 Weight in 10mg of HFB (mg) VS. Day

The weight of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank 2 (10mg Herbal Fortified Biscuit) and a graph was to check the growth rate.

Fig: 5.10 : Weight of zebrafish in 10mg of HFB (mg) VS. Day



5.3.3. 20MG HFB - FISH GROWTH

The length of the zebrafish was measured using a ruler and the weight of the zebrafish was measured using a weighing balance.

Fig: 5.11 : Day 05 in 20mg HFB



Fig: 5.12 : Day 10 in 20mg HFB



Fig: 5.13 : Day 15 in 20mg HFB

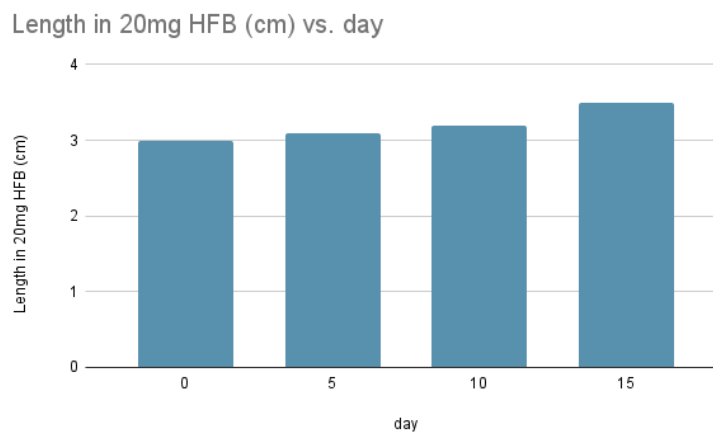


The length of the zebrafish on day 5, day 10 and day 15 are 3.1 cm, 3.2cm and 3.5 cm respectively. The weight of the zebrafish on day 5, day 10, day and 15 are 25mg, 29 mg and 31mg respectively. The length and weight of the zebrafish was ideally increased in comparison to the control and 10mg HFB supplemented zebrafish.

5.3.3.1 Length of Zebrafish in 20mg of HFB (cm) VS. Day

The length of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank 3 (20mg Herbal Fortified Biscuit) and a graph was to check the growth rate.

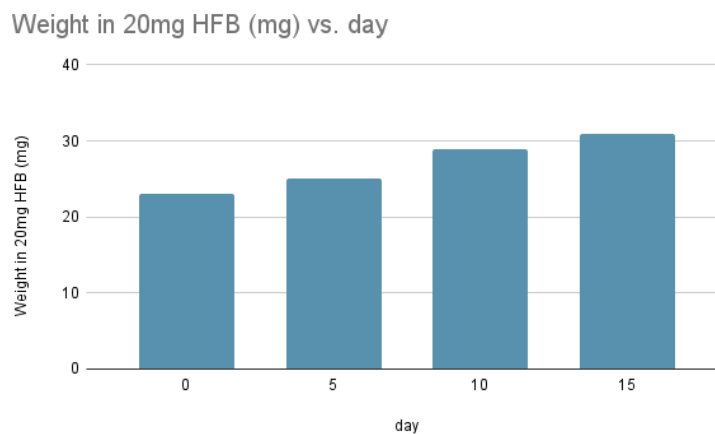
Fig: 5.14 : Length of zebrafish in 20mg of HFB (cm) VS. Day



5.3.3.2 Weight of Zebrafish in 20mg of HFB (mg) VS. Day

The weight of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank 3 (20mg Herbal Fortified Biscuit) and a graph was to check the growth rate.

Fig: 5.15 : Weight of zebrafish in 20mg of HFB (mg) VS. Day



5.3.4. 30MG HFB - FISH GROWTH

The length of the zebrafish was measured using a ruler and the weight of the zebrafish was measured using a weighing balance.

