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DEVELOPMENT AND QUALITY EVALUATION OF ANTIOXIDANT RICH AND HIGH PROTEIN BISCUITS

A THESIS SUBMITTED IN FULLFILMENT OF THE REQUIREMENT OF

MASTER OF RESEARCH

By

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STATEMENT OF AUTHENTICATION

This is to certify that the thesis entitled, **"Development and Quality Evaluation of Antioxidant Rich and High Protein Biscuits"** to the best of my knowledge and belief this thesis contains no material that has been previously published by any other person, except as acknowledged in the text. I hereby declare that this work has not submitted either in full or in part, for the award of degree/diploma at this or any other University.

Signature: 

Date: 10/12/2018

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Abstract

This research was undertaken with a view to develop antioxidant rich high protein biscuits with health promoting benefits. The flour used for this purpose was procured from Manildra Group of Pty Ltd Company. In order to enhance the antioxidant properties of the biscuits, four traditionally well-known underutilized plants with several health benefits have been chosen. These are Moringa leaves, Gotu kola leaves, Schisandra berries, and Goji berries that are known to exhibit antioxidant, antidiabetic, anticancer, anti-inflammatory, immunostimulatory, and cardioprotective properties. The dried form of leaves and berries were used in this research. The research was designed to prepare biscuit samples with different substitutions of the selected plant's material. Sample 1 was substituted with 1% of all the four plants in equal proportion Sample 2 was substituted with 2% of all the four plants in equal proportion; Sample 3 was substituted with 3% of all the four plants in equal proportion; Sample 4 was substituted with 4% of all the four plants in equal proportion; Sample 5 was substituted with 1% of Moringa and Gotu kola leaves in equal proportion; and Sample 6 was substituted with 1% of Schisandra and Goji berries in equal proportion. A control sample without any plant material was also prepared as a reference. Amongst the different biscuit samples developed in this research, sample 4 had the highest proportion of plant's material.

Nutritional and chemical analysis of the four plants used in this study revealed that all of them exhibited highly significant radical scavenging activities that are superior to many well-known anticancer traditional herbs. Particularly, Schisandra berries displayed extremely high antioxidant activities. These activities were well correlated to the total phenolic and flavonoid contents of the plants. The total phenolic and flavonoid contents of the six biscuit samples were

in accordance with the amounts of plant's material incorporated. This is consistent with the highest phenolic and flavonoid contents observed in sample 4. The antioxidant activities of the six biscuit samples were also proportional to the amounts of plant material incorporated showing a highly significant correlation of the activities with their polyphenol contents.

The biscuits (Sample-4) with 4% plant material are found to be the best substitution with the highest polyphenol contents that offer an overall health benefit. Therefore, the antioxidant rich high protein biscuits developed in this research are expected to provide health benefits including alleviation of oxidative stress and associated diseases.

CHAPTER 1
INTRODUCTION

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Introduction

1.1 Introduction

Increasing awareness and scientific evidence clearly indicates a strong relationship between health and diet. These factors have generated new concepts for researchers in the field of nutrition science targeting the development and promotion of functional foods (Carnes et al., 2013). The rapid increase in diseases is now increasing interest in more intensified research regarding the antioxidant activity of bioactive components present in the food products and their contribution to the prevention and treatment of several ailments. Functional foods are prominent junctions in the field of health and nutrition. Most of the research studies have clarified their scope and importance. The demands of functional food products are high in global health and wellness market and also functional ingredient rich food products are now representing one of the fastest growing sectors of food manufacturing an observed during the past few years (Kraus, 2015). Kulczynski & Gramza-Michalowska (2016) have reported in his study that, the American Dietetic Association (ADA) has defined bioactive ingredients of food as "Bioactive food components are physiologically active constituents in food or dietary supplements derived from both animal and plant sources, including those needed to meet basic human nutrition needs that have been demonstrated to have a role in health and to be safe for human consumption".

Antioxidants have free radical scavenging activity and believed to offer a defense to the living organism in protecting against reactive oxygen species (Dong-Ping et al., 2017; Ou et al., 2002). Even though, living organisms have their own antioxidant defense and repair system in the body,

these systems are not enough to overcome the damage caused by reactive oxygen species. Therefore, dietary antioxidant supplementation can help to maintain sufficient amount of antioxidant status that is promising in strengthening the normal physiological function in all living organisms (Almeida et al., 2011; Speroni et al., 2011; Thaipong et al., 2006). The bioactive ingredient rich foods, vegetables, and herbal plants are the good source of exogenous antioxidants.

The various research studies have demonstrated that the scope and benefits of antioxidants constituents vary in every discipline. In food and nutrition science, antioxidants take a wider scope that the field includes dietary antioxidants, such as functional foods that significantly improve the physiological function of the body by decreasing the adverse effects of reactive intermediate species in oxidation mechanisms in human beings. Moreover, natural antioxidants also help to prevent the oxidation process which helps to improve the food products quality and also nutritional values by interrupting the chain reactions of chelating and oxidative agents and decomposed hydroperoxides (Antinio, 2018; Aruoma, 1994).

In biological systems, the reactive oxygen spices (ROS) and reactive nitrogen spices (RNS) play a dual contributory role in the biology of various chronic diseases (Chen et al., 2012; Fang et al., 2002). Consequently, antioxidants provide counteracting protection against the free radicals and the oxidative stress by scavenging these radicals (Liu, 2013; Wang et al., 2010; Zhong et al., 2013). Due to the intense impact of environmental and endogenous radical-initiating factors, including UV-radiations, dust, and pollutants, and autoxidation reactions can contribute to

oxidative stress leading to the structural damage of biomolecules and loss of function (Yousuf et al., 2016).

Oxidative stress plays a key role in the generation of different pathologies, such as pathogenesis of aging, inflammatory processes, cancer, cardio and neurodegenerative diseases (Tsang & Chung, 2009). The exogenous antioxidants with the human body system may help to keep the antioxidant status balance between oxidation and anti-oxidation by ameliorating the damage caused by free radicals or oxidative stress generated during the physiological processes, functioning as the scavengers of reactive radical species and diminishing harmful effects of oxidative stress (Niki & Noguchi, 2008; Rohman et al., 2010; Valko et al., 2007). In the body system, bioactive food compounds are important part of the antioxidant system for defense after oxidative depletion via enzymatic antioxidant shortcomings or the reduction of other antioxidants taken as diet and supplements that includes vitamin C, fatty acids polyphenols, flavonoids, Gallic acid, phenolic acid (Bhagwat et al., 2010; Milner, 2004).

Scientific research studies have reported that synthesized antioxidant compounds, especially butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), and tert-butyl hydroquinone (TBHQ) have been used to prevent the lipid peroxidation of food products. In recent years, the increasing interest to search alternatives for synthetic antioxidants from food components has promoted research on fruits, vegetables, and other plant sources to identify the natural antioxidants in them (Jayathilakan et al., 2007).

The exogenous antioxidants are present in various types of plants, such as fruits, cereals, spices, and herbs. These natural antioxidants from plant materials are mainly polyphenols, flavonoids, β-carotene, vitamin C, anthocyanins, and polysaccharides which display significantly higher antioxidant properties. Antinio (2018) reported that natural antioxidants from plant materials, especially polyphenols, carotenoids, and flavonoids exhibit an extensive range of health-promoting bioactivities, such as cardioprotective, neuroprotective, antidiabetic, anti-inflammatory, antimicrobial, anti-aging, cytotoxic, anticancer, antitumor, regulating of hormone balance, immune system enhancement, and memory-enhancing activities, and also weight reduction in humans.

Many observational studies have investigated that supplying to human beings with sufficient quantity and ratios of nutrient is necessary to maintain the proper functioning of the body and health. In addition to that, nutritional quality, the nutraceutical rich foods are expected to have disease preventative properties. In this context, underutilized edible plants play an important role as they can be added to improve functional /nutraceutical value of food products. Four underutilized plants studied in this research, namely Moringa, Gotu kola, Goji berry, and Schisandra berry are edible and also have been used as traditional medicine for centuries (Benchennouf et al., 2017; Donno et al., 2015). Recently, these plants have also been well studied for their antioxidant and functional properties (Damor et al., 2017; Oyeyinka & Oyeyinka, 2018). These pharmacological studies have found that all the four plants display excellent antioxidant and nutraceutical potentials and hence, are expected to have health-promoting properties (Guo et al., 2008a; Gupta et al., 2007; Li & Zhou, 2007; Mocan et al., 2014b; Pakade et al., 2013b; Panossian & Wikman, 2008; Wang et al., 2010). Based on their

antioxidant properties as well as their important health-promoting benefits, utilization and supplementation of these under-utilized plants have received great attention in food science and nutrition. It has indeed been demonstrated that these four plants can be used as ingredients for the preparation of properly engineered foods (Alam et al., 2014).

Biscuits are popular snack foods and are consumed extensively among all age groups all over the world especially in developing countries where protein and malnutrition are prevalent (Chinma & Gernah, 2007; Chinma et al., 2012). Food industries are faced with the challenge of the production of foods that are rich in bioactive ingredients in order to meet the current demands for functional foods. Considering the nutritional and functional requirements, protein, and fibre enriched flour together with plants' parts (all from natural sources) have been rated as highly nutritious, as these are rich sources of protein and antioxidants, easy to substitute and available at reasonable cost (Kulthe et al., 2014).

1.2 Research Objectives

This project aims to develop and evaluate the physical, chemical, biological and nutritional characteristics of antioxidant rich, high protein biscuits. Therefore, the present investigation was planned to develop antioxidant rich, high protein biscuits by using under-utilized plants' parts along with protein enriched biscuit flour, sugar, pure maple syrup, Omega-3 rich canola oil, baking powder, and other general ingredients. All these food ingredients will impart good physical and nutritional characteristics, such as color, texture, and nutraceutical content that have

significance modern bakery products and other foods. The present research was conducted to address the following objectives:

1. To develop value-added biscuits by using underutilized plants.
2. To study the physical and nutritional aspects of under-utilized plant based biscuits
3. To evaluate the antioxidant activities and antioxidant contents of the underutilized plant's based biscuits.

CHAPTER 2
LITERATURE REVIEW

Chapter 2

Literature Review

2.1 Bakery products

Bakery industry is one of the largest and highly fragmented global industries in the world. In the past five years, demand has risen for high quality bakery goods due to consumers' health consciousness. The introduction of biscuits that have been enriched with value-added bioactive nutrients has also contributed to the growing demand of premium bakery products with functional benefits. Biscuits are popular snack food due to a wide range of high quality products that are readily available have a longer shelf life, and different taste and texture profiles at a reasonable cost. At present, there is an ever-growing demand for high quality protein enriched biscuits (Sonone et al., 2015).

Today, the consumers are aware of the relationship between diets and disease development so they are more health conscious and prefer low calorie, low fat, and high protein foods that are ready-to-eat. In this context, development of recipes that satisfy nutritional requirements and also display diseases preventative function will play a key role in securing consumer health. Among baked products, biscuits are especially popular being perceived as delicious products with special organoleptic properties (Khan et al., 2014).

Recent studies suggest that people are aware of the benefits of consuming nutritious foods. The development of an antioxidant rich and high protein containing biscuit is a worthwhile challenge when considering the overall nutritional status of the population (Klunklin & Savage, 2018). In

considering the demand for the development of healthy food products, researchers have recently become interested in functional foods that are high in protein and antioxidants as well as other bioactive compounds due to their health-promoting benefits (Pasqualone et al., 2015).

Biscuits are one of the most popular bakery foods in the world and popular among all age groups, due to convenience, taste, and low cost (Li, 2009). Fortification of biscuits with various types of vitamins, minerals, herbs is a common practice (Karklina et al., 2012). Biscuits are known for good palatability and have generally longer shelf-life and good eating quality. Also, due to less moisture content in biscuit products, these are less susceptible to microbiological spoilage. Nowadays, people emphasis is on healthy natural food products with low calories and carbohydrates, protein enriched, more dietary fibre and antioxidant rich (Emire & Arega, 2012). Herbal Biscuits are made by incorporation of underutilized plants' parts in a mixture of biscuit flour, powder sugar, canola oil, baking powder, sweetener, and other general ingredients which have beneficial effects on overall health. Biscuits have become extremely popular due to their sensory properties and texture, easy to eat and cost-effectiveness. Hence, antioxidant rich, high protein biscuits will also be beneficial to achieve the nutritional security of any target population. Considering these factors, the present study was designed aimed at formulating biscuits using under-utilized plants' materials in different proportions, and to evaluate the physicochemical, nutritional and nutraceutical quality characteristics.

2.2 Need and significance of protein enrichment

Modern population is extremely health conscious due to some of the major world health problems. In addition, increased awareness of the link between diet and health has motivated

new trends in nutrition science. As a result, more attention is given to the health benefits of individual foods. In particular, attention is given to the development of foods that contain nutraceuticals for the prevention of degenerative diseases and improving body function (Steijns, 2001).

Protein is an endogenous component of healthy diets. Protein source includes parts of plant material, cereals and legumes, roots and vegetables, and animal foods. Animal foods are considered as the best source of protein as they contain essential amino acids. However, over the past few years, there is a growing demand for other alternative protein sources due to high the cost of animal food products. New Scientific technologies have helped to isolate the readily digestible fragments of food components from the non-digestible fractions such as protein, bioactive ingredients, and fibre. It is known that many communities have changed eating habits towards high quality healthy foods (Bunde et al., 2010).

Protein enriched flour has diversified uses in the food industry, especially in the preparation of baked goods such as bread, cookies, and biscuits. Moreover, such preparations are characterized by healthy nutritional properties with less fat content and high protein (about 13.3%) and other essential micronutrients. Protein enriched flows can also be combined with other food ingredients, such as natural antioxidants in the preparation of biscuit products because it helps to enhance the physical, nutritional, functional, and sensorial quality characteristics of the baked goods and also helpful the food industry to find it economical to use in high protein bread preparations and biscuit manufacture (Oyeyinka & Oyeyinka, 2018). Such protein enriched flour

can be produced by mixing wheat, barley, and other ingredients. An example is provided in Table 2.1. Protein enriched flour with similar composition (Table 2.1) has been purchased from Manildra Group of Companies, Australia and used for this research.

Table 2.1: Nutrient composition of Protein Enriched Flour

Constituents	Quantity g/100g
Moisture	14.0
Protein	13.5
Total Fat	1.6
Saturated Fat	0.2
Trans Fat	<0.1
Carbohydrates	66.8
Sugar	2.3
Dietary Fibre	3.5

In the bakery industry, the concept of incorporation value-adding composite flour is not new and has been the subject of several studies. However, composite flour blends materials should preferably be readily available and also provide high quality nutritional potential (Olaoye & Idowu, 2018). Biscuits have been suggested as a better utility for high quality protein enriched flour than bread because of their wide consumption, good eating quality and relatively ready-to-eat form along with longer shelf-life. Recently, there has been an increased trend of producing cookies using natural antioxidants to improve their nutritional qualities. A variety of protein rich flours such as, soybean, sweet potato, oats, and cowpea were utilized in biscuit preparations that

showed improvement in their antioxidative qualities (Sneha et al., 2012). Plant and fruits components in the powdered form were also utilized to prepare nutritive cookies. New strategies (NPD process, FFPD model) are also used where cookies and biscuit were prepared with plants and fruits powder extracts, with the objectives to improve their quality with fortification of protein, dietary fibre, antioxidant activity, and polyphenolic content in a cost-effective manner (Raymond, 2013; Reid & Brady, 2012; Youssef, 2012).

Food products rich in protein and antioxidants can satisfy the demands of people for health benefits. The intake of plant based antioxidants has been related to the maintenance of health and reduction of the incidence of chronic diseases. A different variety of plant sources (Soya proteins, quinoa, seitan, edamame beans, chia and hemp seeds) has been used in bakery products to improve the texture, color, and aroma with a reduced calorie content of the final products (Marijana, 2009). Antioxidants are bioactive constituents that have the ability to inactivate free radicals, which are instable species that could cause several chronic diseases (Bolanho et al., 2014). Underutilized plants parts that can be consumed by humans, and have high antioxidant potential and several other therapeutic properties with major health benefits, such as the ability to improve circulatory functions and mental acuity, antitumor activity against cancer, helps to improve cardio-protective actions, ability to boost anti-diabetic, and anti-inflammatory actions (Baldermann et al., 2016; Jansen et al., 2014). Moreover, the functional properties of new products have therapeutic effects for selected diseases, such as anticancer, anti-ulcerogenic, antiviral, wound healing, blood pressure-reducing, anxiolytic, arteriosclerosis reducing, and improve venous insufficiency effects were ascribed as skin healing effects (Starowicz et al., 2015). Towards this goal under-utilized plants' based products can be included in various food

formulations. As discussed above oxidative mechanisms have the ability to produce undesirable health effects in living systems, and lead to serious diseases. A brief description of oxidative stress and formation is thereby provided below.