Fig: 5.16 : Day 05 in 30mg



Fig: 5.17 : Day 10 in 30mg



Fig: 5.18 : Day 15 in 30mg

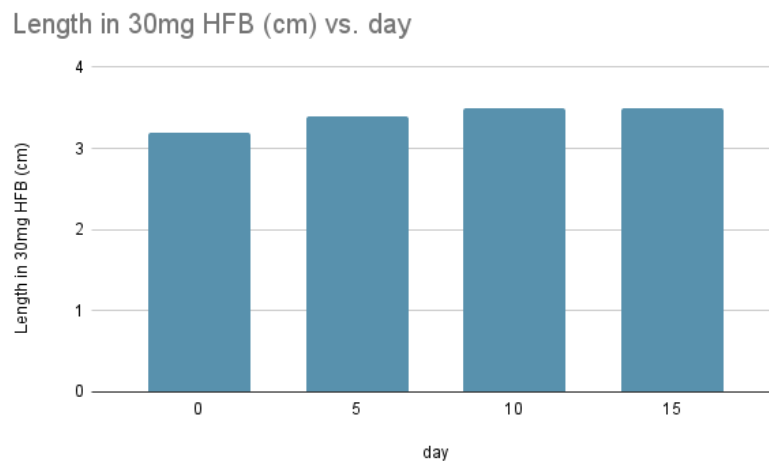


The length of the zebrafish on day 5, day 10 and day 15 are 3.4 cm, 3.5cm and 3.5 cm respectively. The weight of the zebrafish on day 5, day 10, day and 15 are 24mg, 34 mg and 35mg respectively. The length and weight of the fish was massively increased when compared to the control and 10mg HFB supplemented zebrafish and ideally increased when compared to the 20mg HFB supplemented zebrafish.

5.3.4.1 Length of zebrafish in 30mg OF HFB (cm) VS. Day

The length of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank 3 (30mg Herbal Fortified Biscuit) and a graph was to check the growth rate.

Fig: 5.19 : Length of zebrafish in 30mg of HFB (cm) VS. Day



5.3.4.2 Weight of Zebrafish in 30mg of HFB (mg) VS. Day

The weight of the fishes from Day 0, Day 5, Day 10, Day 15 were taken from Tank 3 (30mg Herbal Fortified Biscuit) and a graph was to check the growth rate.

Fig: 5.20 : Weight of zebrafish in 30mg of HFB (mg) VS. Day

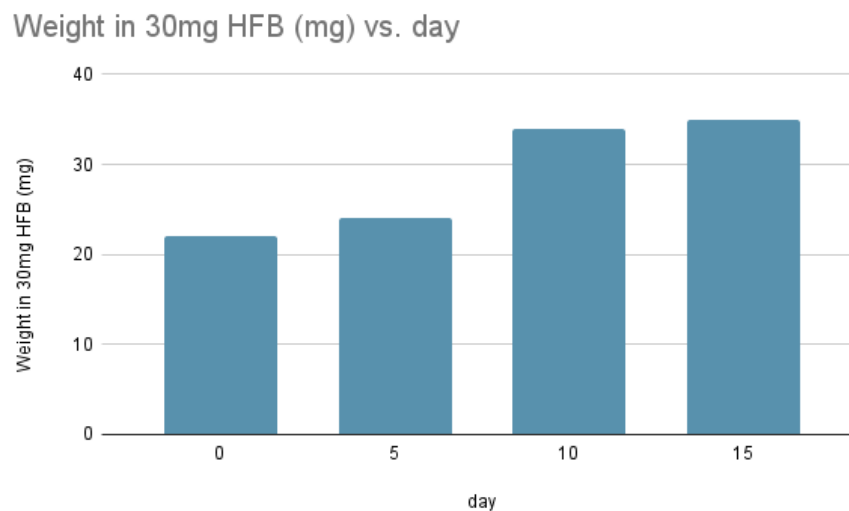


Table:5.3. Overall Growth rate of zebrafish

<u>TANK</u> <u>\</u> <u>DAYS</u>	LENGTH (cm) AND WEIGHT (mg) OF ZEBRAFISH IN CONTROL (TANK 1)	LENGTH (cm) AND WEIGHT (mg) OF ZEBRAFISH IN 10MG OF HFB (TANK 2)	LENGTH (cm) AND WEIGHT (mg) OF ZEBRAFISH IN 20MG OF HFB (TANK 3)	LENGTH (cm) AND WEIGHT (mg) OF ZEBRAFISH IN 30MG OF HFB (TANK 4)
00	2.8cm 16 mg	3.0cm 18 mg	3.0cm 23 mg	3.2cm 22 mg
05	3.0cm 17 mg	3.1 cm 19 mg	3.1 cm 25 mg	3.4 cm 24 mg
10	3.0cm 23 mg	3.1cm 29mg	3.2cm 31mg	3.5cm 34mg
15	3.4cm 25 mg	3.4cm 30mg	3.5cm 29mg	3.5cm 35mg

AVERAGE DAILY WEIGHT GAIN = FINAL WEIGHT – INITIAL WEIGHT / NO. OF DAYS

For control = $9 / 15 = 0.6\text{mg}$

For 10mg = $12 / 15 = 0.8\text{mg}$

For 20mg = $6 / 15 = 0.4\text{mg}$

For 30mg = $13 / 15 = 0.8\text{mg}$

AVERAGE DAILY LENGTH GAIN = FINAL LENGTH – INITIAL LENGTH / NO. OF DAYS

For control = $0.6 / 15 = 0.04\text{cm}$

For 10mg = $0.4 / 15 = 0.02\text{cm}$

For 20mg = $0.5 / 15 = 0.03\text{cm}$

For 30mg = $0.3 / 15 = 0.02\text{cm}$

SPECIFIC GROWTH RATE =

$\ln (\text{ FINAL WEIGHT }) - \ln(\text{ INITIAL WEIGHT}) / \text{ NO. OF DAYS}$

$$\text{For control} = \ln(25) - \ln(16) / 15$$

$$= 3.03$$

$$\text{For 10mg} = \ln(30) - \ln(18) / 15$$

$$= 3.20$$

$$\text{For 20mg} = \ln(29) - \ln(23) / 15$$

$$= 3.15$$

$$\text{For 30mg} = \ln(35) - \ln(22) / 15$$

$$= 3.34$$

With the values, In 10mg dose, we could witness a spike in weight. But when it comes to the length of zebrafish in 10mg dose, it was not impressive. In 30 mg doses, we could witness a spike in weight as well . But like the 10 mg dose, 30mg dose also doesn't show impressive results when it comes to the length of zebrafish in 10mg dose, it was not impressive It was concluded that 20mg dose is ideally acceptable due to its moderate length and weight increase when compared to 30mg dose, 10 mg dose in which the length was not increased as much as it was in 20mg dose.

6. SUMMARY AND CONCLUSION

In this project our intention is to fabricate an herbal fortified biscuit as a nutritional supplement. The main purpose of herbs used in the biscuit was to provide the necessary nutrients the body requires. In this method, The biscuit is made using Finger millet flour, Jaggery powder, Coconut oil and Cocoa powder and the herb extracts (Spinach, Moringa Leaves and Holy Basil). The herbs used here are high in folic acids, iron, calcium, vitamin B and C which would help in the welfare of pregnant women. It can be consumed by Gluten- and lactose-intolerant people and also people who follow a vegan diet. The nutritional analysis and microbiological analysis results were highly likely and it was the way it was expected to be, which is more nutritional when compared with regular biscuits. Herbal fortified biscuits contain surprisingly high levels of protein without containing soy or milk protein, with just herbs and other ingredients, this factor can positively affect the growth of children. An average biscuit contains 2.9 mg of iron per 100 grams, whereas the herbal fortified biscuit has 6.92 mg of iron per 100 grams, which makes it ideal for treating anemic patients. The stats prove that the herbal fortified biscuits were found to promote better growth in the zebrafish than normal feed in the experiment. To estimate the growth rate zebrafishes had been treated with Herbal Fortified Biscuits for a short period of 15 days to conclude its growth. In the result, after 15 days of maintenance, there is a better growth rate in the 20g treated fishes than other treated and untreated fishes. From these results it is known that mg treated fishes had the highest growth (Length 3.5cm, weight 35mg) which is also a stable growth from beginning. As it has stable growth in fishes it is concluded that intake of moderate dose of Herbal Fortified Biscuits gives better results in growth and it is a safe dose without any negative symptoms to the health. Compared with high and low doses intake of moderate dose will give good results in the growth rate.

7. REFERENCES

- [1] Ortiz-Andrellucchi, A., Ngo, Q. A., & Serra-Majem, L. (2016). Malnutrition: Concept, classification and magnitude. *The Encyclopedia of Food and Health*, 3, 610–630.
- [2] Lopez, A. D., Mathers, C. D., Ezzati, M., Jamison, D. T., & Murray, C. J. (2006). Global and regional burden of disease and risk factors, 2001: Systematic analysis of population health data. *The Lancet*, 367, 1747– 1757.
- [3] Shetty, P. (2009). Community-based approaches to address childhood undernutrition and obesity in developing countries. In S. C. Kalhan, A. Prentice, & C. S. Yajnik (Eds.), *Emerging societies-coexistence of childhood malnutrition and obesity* (Vol. 63, pp. 227–257). Basel, Switzerland: Karger Publishers.
- [4] Allen, L. D., de Benoist, B., Dary, O., & Hurrell, R. E. (2006). Guidelines on food fortification with micronutrients (p. 376). Geneva, Switzerland: World Health Organization/Food and Agriculture Organization.
- [5] Bain, L. E., Awah, P. K., Geraldine, N., Kindong, N. P., Siga, Y., Bernard, N., & Tanjeko, A. T. (2013). Malnutrition in sub-Saharan Africa: Burden, causes and prospects. *Pan African Medical Journal*, 15.
- [6] Bryce, J., Boschi-Pinto, C., Shibuya, K., & Black, R. E. (2005). WHO estimates of the causes of death in children. *The Lancet*, 365(9465), 1147–1152.
- [7] Black, R. E., Allen, L. H., Bhutta, Z. A., Caulfield, L. E., de Onis, M., Ezzati, M., ... Rivera, J. (2008). Maternal and child undernutrition: Global and regional exposures and health consequences. *The Lancet*, 371(9608), 243–260.
- [8] Black, R. E., Morris, S. S., & Bryce, J. (2003). Where and why are 10 million children dying every year? *The Lancet*, 361(9376), 2226–2234.