2.3 Oxidative stress in humans and disease formation

Reactive radical species are formed in the human body due to metabolic processes and external factors such as exposure to radiation, pollutants and other factors (Akinmoladun et al., 2010) Prolonged presence of excessive amounts of free radicals in the body can cause oxidative stress that has the potential to impact on various metabolic pathways in cells (Apel & Hirt, 2004; Aruoma, 1994). These effects can lead to oxidative damage to the structures of functional biomolecules, such as DNA, proteins, and lipids. Such damage to functional biomolecules has the potential to cause serious illnesses (as shown in Fig 2.1), such as cancer, cardiovascular and neurodegenerative disorders (Athina & Antonios, 2006; Li et al., 2014; MacNee & Rahman, 1999; Malta et al., 2013). It is therefore essential to alleviate oxidative stress by scavenging the reactive free radicals formed in the body. Biological systems possess their own antioxidant defense; however, this is not sufficient to cope with excessive oxidative damage. In this context, natural antioxidants consumed in the form of functional foods and dietary supplements are extremely important for improving oxidative defense and restoration mechanism in living cells (Aruoma, 1994; Lobo et al., 2010).

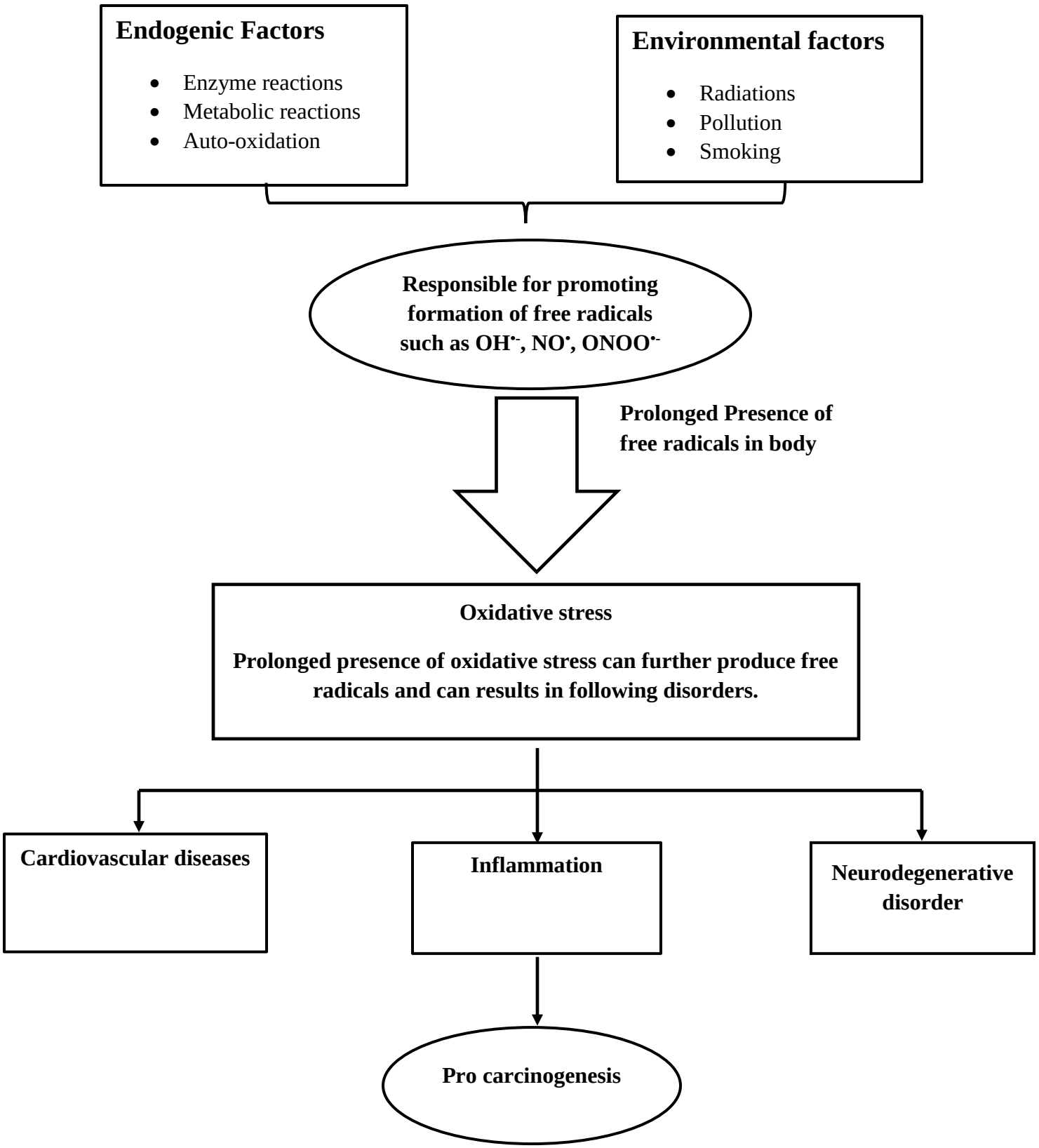


Fig 2.1: Flowchart showing the factors leading to oxidative stress and formation of different diseases

As can be seen from the Figure, prolonged oxidative stress leads to a severe imbalance between the production of free radicals and antioxidant protection in living systems. This situation can ultimately affect the cells, tissue and the whole animal (Lushchak, 2015; Milner, 2004; Mohamad et al., 2011; Rahman & Hussain, 2015). Continuous stress leads to a further increase of free radicals and the formation of inflammation in the body (Diaz et al., 2012; Zhang et al., 2011a). In such a situation, if the person's innate immunity is frail and also not sufficient antioxidants are consumed, inflammation can last for a longer period and a chronic condition is established. Such chronic oxidative stress and inflammation lead to the formation of several serious diseases like cancer (Karker et al., 2016; Shaikh et al., 2014).

Regular consumption of antioxidants such as polyphenols will effectively scavenge the reactive free radicals in the body and prevents oxidative damage and associated diseases (Dance, 2017; Fuente et al., 2005; Malta et al., 2013). Antioxidants are present in many plant based foods, such as fruits and vegetables or they can also be manufactured by synthetic methods. Many scientific studies demonstrated that synthetic antioxidant (such as BHT, BHA, TBHQ, and gallates) have been used in the food industries to prevent the oxidation process and also have been included in the human diet. However, their uses have raised concerns about high cost; cumbersome synthetic procedures that make them least accessibility and more importantly they exhibit adverse side-effects. Hence, natural antioxidants are the attractive alternative due to their cost-effectiveness, least side-effects and high natural abundance in plants (Arulselvan et al., 2016; Ramana et al., 2018).

Therefore, consumption of natural antioxidant rich food with sufficient amounts of polyphenols (phenolic compounds and flavonoids) is expected to show a beneficial effect and provide protective health advantage (Cai et al., 2004; Hashemzaei et al., 2017). Consequently, the use of plant based bioactive compounds as antioxidants to induce a protective effect against the disease causing free radicals has received more interest in food and nutrition science discipline.

In the body system, bioactive food constituents support internal antioxidant system in defense against the reactive free radicals and the natural antioxidants that are consume through our diet include glutathione, thiols, sterols, vitamin C, vitamin E, phytoestrogens, fatty acids, bioactive peptides, carotenoids, stanols, polyphenols, such as flavonoids, isoflavones, Gallic acid, phenolic acid, kaempferol, anthocyanins, and many more (Bhagwat et al., 2010; Guo et al., 2008c; Milner, 2004). Many of these antioxidants are present in plant based foods and herbs. A brief outline of important antioxidant constituents present in various plants is given in the following sections.

2.4 Important classes of antioxidant constituents in plants

2.4.1 Polyphenols in plant sources

Polyphenols are secondary metabolites produced by plants (Molan et al., 2012). The literature demonstrates that many polyphenols possess antioxidant, immunomodulatory, anti-inflammatory, antitumor, antidiabetic, antiviral and many other activities (Cai et al., 2004). It is known that the antioxidant activities are related to the structures of these molecules. Generally, the number of hydroxyl groups and their position and the presence of other functional groups are linked to the antioxidant activity of these compounds. Glycosylation of flavonoids is also linked

to the improved antioxidant action (Cai et al., 2004; Choudhary & Swarnkar, 2011; Guo et al., 2008c).

Flavonoids are an important class of polyphenols and occur extensively in plant tissue and exhibit different colors (blue, purple, yellow, orange and red). Flavonoids are structurally rigid with three-rings and contain many hydroxyl groups, and other functional groups (Hashemzaei et al., 2017; Wolfe & Liu, 2008). They display the antioxidant function by scavenging the free radicals as well as by chelating with metal ions. Owing to the tremendous importance and health benefits of these compounds, the total phenolic content and total flavonoid contents of plant based foods are measured on their water/ethanol extracts. These contents are generally used as a measure of their activities and health benefits of the associated foods.

2.4.2 Trace metals in plants

In addition to polyphenols, plants also contain several trace minerals (Sium et al., 2016). Literature demonstrates that trace metals such as Iron (Fe), Copper (Cu), zinc (Zn), Potassium (K), Magnesium (Mg), Calcium (Ca), Rubidium (Rb), and many other metals display health benefits against several diseases including oxidative stress and cancer (Schwartz, 1975). For instance, it has been demonstrated that sufficient intake of foods rich in zinc is important to alleviate oxidative damage and DNA repair (Nielsen, 2014; Prasad, 2014a), whereas, intake of a diet deficient with zinc can potentially lead to cancer formation (Prasad, 2014b). Selenium rich diet or selenium supplementation lowers the risk of several types of cancers (Dennert et al., 2011; Etani et al., 2014; Hatfield & Gladyshev, 2009).

A brief outline of the principle involved in the measurement of antioxidant activities is provided below. These measurement methods help to grade various plant extracts according to their antioxidant potentials.

2.5 Methods of measurement of antioxidant activities

Generally, radical scavenging abilities of phytochemicals are used as a measure of their antioxidant activities. In this project, the antioxidant activities of the selected underused plants and antioxidant rich biscuit samples were evaluated using the $ABTS^{\bullet+}$ radical scavenging assay. The bioactive compounds from the plants were evaluated through the Folin-Ciocalteu colorimetric method and aluminium chloride method. Basic principles and the theory behind the $ABTS^{\bullet+}$ free radical scavenging activity method is described in the following section.

2.5.1 Principles of $ABTS^{\bullet+}$ free radical scavenging activity test

The $ABTS^{\bullet+}$ free radical scavenging activity assay is the most popular peroxidase substrate for the estimation of total antioxidant activity. ABTS assay is an easier and generally used method and involves the colorimetric approach. ABTS displays an absorption band that has maxima in the range from 414 nm to 815 nm and shows a green-blue color reaction with PBS buffer (Rubio et al., 2016). In this test, metmyoglobin was first reacted with H_2O_2 to produce the ferrylmyoglobin radical, which is subsequently treated with was ABTS to make $ABTS^{\bullet+}$ (Re et al., 1999a). Moreover, several methods were employed for $ABTS^{\bullet+}$production, including treatment with potassium persulphate. In addition, different conditions are also considered in this particular assay. For instance, reaction times (incubation) ranging from 1 to 30 minutes (Almeida et al., 2011). Absorbance measurement usually made at 734 nm to minimize sample turbidity. In

general, the test sample has to be added after the $ABTS^{\bullet+}$ has been generated and amount of $ABTS^{\bullet+}$remaining after reaction with antioxidants in the sample is measured after a certain reaction time; expressed as a standard for comparison scavenging activity of the specific sample. Due to the ease and quick analysis, ABTS assay is the most preferred method for assessing radical scavenging potentials of plant extracts (Re et al., 1999b). In this project, free radical scavenging activities of aqueous extracts and their ethanol solubles of the selected plant species were carried out using this method.

With the knowledge on how natural antioxidants alleviate oxidative stress and help to prevent diseases formation, it is pertinent at this stage to discuss some of the underutilized plants and their antioxidant potentials. Their use in bakery products to improve functional properties is also outlined below.

2.6 Improving the antioxidant quality of bakery products with underutilized plants

Recent research demonstrated that, in addition to incorporating protein into bakery products, the inclusion of suitable quantities of plant material into these foods improves their therapeutic benefit (Chavan & Kadam, 1993; Ranawana et al., 2016). In this context, plants with antioxidant potential are of high value as they offer medicinal value to prevent a number of lifestyle disorders related to oxidative stress (Eddouks et al., 2014).

Increasing human population, changes in the environment and the world food shortage in the past few years has raised the concerns about terrestrial food security (Birch et al., 2011;

Chakraborty et al., 2016; Gross, 2014). In order to address these concerns and also to improve nutritional/medicinal quality, Food Science and Technology researchers have explored the use of natural plant resources to manufacture novel and value-added foods (Carocho et al., 2015; Hintz et al., 2015). Such foods will not only offer great nutritional value but also possess disease preventative and therapeutic importance (Bisla et al., 2014).

Bioactive compounds are abundantly present in plants and these form significant constituents of functional foods in the diet (Hasler, 2002; Rivera et al., 2010). Many researchers strongly suggested that plant food based natural antioxidants and phytonutrients are more effective than synthetic food additives (Kumar et al., 2015; Li et al., 2014). Thus, these sources based antioxidants are more effective to prevent oxidative stress and associated disorders (Scalbert et al., 2011). It should be noted that organic plant based bioactive compounds play a special role in this context (Ganesan & Xu, 2018; Mgbeahuruike et al., 2017; Sasidharan et al., 2010).

There are a number of underutilized plants throughout the world (Mavlyanova, 2013) and their use in bakery products is of enormous value. This project has considered four such underutilized plants to develop and manufacture “high protein and antioxidant rich biscuits”. These plants include Moringa, Goji berry, Schisandra berry, and Gotu kola. Traditional uses and biological activities of these plants along with their scientific names are provided in Table 2.2. Importance of these additions and the antioxidant potentials of the four plants studied in this research are outlined below. A brief description of the traditional knowledge and antioxidant potentials of these plants is also given.

Table 2.2. Traditional uses and biological activities of the plants used in this study

Name of plants	Scientific names	Family names	Traditional uses and scientific findings	References
Moringa	*Moringa oleifera*	Moringaceae	Anticancer, anti-fibrotic, anti-inflammatory, antimicrobial and anti-tumor Antihypertensive, anti-inflammatory, anti-obese, anti-hypothyroidism and antidote to poisonous bites of snakes and scorpion	Abdull et al., 2014 Chippada & Vangalapati, 2011
Gotu Kola	*Centella asiatica*	Apiaceae	Antidiabetic, anti-inflammatory, wound healing, anti-aging, a therapeutic agent for Alzheimer's diseases, varicose veins, venous insufficiency and memory enhancing Stimulate cell rejuvenation, nerve tonic, memory enhancing properties, neuroprotective and improve physical work capacity	Hamidpour, 2015 Hashim, 2011
Schisandra Berry	*Schisandra chinesis*	Schisandraceae	Anti-tumour, anti-inflammatory, anti-stress effects, reduce vasodilatory and blood pressure and stimulate physical work capacity	Gopalakrishnan et al., 2016
Goji Berry	*Lycium barbarum*	Solanaceae	Anticancer, anti-cardiovascular, antidiabetic, immunostimulatory and neuroprotective Anti-fungal, treatment of solavetivone and cervical cancer, and alleviate menstrual discomfort	Kulczyński et al., 2016 Gogoasa *et al*., 2014

2.6.1 Moringa *(Moringa oleifera)*

It is well established in the scientific literature that synthetic antioxidants are non-compatible for human consumption as they cause several side-effects (Chen et al., 2012). In this context, plant based natural antioxidants are considered to be the best alternatives for human use. Particularly, the plants that have been used traditionally for treating various ailments are considered to be important for direct consumption as antioxidants as well as to prepare foods enriched with dietary antioxidants and these foods are expected to possess therapeutic value (Schwarzinger & Kranawetter, 2004; Wang et al., 2011).

Moringa oleifera plant is also known as “Miracle Tree” that belongs to a monogenetic family (Bennett et al., 2003). Moringa is a small ornamental tree which has originated in India. The high nutritional value of its leaves is most useful and the plant grows well in all seasons as it is a tropical drought tolerant tree (Gopalakrishnan et al., 2016). Phytochemicals present in this plant display several biological activities such as analgesic, blood pressure control, and anti-inflammatory potentials (Mbikay, 2012). Alam et al., (2014) have demonstrated that the tea prepared with Moringa plant possess the antidiabetic property and is useful to treat hyperglycemia.

Moringa leaves possess high antioxidant capacity due to the presence of large quantities of minerals, vitamins and phenolic constituents such as Quercetin and kaempferol (Coppin et al., 2013). Animal food enriched with Moringa leaves is known to defend them from diseases associated with oxidative stress and contributes to the improvement of meat quality for consumption by humans (Damor et al., 2017; Karthivashan et al., 2015; Oyeyinka & Oyeyinka,

2018). In fact, it was demonstrated that the water extracts of Moringa leaves exhibit significant antioxidant potential (Richter et al., 2003; Siddhuraju & Becker, 2003). Literature revealed that the extracts of Moringa leave exhibit cholesterol-lowering activity and inhibited the formation of atherosclerotic plaque in coronary arteries in rabbits on high cholesterol food, indicating that these leaves contain preventative agents for cardiovascular diseases (Chumark et al., 2008; Stohs & Hartman, 2015). Proximate analysis and nutritional study of plant based foods play a critical role in evaluating their nutritive importance. Standard analytical techniques have been used to estimate various nutrients in the powdered form of dry Moringa leaves and these results are presented in Table 2.3.

Table 2.3: Nutritional analysis of Moringa leaves

Constituents	As per edible portion % / 100g
Moisture	6.12
Ash	11.50
Protein	24.31
Carbohydrates	55.97
Total fat	9.22
Saturated fatty acids	3.77
Unsaturated fatty acids	5.45
Monounsaturated fatty acids	0.87
Dietary fibre	10.28

Source: (Saini et al., 2016)

Moringa is extremely rich in antioxidant constituents and proven to be better than many well-known antioxidant rich fruits and vegetables. For instance, Moringa stands out in comparison to strawberries with high phenolic content, hot pepper with high ascorbate content, carrot with high β-carotene content and soybean with high α-tochopherol content (Yang et al., 2006b). The literature clearly demonstrates that Moringa provides a wide range of dietary antioxidant constituents (Abdull et al., 2014; Alam et al., 2014; Muthukumar et al., 2014; Thurber & Fahey, 2009; Vergara-Jimenez et al., 2017). Moringa possesses remarkable therapeutic/preventative potential including antiulcer, antibiotic, antioxidative and anticancer activities (Siddhuraju & Becker, 2003; Yang et al., 2006a).

Several studies on the antioxidative activity of the leaves and flowers of the *Moringa oleifera* have shown that results are comparable to many well-known nutrient rich and health beneficial vegetables. Pakade et al., (2013a) have determined the polyphenol contents of Moringa leaves that are found to be superior to many vegetables. Moringa has also been reported to contain different types of tochopherols that are α, γ and $^{\delta}$ at fairly high amounts. Both tender and mature leaf extracts are found to display DPPH radical scavenging activity. The activity was more in the mature leaves than the tender ones (Mohamad et al., 2011; Siddhuraju & Becker, 2003; Sreelatha & Padma, 2009; Stohs & Hartman, 2015).

As can be seen from these findings, high nutritional value and remarkable health beneficial activities of Moringa leaves make them extremely important ingredient for mixing with novel bakery products.

2.6.2 Goji Berry (*Lycium barbarum*)

Goji berries (*Lycium barbarum*) also known as wolfberries, belonging to the Solanaceae family have been used as a traditional medicine for centuries (Kulczyński & Michalowska, 2016). Dry Goji berry fruits have several beneficial health effects because of their rich nutrients, such as phenolic compounds and other antioxidant constituents with high biological activity (Benchennouf et al., 2017; Donno et al., 2015; Guo et al., 2008b; Islam et al., 2017; Jiang, 2014; Kulczyński & Michalowska, 2016; Wang et al., 2010; Xin et al., 2017; Zhang et al., 2011b). Dietary fibre and other phytonutrients present in this fruit help to reduce the risk of several diseases including cardiovascular, diabetics, high blood pressure, obesity, and certain gastrointestinal ailments (Wang et al., 2010). Recent scientific literature demonstrates that polysaccharides and other phytochemical constituents from goji berries display several biological activities such as antioxidant, anti-tumor, immunomodulatory effects (Amagase et al., 2009; Anon, 2015; Cheng et al., 2014; Yan et al., 2014). These studies indicate tremendous health benefits of goji berries which are consistent with their traditional use (Amagase & Nance, 2011; Paul-Hsu et al., 2012). It is therefore expected that Goji berries are an extremely important source of dietary antioxidants that may be beneficial to prevent diseases related to oxidative stress (Islam et al., 2017). It is important to note that a few cases of side effects have been reported, however, such negative effects are very rare (Xin et al., 2017). Some side effects have been noticed in the patients on anticoagulants and these people need to exercise caution due to possible drug interaction (Rivera et al., 2012).

The Goji fruit market is significantly expanding because of an increased awareness of the health benefits. Carotenoids present in Goji berries possess significant bioactivities (Donno et al., 2014;

Endes et al., 2015) that are widely used as coloring agents for a wide range of foods (Fratianni et al., 2015). Table 2.4 provides the nutritional composition of dried Goji berries (Niro et al., 2017).

Table 2.4: Nutritional composition of dried Goji berries

Constituents	As per edible portion/100g
Moisture	9.3
Protein	10.2
Fat	4.4
Carbohydrates	61.3
Dietary Fibre	8.8

Source: (Niro et al., 2017)

2.6.3 Schisandra berry *(Schisandra chinensis)*

The fruits of Schisandra (*Schisandra chinensis*) are well-known for their use in traditional Chinese medicine for treating chronic cough, palpitation, memory loss, protecting the skin, repair skin damage and many other ailments (Chen et al., 2012). Recent scientific studies clearly demonstrate the high antioxidant potential of these fruits and the bioactive constituents derived from them (Chen et al., 2012; Mocan et al., 2014a; Wang et al., 2011). It has also been demonstrated that the antioxidant activities of extracts of Schisandra berries correlate well with phenolic and flavonoids contents (Wang et al., 2011). Very recently, Lee et al., (2018) have isolated a novel anticancer agent (deoxyschizandrin) that exhibits significant activity against

ovarian cancer. Owing to their tremendous health beneficial effects as traditional medicine and also due to their antioxidant/therapeutic potential, it may be concluded that Schisandra berries are extremely suitable candidates to prepare antioxidant rich bakery foods (Lobo et al., 2010). In addition, no negative health effects on humans were identified for this fruit and this is understandable for a traditional medicine that was used for thousands of years.

In addition to having significant quantities of polyphenols, Schisandra berries also contain other antioxidants such as essential oils, polysaccharides, vitamin C, vitamin E, and other phytonutrients (Chen et al., 2012; Mocan et al., 2014a; Wang et al., 2011). Highly active flavonoids present in these fruits include isoquercitrin, quercetin, and rutin. These fruits are currently used in foods such as jam, yogurt, cakes, wine, and a few other products as functional additives (Chen et al., 2011; Lu & Chen, 2008).

2.6.4 Gotu Kola (*Centella asiatica*)

Gotu Kola (*Centella asiatica*) is a tropical medicinal plant that has been extensively used in Indian Ayurvedic medicine for centuries (Hamidpour, 2015). According to Ayurveda literature, this plant is used to treat several ailments including skin disorders, enhancing memory and cognition, ulcers, leprosy, diarrhoea and many other conditions (Hamidpour, 2015; Hashim, 2011). Several clinical studies have revealed its therapeutic potential to treat Alzheimer's, high blood sugar, wound healing and also as an anti-inflammatory agent (Chippada & Vangalapati, 2011; Hamidpour, 2015; Hashim, 2011). It is an extremely useful plant to improve learning and memory. Presence of large quantities of antioxidant constituents such as phenolic compounds

and flavonoids make this plant an extremely useful herb to treat diseases associated with oxidative stress (Hamidpour, 2015; Hashim, 2011). In many countries, Gotu kola leaves are used as vegetables and also as a tonic to improve health. These drinks are processed as ready to drink juices and cordials (Hashim, 2011).

Extracts of Gotu kola and also its fermented herbal tea exhibit high antioxidant activities and these activities correlate well with their phenolic content (Ariffin et al., 2011; Hashim, 2011; Zainol et al., 2003). Tremendous health benefits together with extremely low toxicity make this plant an ideal candidate for functional and therapeutic applications (Ariffin et al., 2011; Hashim, 2011; Shakir Jamil et al., 2007; Zainol et al., 2003). Shukla et al., (1999) and Somboonwong et al., (2012) have reported that triterpenoid compounds present in this plant are effective for treating skin problems and wound healing (Somboonwong et al., 2012).

Literature reports that the leaves of this plant contain several important and highly active flavonoids, such as quercetin, kaempferol, patuletin, rutin (Ariffin et al., 2011; Das, 2011). These phytochemicals are responsible for radical scavenging and metal chelating activities (Schmitt-Schillig et al., 2005).

Nutritional composition found in Gotu kola leaves are mainly proteins, carbohydrates, and fibre and these details are presented in the Table 2.5 below.

Table 2.5: Nutritional composition of Gotu kola

Constituents	As per edible portion/100g
Moisture	8.7
Protein	2.4
Fat	0.2
Insoluble Fibre	5.4
Carbohydrates	6.7
Phosphorus	17.0 mg
Iron	14.9 mg
Sodium	107.8 mg

Source: (Chandrika & Prasad-Kumara, 2015)

Tremendous health benefits as evidenced by traditional, as well as scientific knowledge on the four plants, leads to the conclusion that they are improved candidates to be used to manufacture antioxidant rich biscuits. This research, therefore, aimed to manufacture biscuits using these four plants' parts and their nutritional and antioxidant potential has been evaluated.

CHAPTER 3

MATERIALS

&

METHODS

Chapter 3

Materials and Methods

3.1 Procurement of raw materials

Moringa leaves, Gotu kola leaves, Schizandra berry fruit, and Goji berry fruit samples were procured from Sunrise botanicals Austral Herbs and Spices Australian Company PTY LTD. The bulk sample of flour was procured from Manildra flour mills PTY LTD, Australia and other food ingredients, such as Queen pure maple syrup, CSR powdered sugar, Gold'n Canola oil (Omega3), and McKenzie's baking powder was procured from Australian local supermarket. All the plants' sample and food materials samples were stored in the food research laboratory. The plants' samples were powered and subjected to hot water extraction procedure.

Table 3.1: Description of Pants' Sample

Sample Name	Scientific Name	Sample Description
Moringa	*Moringa oleifera*	Dry leaves
Gotu Kola	*Centella asiatica*	Dry leaves
Goji Berry	*Lycium barbarum*	Dry berries
Schisandra Berry	*Schisandra chinensis*	Dry berries

3.2 Chemicals and reagents

Quercetin, potassium chloride, gallic acid, disodium orthophosphate, sodium chloride, potassium phosphate monobasic, potassium persulphate, sodium nitrite, sodium phosphate

dibasic, aluminium chloride, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Folin–Ciocalteu reagent (FCR), Sodium carbonate, ethanol, methanol, ascorbic acid (Vitamin C). Sodium hydroxide, petroleum ether, Ethylenediaminetetraacetic Acid (EDTA) was purchased from Sigma, Australia and Lomb Scientific Pty Ltd, Australia.

3.3 Preparation of plant powder (raw materials)

The Schisandra berries and goji berries were dried in an air oven (Rational CPC, Germany) at 110 °C for 24 h. After that, dried forms of all four plants material were ground in the mechanical grinder (Breville – The Boss^{TM} – BBL915BAL) to form a powder. The powdered plant samples were stored in airtight bags at 4 °C in a refrigerator until further analysis.

3.4 Development of biscuits

The biscuit was also made from the flour without plant samples to serve as the control. Biscuits with different proportion of the selected four plant powders were mixed with the same flour. In addition to antioxidant rich plant material and high protein flour, the other standard ingredients (sugar, pure maple syrup, canola oil, baking powder, and water) were used for the preparation of these biscuits. These ingredients are expected to improve nutritional as well as the functional value of the biscuits that are desirable in the modern food industry. The antioxidant rich and high protein biscuit samples were prepared in the M10 building of Western Sydney University at Hawkesbury campus; kitchen laboratory using standardized recipes (Chauhan et al., 2016a, Chauhan, 2014) with fortification of under-utilized plants powder is given as follows:

Table 3.2: Ingredients used in different treatments

Ingredients (g)	Control (g)	Sample 1 (g)	Sample 2 (g)	Sample 3 (g)	Sample 4 (g)	Sample 5 (g)	Sample 6 (g)
Protein enriched Flour	1000	990	980	970	960	995	995
Moringa leaves	-	2.5	5	7.5	10	2.5	-
Gotu Kola leaves	-	2.5	5	7.5	10	2.5	-
Goji Berry	-	2.5	5	7.5	10	-	2.5
Schisandra Berry	-	2.5	5	7.5	10	-	2.5
Powder Sugar	125	125	125	125	125	125	125
Maple Syrup	125	125	125	125	125	125	125
Canola oil	250	250	250	250	250	250	250
Baking Powder	8	8	8	8	8	8	8
Water	400	400	400	400	400	400	400

3.4.1 Biscuit Manufacturing Process

Sieve the flour, plants powder, and baking powder and were mixed together to homogenize the blend. Canola oil, maple syrup, and sugar were mixed together in a Hobart commercial dough mixer (Hobart A200-20 QT Mixer) for 30 s at low speed (no. 1) to obtain a shortening cream, the bowl was scraped down and the mixing was continued at higher speed (no. 2) for 3 minutes.

Then the mixture of dry ingredients such as flour, plants' powder, and baking powder was added to shortening cream with the addition of water and mixing for 3 minutes at no. 1 speed for proper mixing of all the ingredients (Hobart A200-20 QT Mixer). After that, scraped down the bowl once more and continued mixing at the same speed for 2 minutes. The kneading procedure developed by (Chauhan, 2014) was employed to prepare the dough with desirable consistency. The dough was then kept at room temperature for 15-20 minutes. Then the sheets were made by making balls with the dough and rolling them and were then cut by specific biscuit cutter (D-Line- Plain Biscuit Cutter S/S). The biscuits were placed on a baking tray coated with baking paper. Each lot was baked at 175 °C for 15-17 minutes in an electric oven (Rational ClimaPlus Combi$^{@}$ CPC). The biscuits were cooled to room temperature and packed in heat-sealed high-density polyethylene (HDPE) bags and stored in the cold room for subsequent analysis.