- [9] Mandelbaum, J. (2004). Vitamin and mineral deficiencies harm one-third of the world's population. *Bulletin of the World Health Organization*, 82(3), 230–231.
- [10] Mishra, R. (2011). Current approaches in food fortification for overcoming micronutrient deficiencies. *Research in Environment and Life Sciences*, 4, 39–48.
- [11] Mannar, V., & Gallego, E. B. (2002). Iron fortification: Country level experiences and lessons learned. *Journal of Nutrition*, 132, 856S–858S.
- [12] Zimmermann, M. B., Muthayya, S., Moretti, D., Kurpad, A., & Hurrell, R. F. (2006). Iron fortification reduces blood lead levels in children in Bangalore, India. *Pediatrics*, 117, 2014–2021.
- [13] Verma, A. (2015). Food fortification: A complementary strategy for improving micronutrient malnutrition (MNM) status. *Food Science Research Journal*, 6, 381–389.
- [14] Brown, K. H., Hambidge, K. M., Ranum, P., & Zinc, F. W. G. (2010). Zinc fortification of cereal flours: Current recommendations and research needs. *Food and Nutrition Bulletin*, 31, S62–S74.
- [15] Brown, K. H., Pearson, J. M., Baker, S. K., & Sonja, Y. H. (2009). Preventive zinc supplementation among infants, preschoolers, and older prepubertal children. *Food and Nutrition Bulletin*, 30, S12–S40.
- [16] Moench-Pfanner, R., Lallou, A., & Berger, J. (2012). Introduction: Large-scale fortification, an important nutrition-specific intervention. *Food and Nutrition Bulletin*, 33, S255–259.
- [17] Manley, D. 2003a. Setting the scene: History and position of biscuits. In *Technology of Biscuits, Crackers and Cookies*, 3rd edn, 1–8. Boca Raton, FL: CRC Press.

- [18] Manley, D. 2003b. Position of biscuits in nutrition. In *Technology of Biscuits, Crackers and Cookies*, 3rd edn, 307–313. Boca Raton, FL: CRC Press.
- [19] Manley, D. 2005. Recipes for dietetic biscuits. In *Biscuit, Cracker and Cookie Recipes for the Food Industry*, 168–173. Boca Raton, FL: CRC Press.
- [20] Sevá-Pereira, A., de Moraes, G.R., de Oliveira, S.P., Ryes, F.G. 1991. Use of fiber enriched biscuit in the treatment of chronic intestinal constipation. *Revista da Associação Paulista de Medicina* 109: 265–268
- [21] Sharif, K., Butt, M.S., Anjum, F.M., Nasir, M. 2005. Improved quality of baked products by rice bran oil. *Internet Journal of Food Safety* 5: 1–8.
- [22] Rababah, T.M., Al-Mahasneh, M.A., Ereifej, K.I. 2006. Effect of chickpea, broad bean or isolated soy protein additions on the physicochemical and sensory properties of biscuits. *Journal of Food Science* 71: S438–S442
- [23] Sudha, N.L., Srivastava, A.K., Vetrmani, R., Leelavathi, K. 2007. Fat replacement in soft dough biscuits—Its implications on dough rheology and biscuit quality. *Journal of Food Engineering* 80: 922–930.
- [24] Savitha, Y.S., Indrani, D., Prakash, J. 2008. Effect of replacement of sugar with sucralose and maltodextrin on rheological characteristics of wheat flour dough and quality of soft dough biscuits. *Journal of Texture Studies* 39: 605–616.
- [25] Jenkins, A.L., Jenkins, D.J.A., Wolever, T.M.S. 2008. Comparable postprandial glucose reductions with viscous fiber blend enriched biscuits in healthy subjects and patients with diabetes mellitus—Acute randomized controlled clinical trial. *Croatian Medical Journal* 49: 772–782.