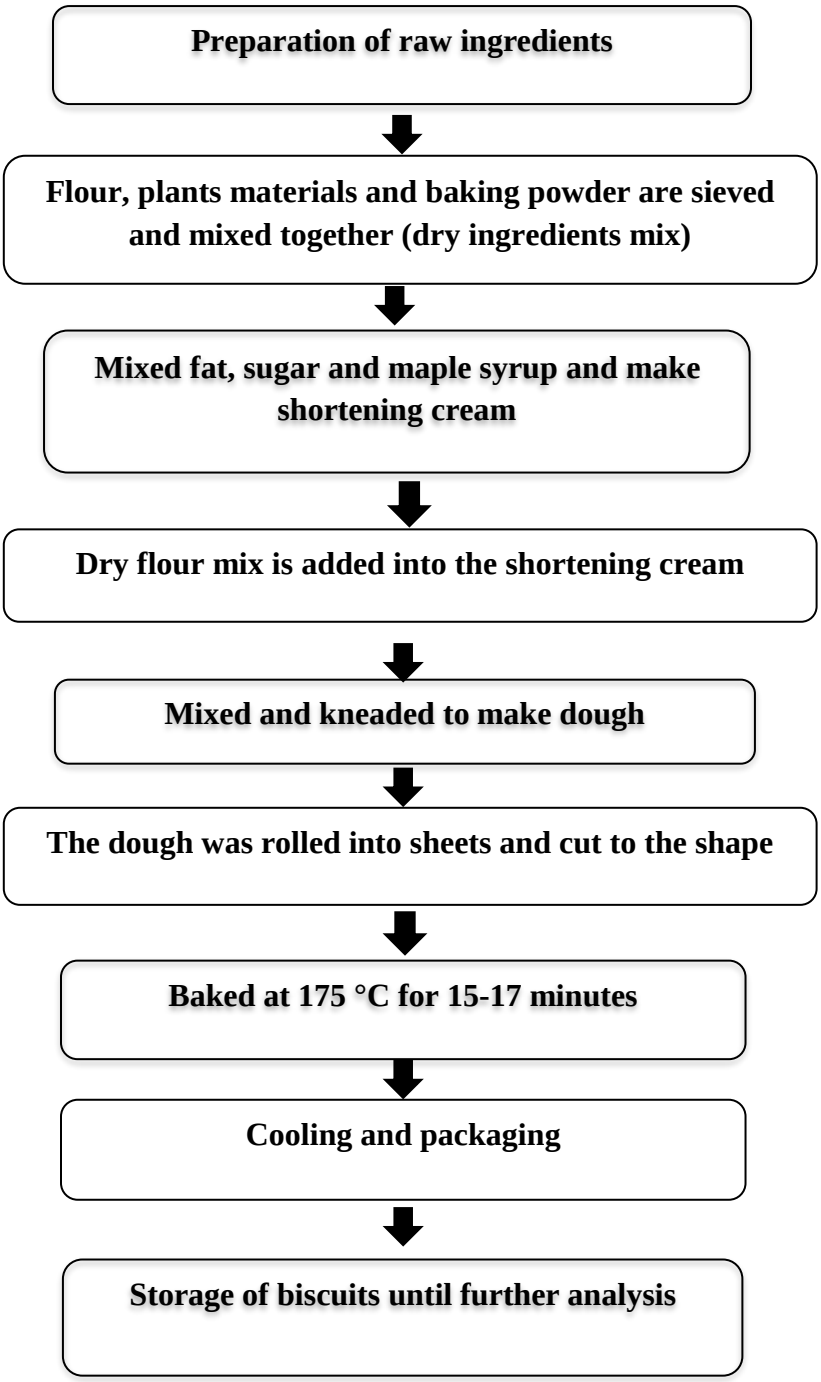


Fig 3.1: Flowchart of preparation of Biscuits

3.5 Proximate and physical analysis of antioxidant rich and high protein biscuits

Analyses were performed in the food science research laboratory at the School of Science and Health, Hawkesbury campus, Western Sydney University. The analyses were carried out to evaluate the biscuit samples in terms of nutritional and physical qualities.

3.5.1 Proximate composition

The biscuits samples were analyzed for moisture, ash, protein, fat, fibre, carbohydrates, and energy. The proximate analyses were determined by using the Official Methods of Analysis of AOAC (AOAC, 1995). Samples were ground to make a powder form before analysis. All samples were analyzed in triplicates.

3.5.1.1 Moisture content

The moisture content was determined by using AOAC Official method, 1995. Five grams (5 g) of biscuit samples were taken into the tared aluminium moisture pans. The moisture content of the biscuit samples was measured as the loss after drying in an air oven (Clayson OM400T oven) at 100 °C for overnight as per standard AOAC method and the lids were placed under the dishes to evaporate moisture until a constant weight was attained. Dried samples of biscuits were removed from the oven and were placed in a desiccator to cool to room temperature and weighed. All the experiments were carried out in triplicates and results were expressed in percentages. The moisture content of the biscuit samples was calculated using the equation below:

$$\%\text{Moisture} = \frac{(\text{Pan \& dry sample} - \text{Pan Wt.})}{(\text{Pan \& wet sample} - \text{Pan wt.})} \times 100$$

3.5.1.2 Total Ash

The total ash content was measured by using the standard AOAC, 1990 method. Three grams (3 g) of biscuit samples were taken in previously weighed porcelain crucibles in triplicates. The samples were charred (to prevent the bubbling over in the muffle furnace) on the hotplate (VULTEK Series Fume Cupboard) heated to 400 °C. The fibreboard try was used to transfer the samples into the muffle furnace (Ceramic Engineering Furnace 721682, Sydney Australia) at 550 °C for overnight until a grey-white ash remains. The samples were cooled in a desiccator to room temperature and the weights of the residues left in the crucibles were weighed on an analytical balance to calculate the ash content.

$$Ash(\%) = \frac{Crucible\ \&\ ashed\ sample - crucible\ wesight}{(crucible\ \&\ wet\ sample - Crucible\ weight\ )} \times 100$$

3.5.1.3 Crude Protein

Protein content was determined using DUMAS Nitrogen Analyser technique (VELP Scientifica, 2013) as per standard AOAC, 1990. Dumas method is an automated, rapid, and safe alternative technique to the Kjeldhal method of analysis for nitrogen (Protein) content of food samples. The biscuit samples were weighed approximately 200 mg in the tin foil on to a plate of the analytical balance using tweezers. The tin foils were closed with samples manually, taking care to do uniform little balls and transferred carefully into a sample holder. After recording position in the holder, the samples were transferred to the autosampler disc of DUMAS apparatus. EDTA was used as a standard, empty tin foils were used as blank 'Check up' to confirm calibration before performing the biscuit samples. The conversion factor of 6.25 was used to convert nitrogen to protein content (AOAC, 1990). The protein present in the biscuit samples was determined using the following details were filled in the database on the computer (DUMASoftTM software) for each sample:

Table 3.3: DUMAS analysis procedure for the determination of protein percentage

Sample name	Name of the biscuit samples treatment, standard, and tin foil
Weight	Weight of all the samples in mg
Method	CEREAL MEAL 1, EDTA, Tin foil
Calibration	15
Test type	Check-up, standard, and sample

3.5.1.4 Crude Fat

Fat content was determined using ANKOM XT15 technology method as per standard AACC Official method, 2001(*Am 5-04*). Approximate 2g weighed of samples were taken into the tared filter bags. The filter bags were sealed within 4mm of their open ends on Heat Sealer (no. 6). The samples were dried in an air oven (Clayson OM400T oven) at 102 °C ±2 °C for 3 hours to remove the moisture before extraction. Dried samples were placed in a desiccator for 15 minutes to cool down and then reweighed. The crude fat present in samples was measured using the ANKOM XT15 extraction system procedure (AOCS *Am 5-04*) and calculated using formula given below.

$$Crude\ Fat\ (\%) = \frac{[WB(2) - WB(3)]}{WB(1)} \times 100$$

Where,

WB (1) = Sample weight (g)

WB (2) = Pre-dried sample weight with filter bag (g)

WB (3) = Dried sample weight with filter bag after extraction (g)

3.5.1.5 Crude Fibre

Due to time and resources limitations, the fibre content of the biscuit samples was calculated using the specification provided by suppliers. The crude fibre content of the biscuit samples was calculated according to the amount of flour and plants powder used in the preparation of biscuit samples since the storage conditions have no significant effect on crude fibre content.

3.5.1.6 Carbohydrates content

For calculating the total carbohydrate content, the following equation was used which is described in AOAC, 2000.

$$\% \text{ Carbohydrates} = 100 - (CP + EF + CF + \text{Total Ash}) \%$$

Where,

CP = Protein (g)

EF = Fat (g)

CF = Fibre (g)

3.5.1.7 Energy

Following equation (fractional method) was used to calculate the energy content of the biscuits:

$$\text{Energy (Kcal)} = [(4 \times \text{Protein}) + (9 \times \text{Fat}) + (4 \times \text{Carbohydrates})]$$

3.5.2 Physical analysis of biscuits

3.5.2.1 Diameter and thickness determination

The weight of the baked biscuits was measured by weighing on an analytical weighing balance (Mettler Toledo Xs 10002s Xs-s Precision Balance). The diameter and thickness of the biscuit samples were determined by using Vernier Calliper (Mitutoya Vernier Caliper, Metric). The diameter of the biscuits was measured by placing six biscuits edge-to-edge horizontally and rotating at a ninety-degree angle for triplicate readings and the average was calculated (Chauhan et al., 2016b). The biscuit samples thickness was determined by placing six well-formed biscuits on the top of each other, followed by triplicate readings recorded by shuffling biscuit samples (Gomez et al., 1997). The diameter and thickness measurement was done in triplicates.

3.5.2.2 Spread ratio (SF) determination

The spread ratio of the biscuits was determined by using the following formula: (Zoulias et al., 2000)

$SF = (D/T \times CF) \times 10$

D= Diameter of biscuits

T= Thickness of biscuits

CF = 1.0 (standard correction factor at atmospheric pressure) (AACC, 2000).

3.5.2.3 Bake loss

The bake loss of the biscuit samples was determined by weighing six biscuits before baking and after baking. The weight difference was regarded as percent bake loss (Gupta and Singh, 2005)

3.5.2.4 Color analysis

Colour analyses of biscuit samples were measured with Minolta Chroma Meter (Konica Minolta CR-410 Chroma Meter) on the basis of the value of *L**, *a**, *b** color parameters. *L** values measure black to white (range between 0-100), *a** values measure redness of the product and *b** values measure yellowness of the sample. The colorimeter was calibrated with the white standard. The color was analyzed using the following equation (ΔE).

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2}$$

Where,

L_0 = Standard reading of L^* value

a_0 = Standard reading of a^* value

b_0 = Standard reading of b^* value

L = Instantaneous reading of L^* value after applying the experiment

a = Instantaneous reading of a^* value after applying the experiment

b = Instantaneous reading of b^* value after applying the experiment

3.6 Antioxidant activities and their correlation with antioxidant contents of high protein biscuits

3.6.1 Preparation of hot water extracts for chemical analysis

Hot water extraction of samples was done followed by the method of (Benchikh & Louailèche, 2014). Five grams (5 g) of the powered plants' materials and biscuit samples (50 g) were added to 40 mL and 400 mL of ultra-pure water respectively. The samples were subjected to hot water extraction using autoclave method for 1 hour at 121 °C and then cooled to room temperature. The extracts were then subjected to centrifugation at 10,000rpm for 30 min. The supernatant was separated by filtration and then made up to 50 mL by adding ultrapure water in a standard volumetric flask. The solutions were transferred into pre-weighed 100mL beakers and then freeze-dried (CHIRST ALPHA 1-4 LDplus). After freeze-drying the samples, the solid extracts were weighed on an analytical balance (to get the dry mass of extracts. Then the dry extracts were stored at -20 °C until further analysis. The entire process of hot water extraction is illustrated in Figure 3.2.

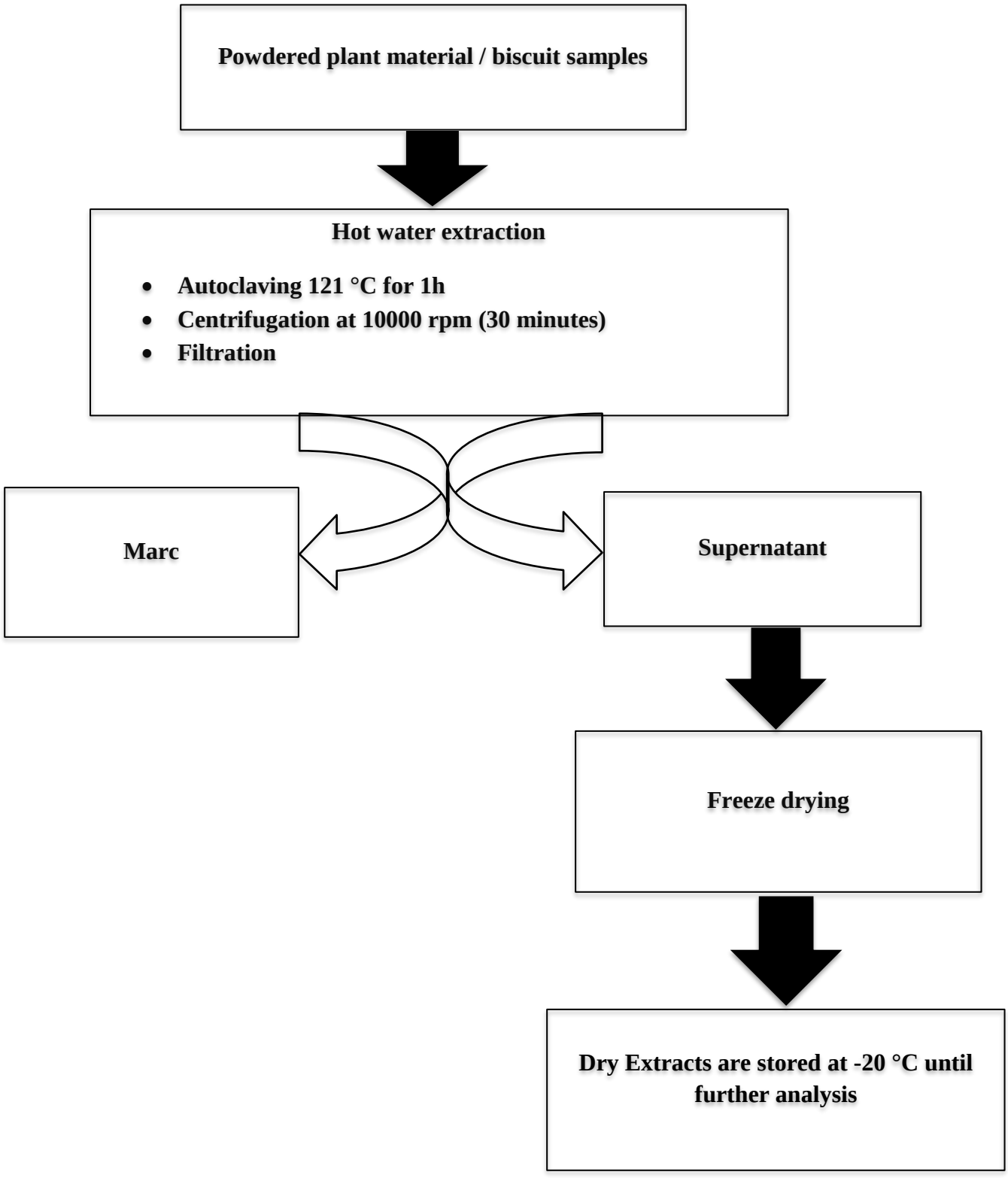


Fig 3.2: Flowchart of Hot water extraction of plants and biscuit samples

3.6.2 Isolation of ethanol soluble small molecules from hot water extracts

Isolation of Ethanol soluble small molecules from hot water extracts was done by using the method (Benchikh et al., 2018). 1.5 grams of hot water extracts of each biscuit samples were subjected to ethanol precipitation in 1:4 v/v ratio (Deionized Water: Ethanol = 20 mL water and 80 mL ethanol) and left them overnight at 4.0 °C to isolate ethanol solubles of extracts. The extracts were centrifuged for 30 minutes at 10000 rpm in a centrifuge at room temperature. The supernatant containing ethanol solubles were separated by filtration. Ethanol was evaporated by using Rotavapor (BUCHI Rotavapor R-II Rotary Evaporator, Switzerland). The samples were then freeze-dried to remove water (CHIRST ALPHA 1-4 LDplus) and keep in a refrigerator at -20 °C for further analysis. The entire procedure to isolate ethanol soluble small molecules is presented in Fig. 3.3.