- [26] Zucco, F., Borsuk, Y., Arntfield, S.S. 2011. Physical and nutritional evaluation of wheat cookies supplemented with pulse flours of different particle sizes. *LWT: Food Science and Technology* 44: 2070–2076.
- [27] Sharma, A., Wang, R., Zhou, W. 2011. Functional foods from green tea. In *Functional Foods*
- [28] James, M., Lattimer and Mark D. H. “Effects of Dietary Fiber and Its Components on Metabolic Health” (2010). *Nutrient*, 2: 1266-1289.
- [29] Dietary fiber, University of California, Berkeley Bancroft Way Berkeley, CA 9472
- [30] Jenkins, D.J.A., Wolever, T.M.S., Leeds, A.R., Gassull, M.A. and Haisman, P.J.B. “Dietary fibres, fibre analogues and glucose tolerance: importance of viscosity” (1978). *Brit. Med. J.* 1:1392-94.
- [31] Holt, S., Heading, R.C., Carter, D.C., Prescott, L. F. and Tothill, P. “Effect of gel fiber on gastric emptying and absorption of glucose and paracetamol” (1979). *Lancet* 1:636-39.
- [32] ADA (American Dietetic Association). Position of the American Dietetic Association: “Health implications of dietary fiber” (2008). *J. Am. Diet. Assoc.* 108: 1716–31.
- [33] James, M., Lattimer and Mark D. H. “Effects of Dietary Fiber and Its Components on Metabolic Health” (2010). *Nutrient*. 2: 1266-1289.
- [34] IFIC (International Food Information Council) (2008). Fiber Fact Sheet. Retrieved Nov. 10, 2010.

- [35] Geiss L. S., Herman W. H. and Smith P.J. "National Institute of Diabetes and Digestive and Kidney Disease" (1995). NIH Publication No. 95-1468; pp. 233-257.
- [36] Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997). *Diabetes Care* 20: 1183–1197.
- [37] Blackburn, N. A., Redfern, J. S., Jarjis, H. and Holgate, A. M. "The mechanism of action of guar gum in improving glucose tolerance in man" (1984). *J. Clin. Sci.* 66: 329-36.
- [38] Pasha, I., Parveen, S., Salim, U.R. and Nawaz, H. "Baking quality of wheat flour cookies supplemented with fiber from different sources" (2008). *Pak. J. Food. Sc.* 18:1-4
- [39] Maheshwari, R., Rani, B. Yadav, R. K. and Prasad, M. "Usage of Holy Basil for Various Aspects" (2012). *Bull. Env. Phar. and Life Sci.* 1: 63 – 65.
- [40] Shama, S. N. "A mine of medicinal uses: *Ocimum sanctum*" (2012). *Int. J. of Phar. Rev. Res.* 2 (2): 69-74.
- [41] Kumari, P., Sharma, P. S. and Srivastava M. M. "Biosorption studies on shelled *Moringa oleifera* Lamarck seed powder: Removal and recovery of arsenic from aqueous system" (2006). *Int. J. Miner. Proc.* 78:131–139.
- [42] Cáceres, A., Saravia, A. S., Rizzo, L., Zabala, E. D., Leon and Nave, F. "Pharmacologic properties of *Moringa oleifera*: screening for antispasmodic, anti-inflammatory and diuretic activity" (1992). *J. of Ethnophar.* 36(3): 233–237.
- [43] Marugandan, S., K. Srinivasan, S. K. Tandon and H. A. Hasan. "Anti-inflammatory and analgesic activity of some medicinal plants" (2001). *J. Med. Aromat. Plant. Sci.* 22:56–58.

- [44] Ples, M. And Howell H., Comparative Effects of Moringa Oleifera Lam. Tea on Normal and Hyperglycemic Patients.
- [45] Kulkarni, S.D. Roasted soybean in cookies. Influence on product quality (1997). J. Food Sci. Technol. 34: 503-505.
- [46] Akubor, P. "Functional properties and performance of cowpea/ plantain/ wheat flour blends in biscuits" (2003). Springer. 58 (3):1-8.
- [47] Pratima, A. and Yadava, M.C. "Effect of incorporation of liquid dairy by products on chemical characteristics of soy fortified biscuits" (2000). J. of Food Sc. and Tech. 37 (2): 158-61.
- [48] AOAC. Official methods of Analysis of AOAC, International, Washington, DC: Association of Official Analytical Chemists (1990). 16: 1546.