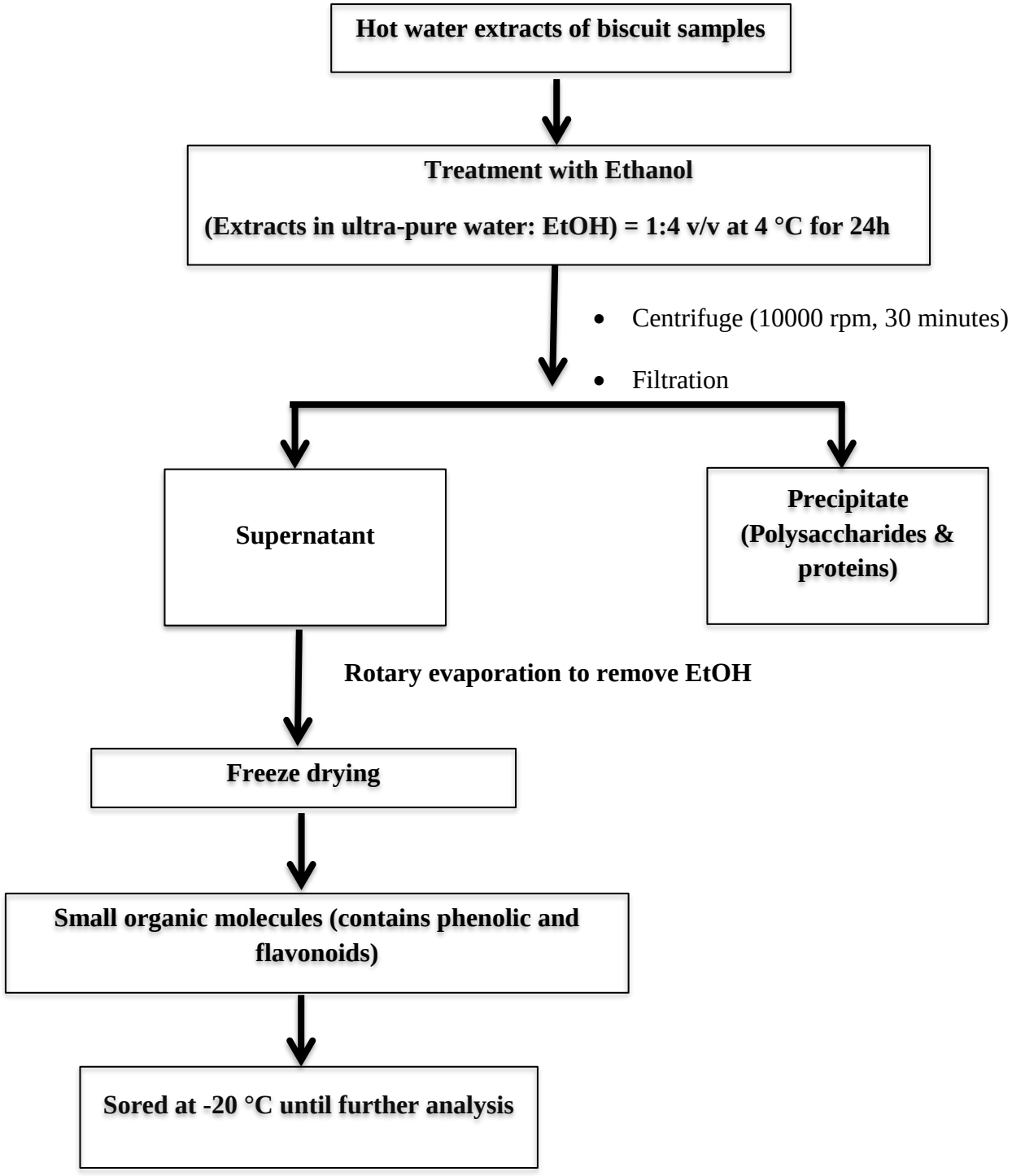


Fig 3.3: Flowchart for the isolation of ethanol solubles from hot water extracts of biscuit samples

3.6.3 Antioxidant activity determination

The antioxidant activity potential of plants powder and biscuit samples was assessed by measuring the abilities of the samples to scavenge $ABTS^{\bullet+}$ radical.

3.6.1 Measurement of radical scavenging activities against $ABTS^{\bullet+}$

Antioxidant capacities of the samples were measured using $ABTS^{\bullet+}$ assay (Ozgen et al., 2006; Ma et al., 2018). In this method, $ABTS^{\bullet+}$ radicals were produced by the addition of 33.11 mg of $K_2S_2O_8$ to 192.04mg of ABTS in 50 ml distilled water and the solution was left in dark for 15 hours (Alam et al., 2013) at room temperature. The absorbance of the generated $ABTS^{\bullet+}$ radicals was adjusted to 0.70± 0.02 to 0.75± 0.02 by addition with PBS buffer. Suitable concentrations of plant extracts, biscuit extracts, and ethanol solubles were prepared in PBS buffer and serially diluted for concentration-dependent study. Vitamin C was used to build the standard curve by taking various concentrations (1000 µM, 400 µM, 200 µM, 100 µM, 80 µM, 60 µM, 40 µM, 20 µM). Briefly, the assay procedure involves, 100µl of the sample was taken in a test tube. 1 mL of $ABTS^{\bullet+}$ radical solution was added. The samples were then incubated for 0.5 h by covering with an aluminium foil. Absorbance values were then measured at 734 nm (Multiskan 141 EX, Thermo Electron, USA). Triplicate measurements were made.

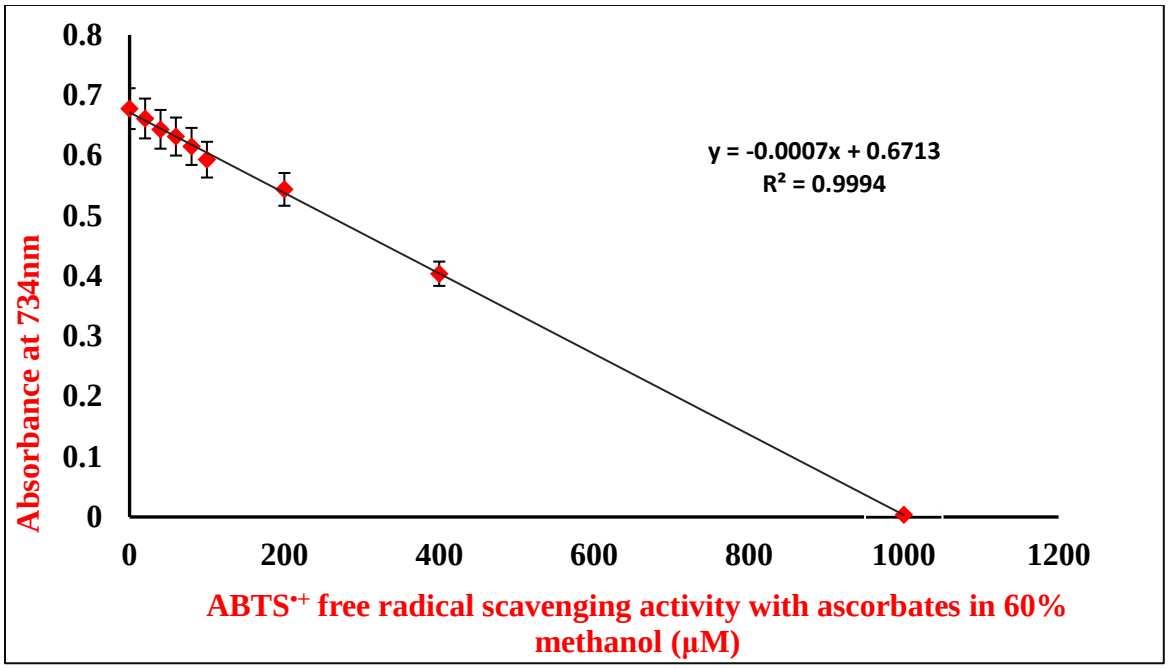


Fig 3.4: Standard curve of ascorbic acid in 60% of methanol.in terms of hot water extracts

The standard curve gave an R^2 value of 0.99 showing high significance. Therefore, the straight line equation y = -0.0007 + 0.6713 was used to measure the radical scavenging activity of the plants and biscuit samples in ascorbate equivalence units.

3.6.4 Measurement of total phenolic content (TPC)

In order to measure phenolic contents of the samples the method of Folin-Ciocalteu was used with minor modifications (Luís et al., 2009). Gallic acid was the standard used in this method. Different concentration of Gallic acid (0, 25, 50, 100, 200, 250, and 300 µg/mL) were made and 50 µL were mixed with Folin-Ciocalteu reagent (50 µL) and then vortexed the contents for 10s. After two minutes sodium carbonate 5% (500 µL w/v) was added. The solution was made up to 1 mL with 400 µL of ultra-pure water. After heating the solution in a water bath at about 45 °C for

half an hour. Then quenched in an ice bath. Absorbance values were determined at 760 nm (Cai *et al*., 2004) in a UV spectrophotometer (GENESYS 10S UV-VIS Spectrophotometer). A Gallic acid standard curve was built (shown in Fig 3.5) using different concentrations (0-300 µg/mL). The regression of standard curve gave a linear equation (y = 0.002x-0.0108 with R^2 = 0.9987). Each sample was analyzed in triplicates.

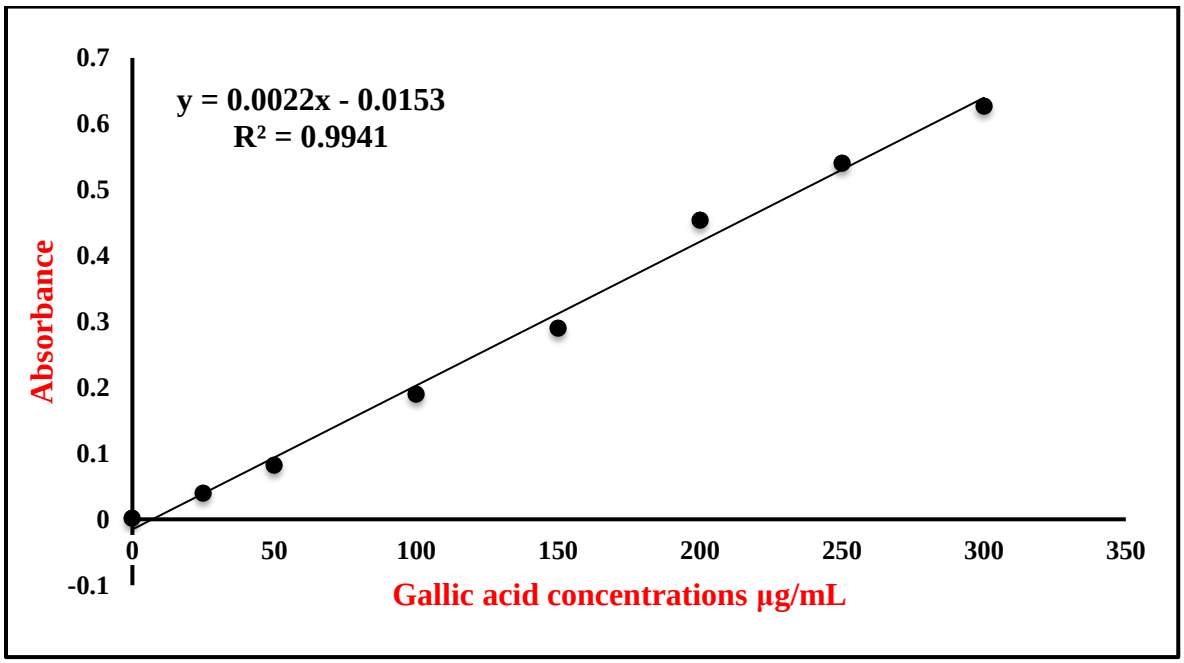


Fig 3.5: Standard calibration curve of Gallic acid.

The straight line equation for the standard curve gave an R^2 value of 0.99 showing high significance. The straight line equation (y = 0.0022x – 0.0153) was used to calculate the phenolic contents of plants and biscuit extracts. The total phenolic contents were estimated as gallic acid equivalent (GAE). The results were expressed as milligrams (mg) of GAE per 1g of the dry weight of extracts (mg GAE/gdw). Each sample was analyzed in triplicates.

3.6.5 Measurement of flavonoid content (TFC)

Total flavonoid content was measured using the aluminium colorimetric method (Khodaie et al., 2012; Zhishen et al., 1999). Quercetin was used as a standard. Different concentrations of Quercetin (0, 200, 400, 600, 800, 1000, 1200 µg/mL) were made. The samples extract 0.5 ml was diluted with methanol (80%) were added to 300 µL of $NaNO_2$ and vortexed contents for 10s and then left at 25 °C for 2 minutes. Then add 300 µL $AlCl_3$ (1:10 w/v) left the contents at 25 °C for 6 minutes. Add 2 mL of 1M NaOH to the reaction mixture and immediately added to 1.9 mL of distilled water. Absorbance values were determined at 510 nm (GENESYS 10S UV-VIS Spectrophotometer). A standard calibration curve was built for various concentrations of quercetin (0-1200 µg/mL) (Honmore et al., 2016). The regression of the standard curve gave a linear equation ($y = 0.0008x-0.0115$, $R^2 = 0.9988$) shown in Figure 3.6. Each sample was examined in triplicates.

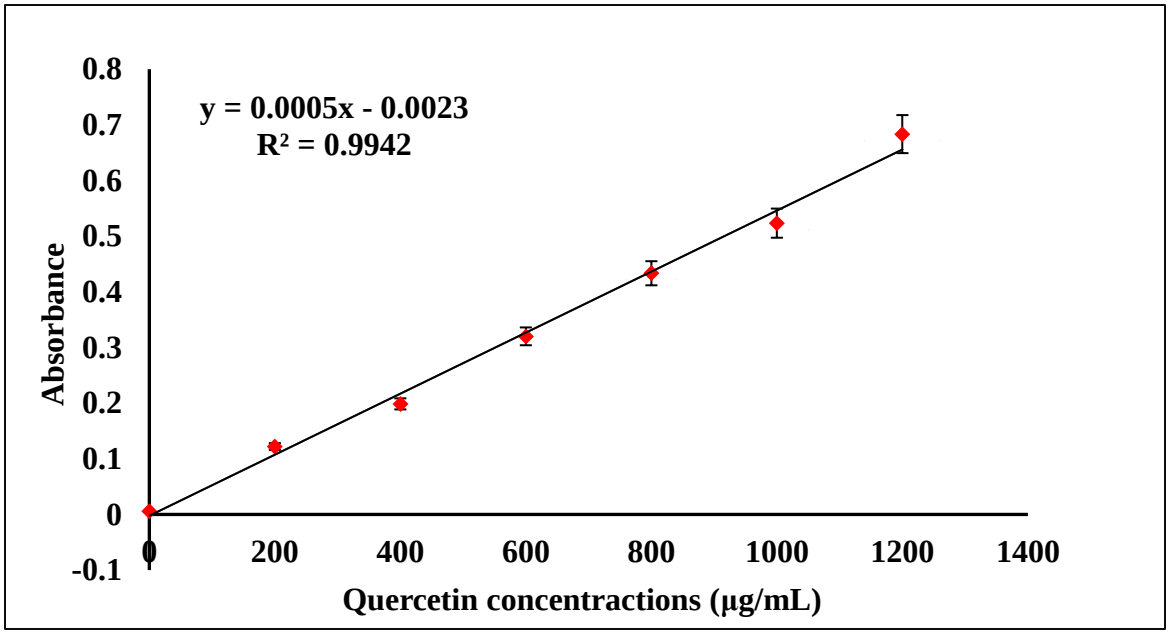


Fig 3.6: Standard curve of Quercetin.

As the correlation coefficient value (R^2 = 0.9942) was highly significant, the straight line equation (y = 0.0005x – 0.0023) was used to determine flavonoid contents of plants and biscuit extracts. The total flavonoid contents of the plant and biscuit extracts were determined as the quercetin equivalent (QE). The results were expressed as milligrams (mg) QE per 1g of dry plant and biscuit extracts weight (mg QE/gdw). Each sample was examined in triplicates.

3.7 Statistical Analysis

All statistical analysis was performed using the SPSS version 22.0. The data collected from physicochemical and nutritional composition tested statistically by using a One-way Analysis of Variance ANOVA to determine the significant difference ($p \leq 0.05$). The mean difference among groups was compared using Turkey's HSD test at 95% confidence level. The results were expressed as mean± standard deviation (SD).

CHAPTER 4

RESULTS

&

DISCUSSION

Chapter 4

Results and Discussion

4.1 Proximate and physical analysis of antioxidant rich and high protein biscuits

4.1.1 Proximate analysis

The results of the proximate analysis of biscuit samples are displayed in Table 4.1. Results showed that there was a significant variance ($p \leq 0.05$) in the moisture, ash, protein, fibre, carbohydrates, and energy samples.

4.1.1.1 Moisture content

The moisture content of the biscuit samples was ranged from 4.56 to 4.89% wwb. Moisture plays a crucial role in terms of quality parameters, acceptability, and shelf-life of fat and sugar based baked products (Adebayo-Oyetoro et al., 2015; Ergun et al., 2010). The incorporation of under-utilized plants powder into biscuits preparation had also shown the effect of increasing the moisture content. The difference in moisture content between samples might be due to the difference in moisture holding capacity of different ingredients (Abu-Salem & Abou-Arab, 2011; Rathnayake & Navaratne, 2017). The Table 4.1 depicts that there was a significant difference in moisture content ($p < 0.02$) between control and treatments made with 1%, 2%, 3%, 4% of plants parts based powder. Figure 4.1 shows that control sample contains 4.79% of moisture on wet wet basis (wwb) while all the samples incorporated with under-utilized plants' material, except sample-4, contains lower moisture contents (ranges between 4.5 to 4.6%) on wet wet basis

compared to the control. (Mamat et al., 2010) reported that the biscuits are very low in moisture contents. The majority of the moisture lies in the thin lamella of material near the centre, whereas the surface and outer periphery of the product of the product are nearly dry. The typical initial moisture content of biscuit dough ranges from 11-30%, comprising both added water at the dough mixing process and water naturally occurring in the ingredients. The baking process reduces the final moisture content to 1-5% in the final products. Alam et al., (2014), reported that plant based herbal biscuits have lower moisture content than normal biscuits. The moisture content of the wheat flour is or around 14%. The high moisture content could result in short shelf-life of the product where microbial spoilage can produce off odours and flavors.

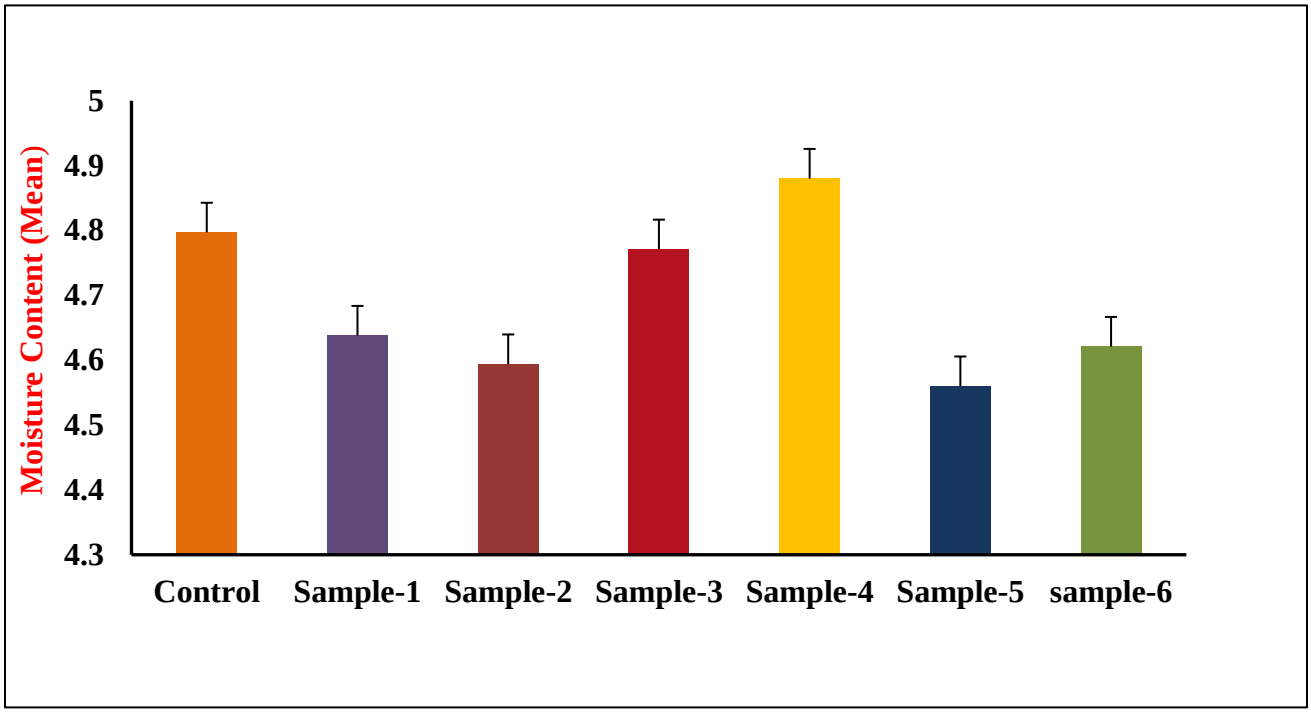


Fig 4.1: Moisture content of biscuit samples

Table 4.1: Proximate compositions and energy values of biscuit samples

Treatments per 100g	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Fibre (%)	Carbohydrates (%)	Energy (Kcal/ 100g)
Control	4.79± 0.05	2.12± 0.03	13.41± 0.02	15.18± 0.10	2.5± 0.01	66.7± 0.12	457.3± 0.60
Sample-1	4.63± 0.05	2.30± 0.05	14.83± 0.05	15.58± 0.45	3.1± 0.03	64.1± 0.43	456.0± 2.1
Sample-2	4.59± 0.11	2.45± 0.03	14.86± 0.12	15.37± 0.06	3.7± 0.004	63.5± 0.08	452.1± 0.40
Sample-3	4.77± 0.06	2.57± 0.01	14.97± 0.14	15.39± 0.23	4.2± 0.02	62.9± 0.40	449.3± 1.33
Sample-4	4.89± 0.11	2.71± 0.03	15.0± 0.08	15.13± 0.09	4.9± 0.01	62.1± 0.22	445.0± 0.30
Sample-5	4.56± 0.07	2.25± 0.03	14.7± 0.07	15.25± 0.50	2.7± 0.004	64.9± 0.54	456.1± 2.65
Sample-6	4.62± 0.08	2.27± 0.02	14.8± 0.08	15.32± 0.39	2.6± 0.003	64.8± 0.38	456.7± 1.89

Note: Data represented are mean± SD (n = 3). Each parameter values for different treatments presented on the same column in the table display are statistically significantly different ($p \leq 0.05$). The incorporation of plants materials is at different proportions are; control, sample-1 (1%), sample-2 (2%), sample-3 (3%), sample-4 (4%), sample-5 (1%), sample-6 (1%).

4.1.1.2 Ash content

The ash content of biscuit samples increased with the increasing under-utilized plants material substitution levels (Fig 4.2). There were significant differences ($p \leq 0.05$) in ash contents between the control and under-utilized plants parts' based biscuit samples at each substitution levels up to 4%. Table 4.1 depicts that ash content of biscuits ranged from 2.1 to 2.7%. The ash content represents the mineral content of the food samples (Agrahar-Murugkar et al., 2015; Mishra et al., 2015). Minerals are essential nutrients in diet which helps in important metabolic functions of the body and are parts of a molecule which serve as a normal metabolism of the other compound, include haemoglobin, adenosine triphosphate (ATP), DNA (deoxyribonucleic acid) (Essuman et al., 2016; Ibidapo et al., 2017). The ash content is the proportion of inorganic residue after removed water and organic materials, such as moisture, protein, fat, and carbohydrates by incineration in the presence of oxidizing agents (Adeyeye, 2016). Ikuomola et al., (2017) reported that milling and soaking could result in lower minerals contents. The results suggest that biscuits prepared with under-utilized plants will provide more minerals to the consumers than the standard biscuits. Alam et al., (2014) observed similar findings that herbal biscuits produced from plant materials had higher ash contents (2.1 to 2.4%).

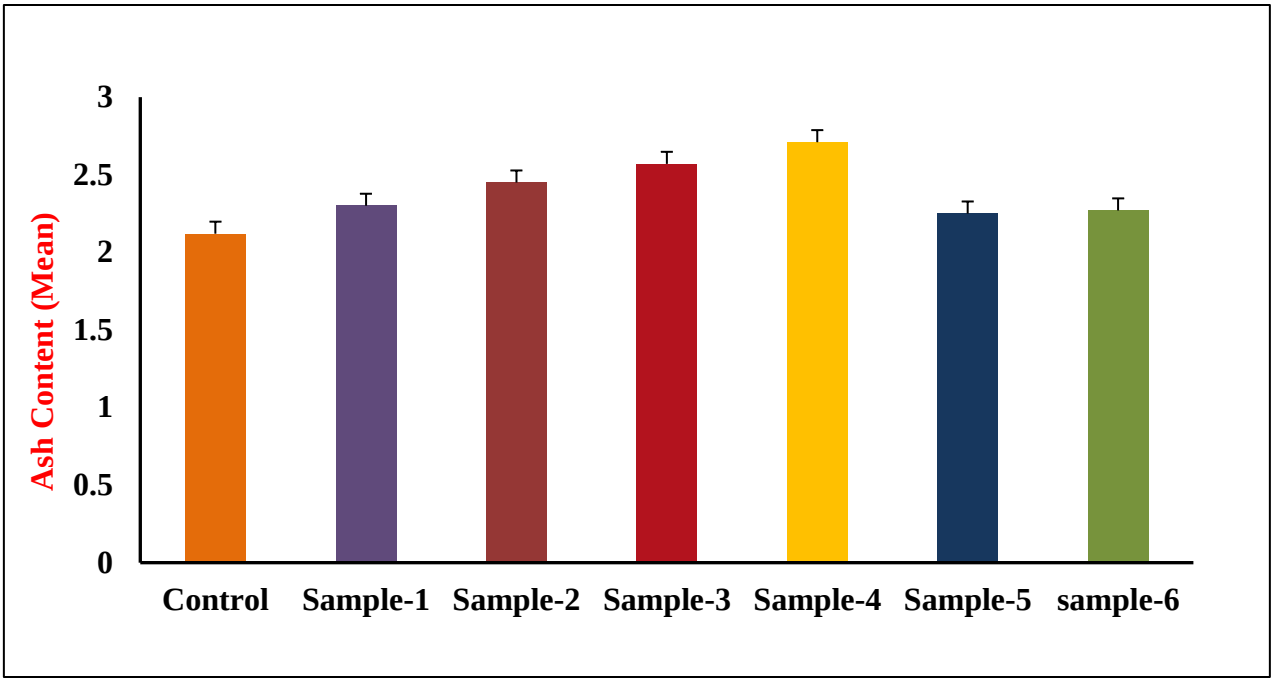


Fig 4.2: Ash content of biscuit samples

4.1.1.3 Protein content

The protein contents of the biscuit samples were ranged from 13.41 to 15%. The sample-4 with 4% plants based materials had the highest protein content (15.0%) while the control sample had the lowest protein content (13.41%). Addition of plants materials shows a significant ($p \leq 0.05$) increase in the protein content among all the biscuit samples. The protein content of biscuits slightly increased in every level of plant materials substitution (14.83, 14.86, 14.97, 15.0, 14.7, 14.8 with $p < 0.001$) shown in Table 4.1. This increase was expected because the under-utilized plants' parts contain a significant quantity of protein. Karki et al., (2016) found a similar trend in the protein content of biscuits and cake made with supplementation of sorghum wheat flour. Alam et al., (2014) reported an increase in the protein content with a corresponding increase in the proportion of plant material supplementation in biscuits produced from refined wheat flour.

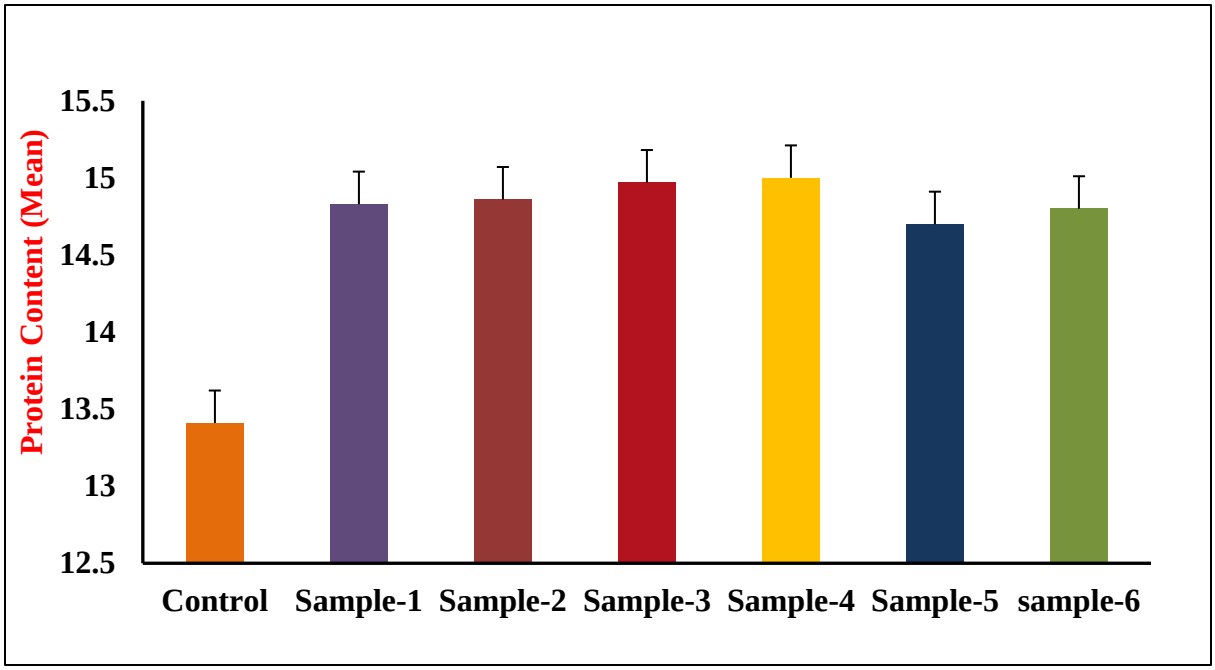


Fig 4.3: Protein content of biscuit samples

4.1.1.4 Crude Fat Content

The fat content of the biscuit samples ranged from 15.18 to 15.37 %. Biscuit sample-4 had the highest fat content (15.58%) while the reference control sample had the least value (15.13%). The results of the fat content showed that there was no significant difference ($p > 0.05$) between biscuit samples. Fat is an important factor the more fat content indicates the high calorific value and also serves as a lubricating agent that helps in improving the texture, rheology, and overall quality of the product. Omeire & Ohambele (2010) reported that fat is a rich source of energy and is essential as carriers of fat-soluble vitamins, such as vitamin A, D, E, and K. Despite fat content in food products should not be >25% because this could lead to oxidative rancidity as well as development of unpleasant and odorous compounds in product (Ikuomola et al., 2017;

Okpala et al., 2013). However, the presence of low-fat content (unsaturated fatty acid) is beneficial to ensure the longer shelf-life for food products (Okpala & Okoli, 2014).

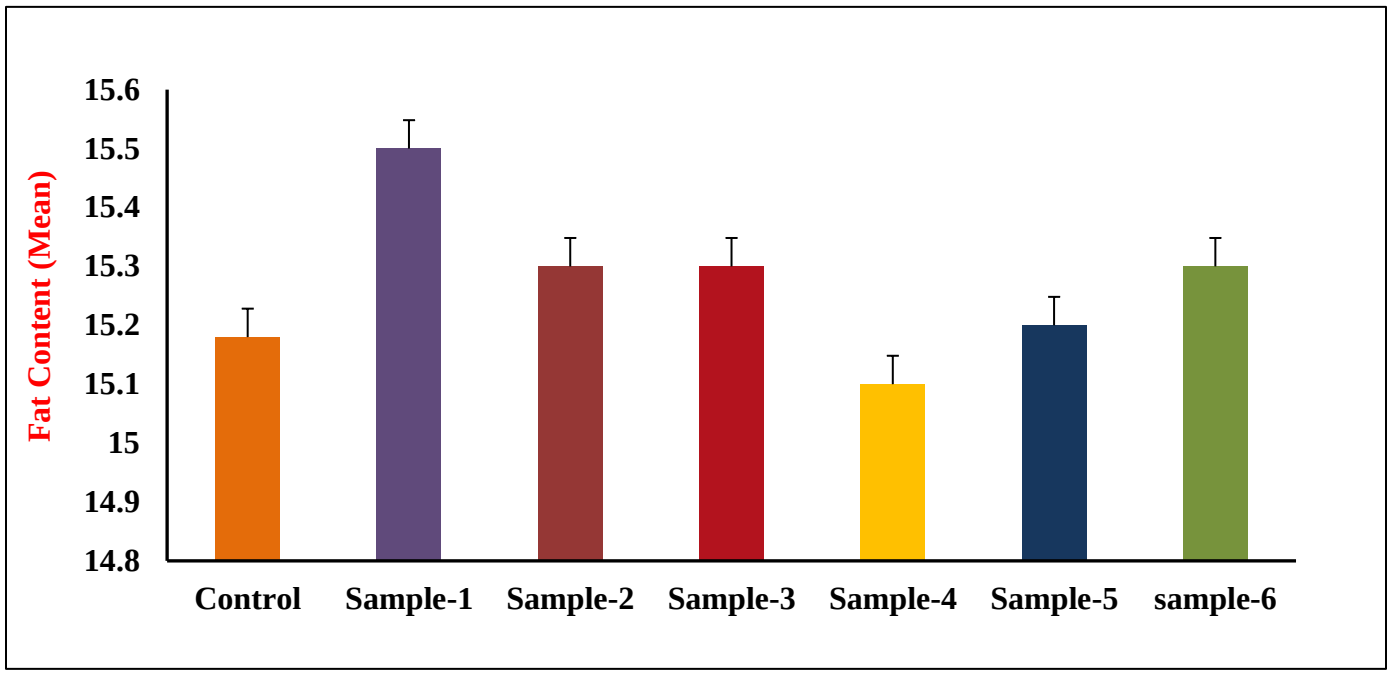


Fig 4.4: Fat content of biscuit samples

4.1.1.5 Crude Fibre

The total dietary fibre content of biscuit samples ranged from 2.50 to 4.95%. The results showed the significant difference ($p \leq 0.05$) between the control and plant based biscuit samples made with the incorporation of 1%, 2%, 3%, and 4% under-utilized plants. The highest proportion of plants materials substitution samples contain the highest dietary fibre (4.95, 4.23, 3.7, 3.1, 2.7, and 2.6 respectively) shown in Table 4.1. The increasing dietary fibre content trend indicates that the use of plants materials had significant effects by increasing the dietary fibre of different proportion of all the biscuit samples. The findings agree with the observations of (Alam et al.,

2014) for the increasing trend in the crude fibre content (2.3 to 4.1%) of biscuits made from herbal plants. However, there was no significant difference between plants based biscuit sample-5 and sample-6 in terms of dietary fibre content which may be due to least amount substitution levels of plants parts. There was an effort by food industries to increase the total dietary fibre content in biscuits and other bakery products by using various wholemeal grains which are rich in dietary fibre (Ktenioudaki & Gallagher, 2012). Ellouze-Ghorbel et al., (2010) and Li & Komarek (2017) reported that dietary fibre has a significant role in the human body. Dietary fibre also has beneficial physiological effects on the body, such as lowering blood glucose level, blood cholesterol level, promotes the prevention of obesity, and reducing the risk of colon cancer. In contrast, (Stevenson et al., 2012; Yang et al., 2012) reported that the presence of high fibre in food products is beneficial to facilitate the bowel movement (peristalsis) and also prevention of many gastrointestinal diseases in man.

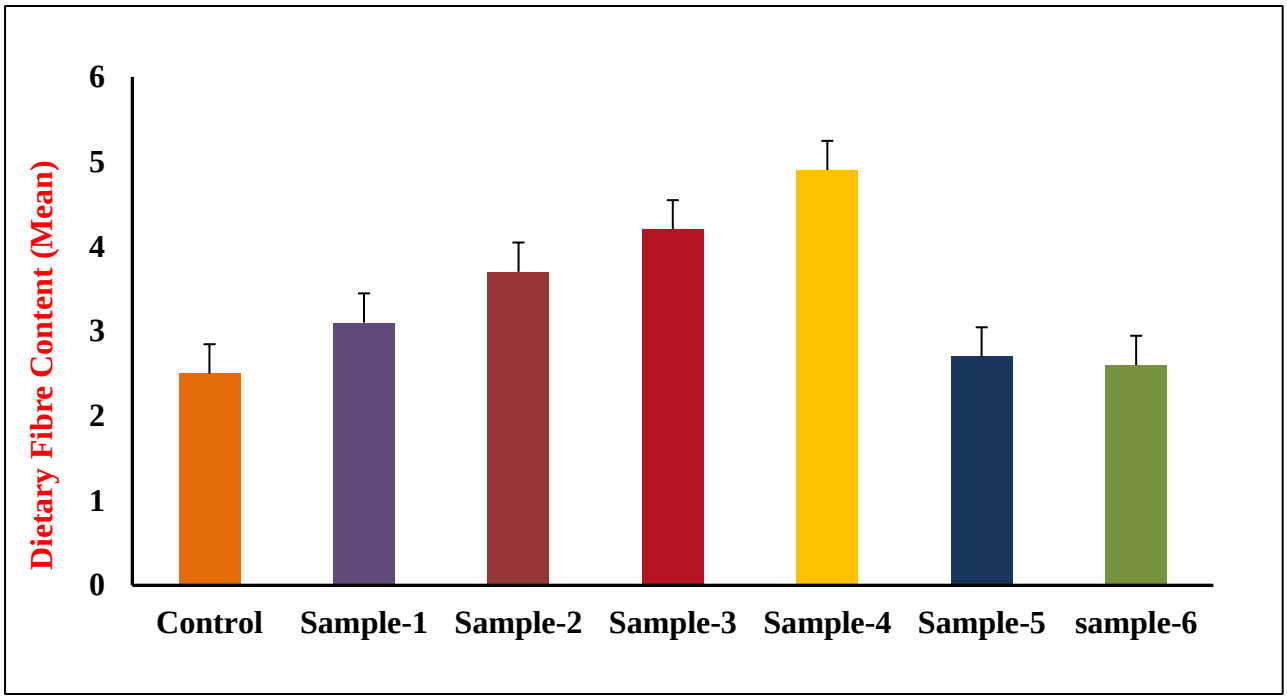


Fig 4.5: Dietary fibre content of biscuit samples

4.1.1.6 Carbohydrates Content

Carbohydrate content of biscuits ranged between 62.1 to 66.7%. Biscuit sample-4 had the lowest carbohydrate content (62.1%) while the reference control sample had the highest content (66.7%). The increase in the proportion of plants materials brought a decrease in the carbohydrate content of the biscuits. There was a significant difference in the carbohydrate content in all the biscuit samples. Similarly, Alam et al., (2014) have found a decreasing trend in the carbohydrate contents (56.6 to 54.6%) of biscuit samples made from plants parts. Carbohydrates are macromolecules (Sugar, starch, and cellulose) that provide a good source of energy value (Thongram et al., 2016). In contrast, Ganorkar (2014) and Stevenson et al., (2012) states that low carbohydrate and high fibre content of food products have protective health effects, such as relief from constipation and also aids digestion in the colon.

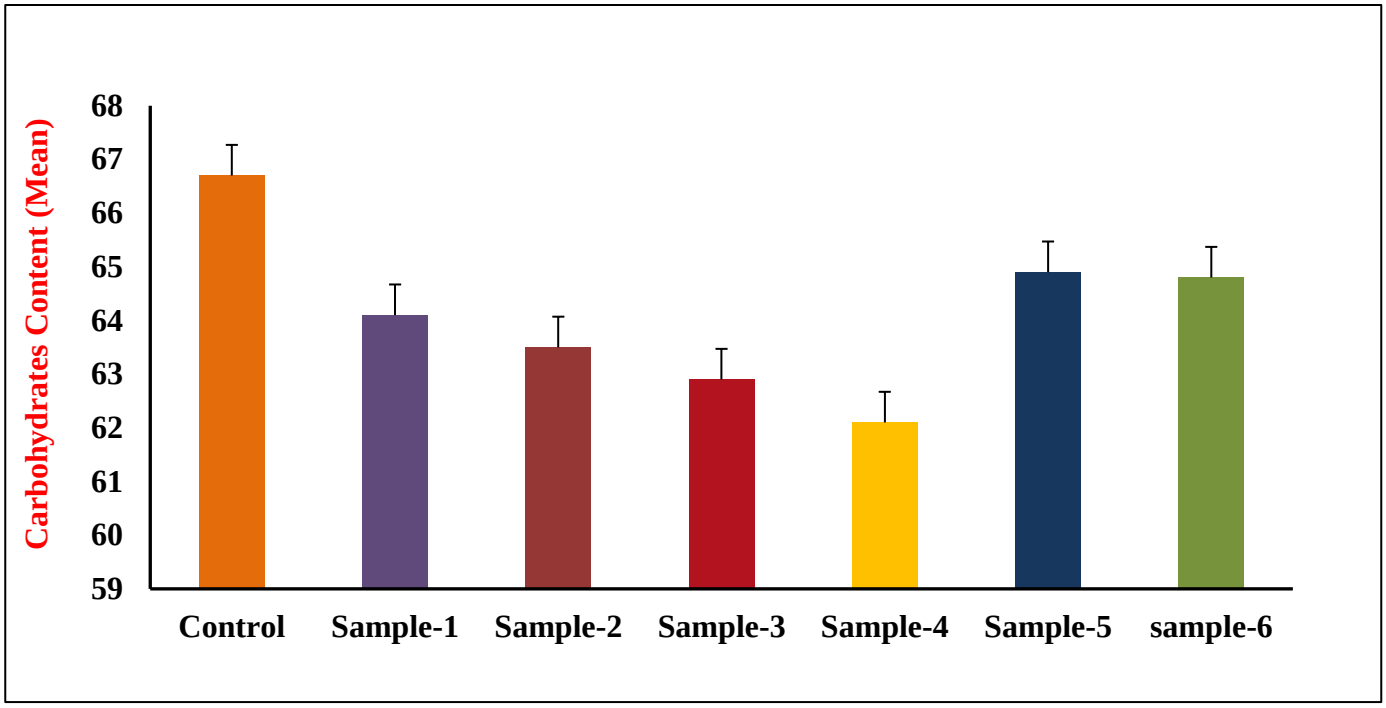


Fig 4.6: Carbohydrate content of biscuit samples

4.1.1.7 Energy

The energy values of the biscuits are shown in Figure 4.7. The energy value of all the samples ranged from 445 to 457.3 Kcal/100g. Biscuit sample-4 had the lowest energy value (445 kcal/100g), whereas the control sample had a higher energy value (457.39 Kcal/100g). The energy values of biscuits were shows a significant difference ($p \leq 0.05$) from the control to sample-6 respectively. Similarly, a decreasing trend in the energy value (458 to 449 Kcal/100g) for biscuits made from different flour blends was reported by (Norhayati et al., 2015). In addition, the protein, fat, carbohydrates constituents all contributed to providing energy. Biscuits are energy giving food products that are consumed as a snack by all age groups in-between meals. The incorporation of plant materials could help to boost the level of protein, antioxidant and fibre content of biscuits (Alam et al., 2014; Ikuomola et al., 2017).

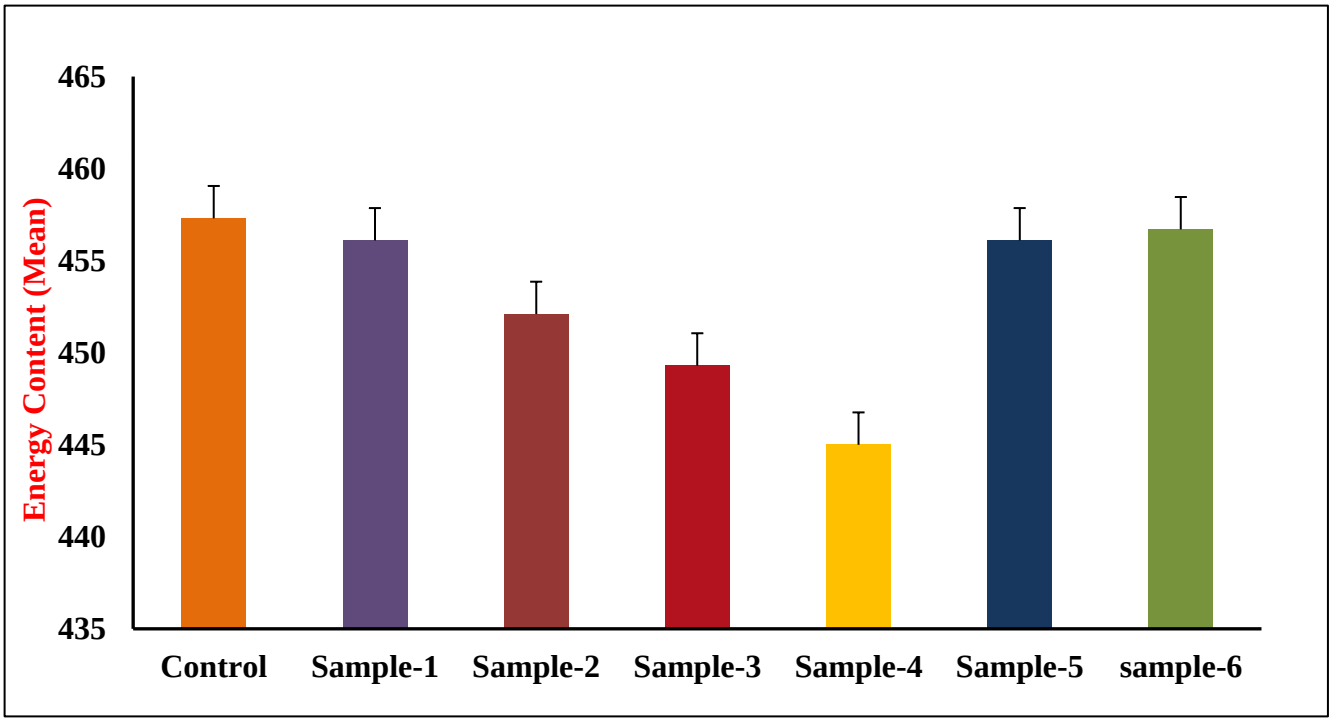


Fig 4.7: Energy Values of biscuit samples.

4.1.2 Physical Analysis of Biscuits

Six formulations of biscuits were prepared with the incorporation of under-utilized plant materials respectively and the results of the physical properties of all biscuit samples are presented in Table 4.2. The mean score of weight, diameter, thickness, and spread ratio and bake loss of the biscuits were significantly different ($p \leq 0.05$) in terms of all the samples from each other. The parameters have been used to determine the quality of flour as well as product (Gul et al., 2014; Mir et al., 2014a; Mir et al., 2014b; Singh et al., 2011).

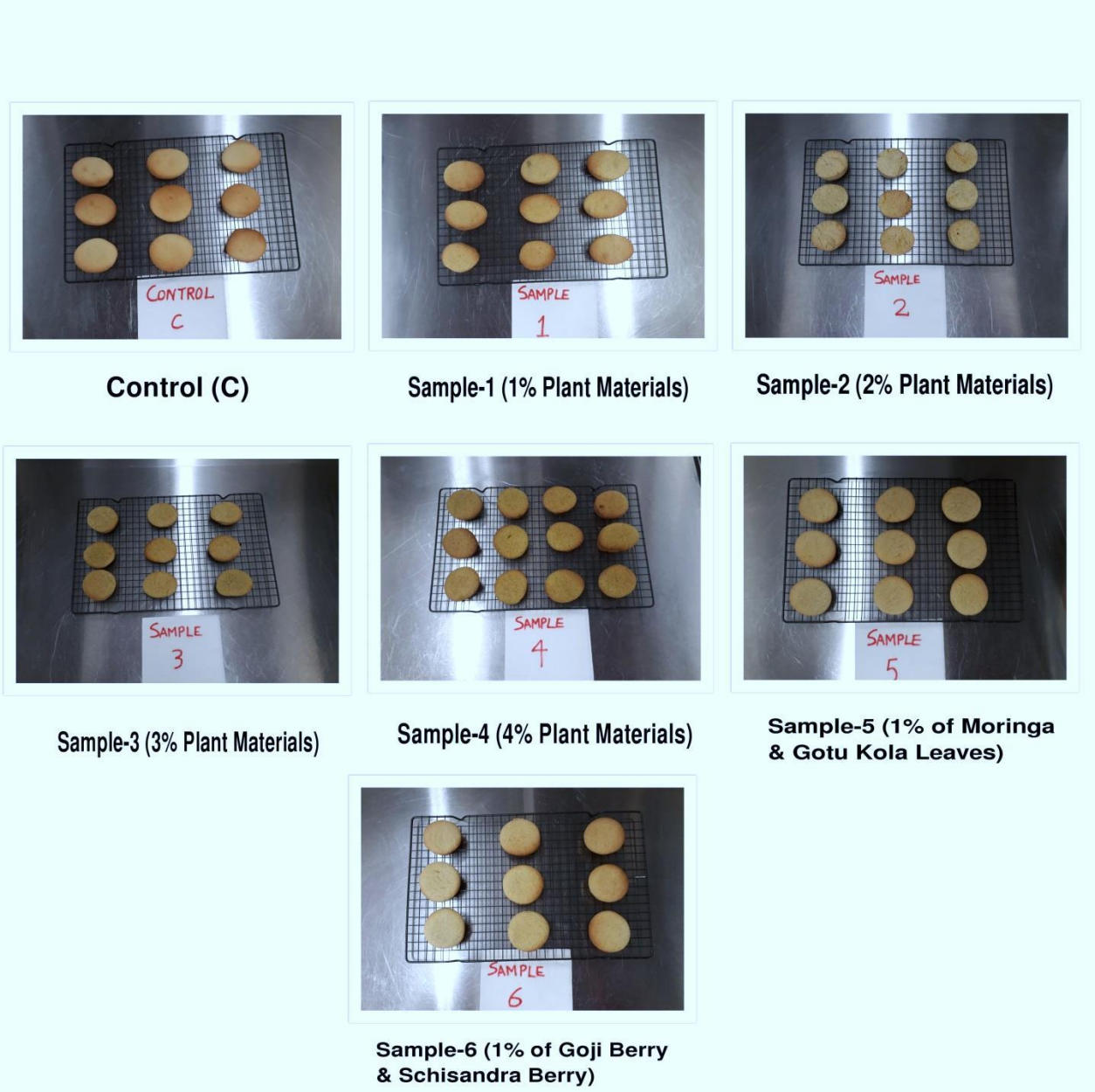


Fig 4.8 Control and Underutilized plants materials incorporated biscuits

Table 4.2: Physical analysis of biscuit samples

Treatments	Weight (g)	Diameter (mm)	Thickness (mm)	Spread ratio	Bake loss (g/100g)
Control	16.28± 0.25	51.33± 0.50	6.54± 0.26	7.83± 0.32	21.00± 0.87
Sample-1	17.19± 0.19	51.39± 0.35	6.50± 0.25	7.99± 0.16	19.82± 0.69
Sample-2	19.70± 0.54	51.48± 0.30	6.37± 0.23	8.12± 0.28	18.45± 0.68
Sample-3	20.45± 0.30	51.78± 0.43	6.26± 0.27	8.24± 0.32	16.47± 0.62
Sample-4	20.81± 0.51	52.23± 0.47	6.15±0.23	8.53± 0.22	15.19± 0.45
Sample-5	17.53± 0.30	51.35± 0.57	6.10± 0.24	8.04± 0.34	18.88± 0.42
Sample-6	18.22± 0.40	51.46± 0.26	6.05± 0.19	8.08± 0.27	19.41± 0.69

Note: Sample-1 (1%), sample-2 (2%), sample-3 (3%), sample-4 (4%), sample-5 (1%), sample-6 (1%) of underutilized plants materials

All the values for different samples; different parameters in the same column presented in the table display are significantly different ($p \leq 0.05$) from one another; values are represented in means± SD (Standard deviations) of three determinations (n = 6)

4.1.2.1 Biscuits Weight

According to Table 4.2, the weight of the biscuits ranged from 16.2, 17.1, 19.7, 20.4, 20.8, 17.5, and 18.2 g respectively. It is evident from results, the addition of plants materials caused a significant difference ($p \leq 0.05$) in the weight between control and under-utilized plants incorporated biscuit samples. Control Sample had a minimum weight which may be due to high water holding capacity (WHC) rather than other biscuit samples as they have more protein

content (Kolawole et al., 2018; Kulthe et al., 2014), while sample-4 had maximum weight (20.8 g). Ho & Abdul (2016) and Thongram et al., (2016) reported that water holding capacity usually higher in wheat proteins which may lead to a low weight of the product, as a result, in this study, it represents that the biscuits have different weights because of different levels of water holding capacities (WHC). The findings were similar to the observation of some researchers who reported increase trend in the weight of biscuits produced from composite flours blends (Okpala et al., 2013), plants material (Alam et al., 2014), flour blends unripe banana, pigeon pea and sweet potato (Adeola & Ohizua, 2018) respectively.

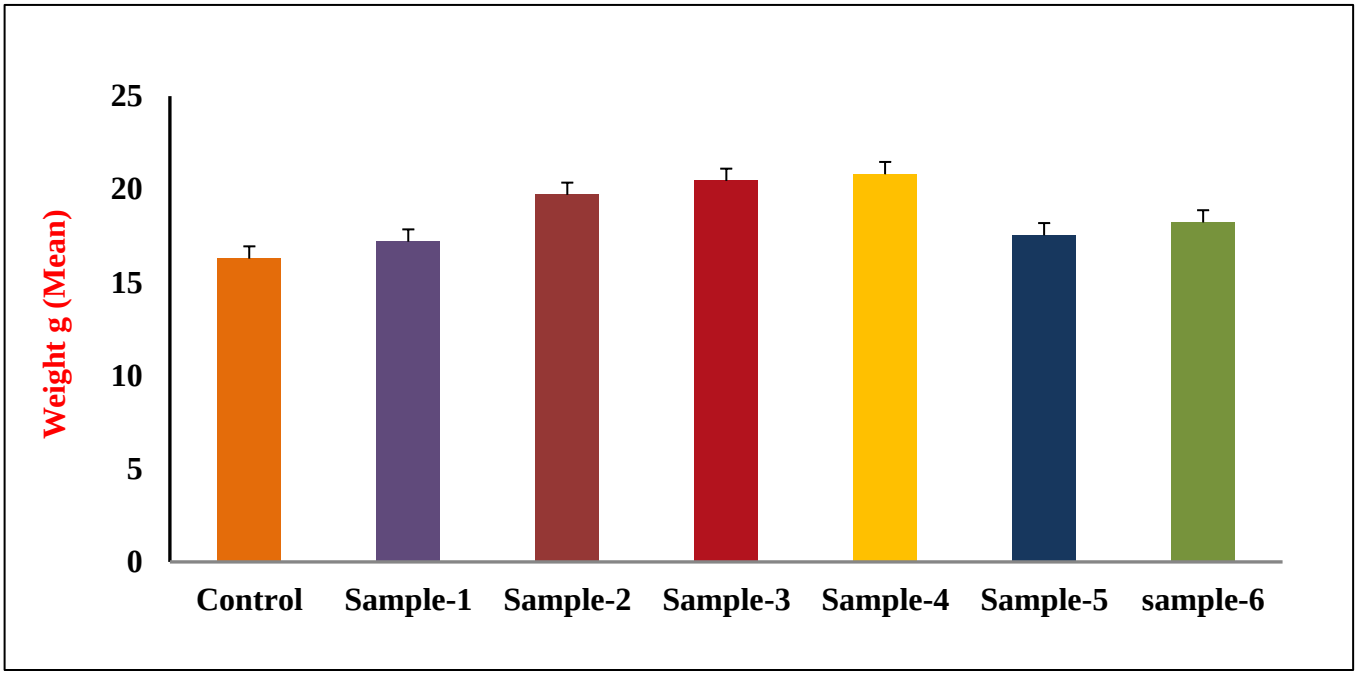


Fig 4.9: Weight of biscuit samples.

4.1.2.2 Diameter

The results from Table 4.2 showed that the diameter of biscuits ranged from 51.1 to 52.2 mm (Table 4.2). As noticed from reference data the diameter of the biscuits showed an increasing trend with the increase of plants materials substitution level in biscuits. According to Chauhan et al., (2016), protein content and diameter have an inverse correlation. During the heating process, protein gluten present in flour undergoes glass transition, therefore gaining mobility that allows it to interrelate and form a web, thus increasing viscosity and stop the flow of dough. In contrast, (Ballabio et al., 2011; Ibidapo et al., 2017) reported that dough with lower viscosity causes the baked product to spread at a faster rate thereby increases the spread ratio. The results of the diameter of biscuit samples showed that all the samples were different significantly ($p \leq 0.05$) from each other. The sample-4 (52.2 mm) had the maximum diameter and minimum in the control sample (51.3 mm). The findings agreed with the observation of Giwa & Ikujenlola (2010) and Ikuomola et al., (2017), who reported an increase in diameter of biscuits with increasing substation level of quality protein maize (QPM) flour and malted barley bran (MBB) blends. However, these results were in contrary to the findings on biscuits produced from soybean supplemented wheat flour (Ayo et al., 2007), cowpea-wheat (Okaka & Isieh, 1990), and millet-sesame flour (Alobo, 2001) respectively.

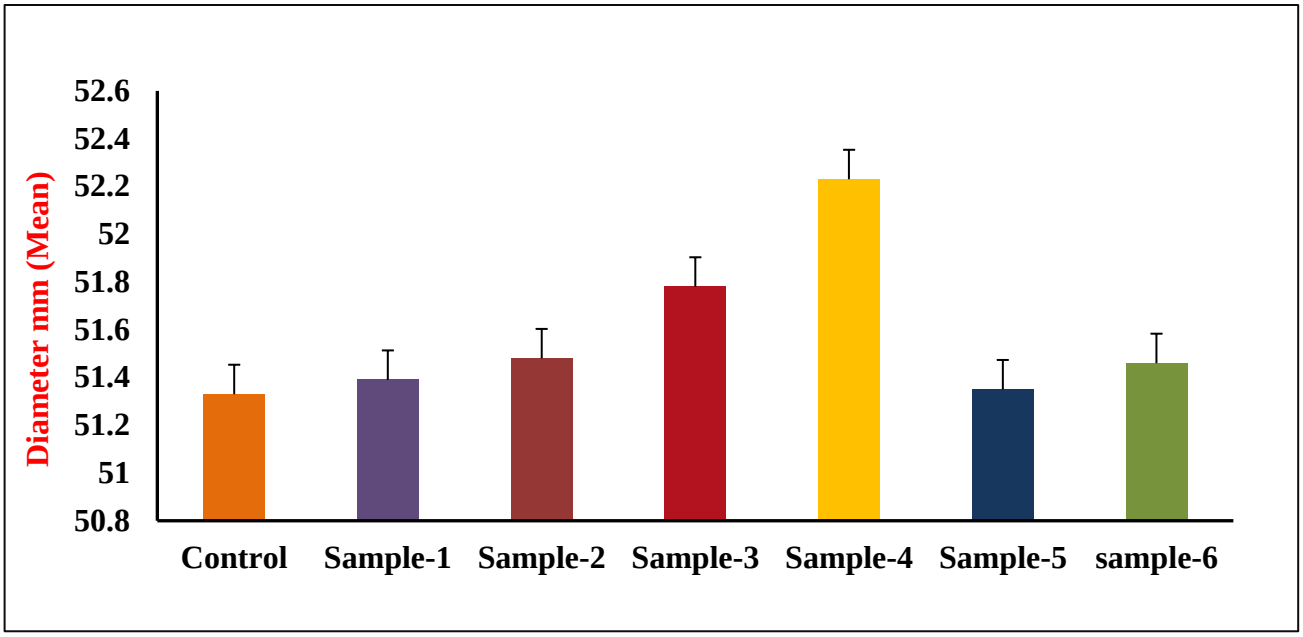


Fig 4.10: Diameter values of biscuits

4.1.2.3 Thickness

The thickness of the biscuits ranged between 6.05 to 6.60 mm (Table 4.2). The results of the thickness showed that there was significant ($p \leq 0.05$) difference between control and under-utilized plants parts biscuit samples. The biscuit sample-2 had the highest height value (6.60 mm), whereas the sample-6 had the least thickness value (6.05 mm). Similar results have been observed by Chauhan et al., (2016) produced from wheat and amaranth flour, wheat flour semi-sweet biscuits (Mamat and Hill, 2014). However, the findings disagree with Abdul et al. (2015), who reported increasing trend as the thickness of the samples produced from composite wheat flour and oat bran blends, wheat flour and malted barley brans blends (Ikuomola et al., 2017). Thongram et al., (2016) reported that the thickness of the biscuits may be affected due to different baking properties, such as lower temperature, the rise of temperature, and high absorption of moisture of the dough (presence of water binding components).

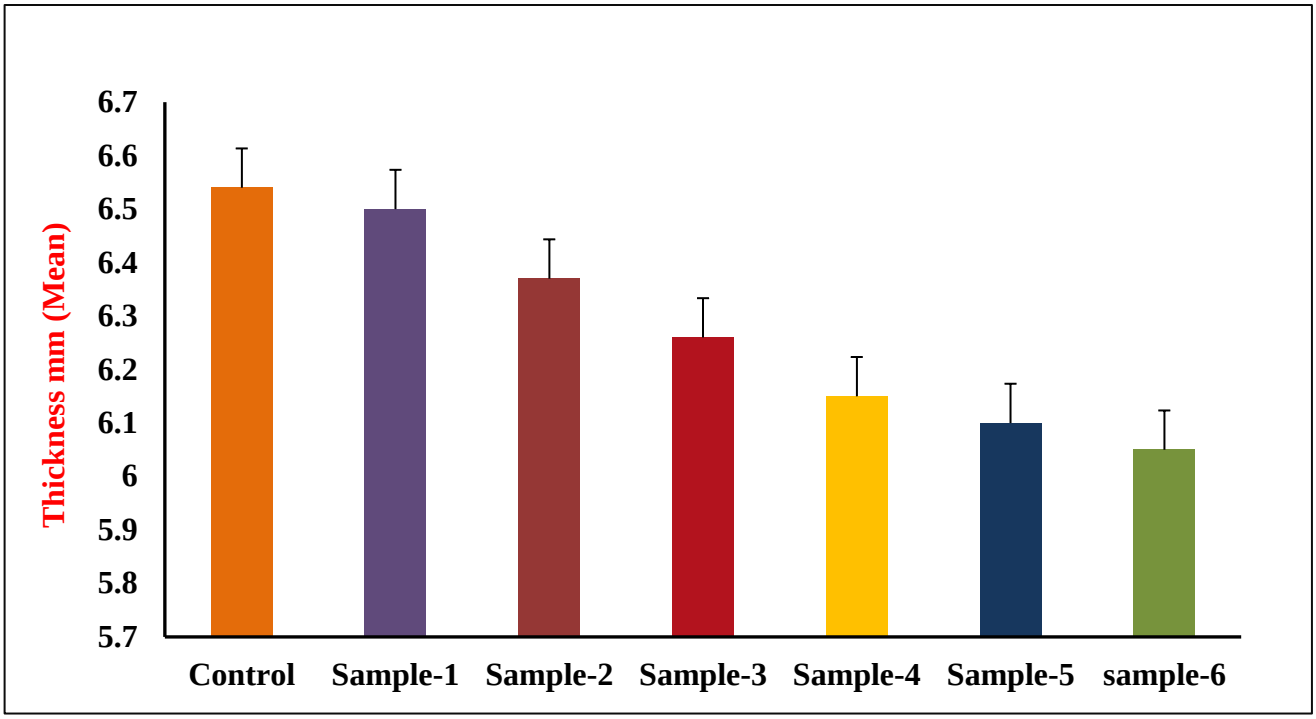


Fig 4.11: Thickness values of biscuits

4.1.2.4 Spread Ratio

Biscuits spread rate stands for a ratio of diameter to thickness. Spread rate of biscuits ranged from 7.83 to 8.53. It is affected by dough viscosity; lower viscosity of dough increases the spread ratio at a faster rate (Miller & Hoseney, 1997). The addition of plants parts caused a significant difference in the spread rate of the biscuits with increasing levels of incorporation from 1 to 4%. From the results (Table 4.2) spread ratio of the biscuit samples revealed an increasing trend along with increasing substitution levels of plants materials. Results showed that biscuit sample-4 had the maximum spread rate value (8.53), while the control sample had the minimum (7.83). A similar trend was observed by Chauhan et al., (2016), who stated that the spread ratio of cookies samples increased with increasing incorporation levels of quality maize flour; wheat and

amaranth flour blends (Chauhan et al., 2016); cassava and water chestnut flours (Bala et al., 2015). According to Chugh et al., (2013) products with higher spread ratios are considered as most desirable. However, findings of this study are inconsistent with the study of Abdul et al. (2015), who observed decreasing tend in the spread ratios for cookies produced from oat bran; legumes flour (Thongram et al., 2016). Moreover, the spread rate values for products produced from unripe plantain and defatted sesame flour blends did not follow a particular trend (Chinma & Gernah, 2007). In contrast, low spread factor of the products due to hydrophilic nature and of the flour used in the production of the products which led to increase in thickness and decrease in spread rate (Yahya, 2004).

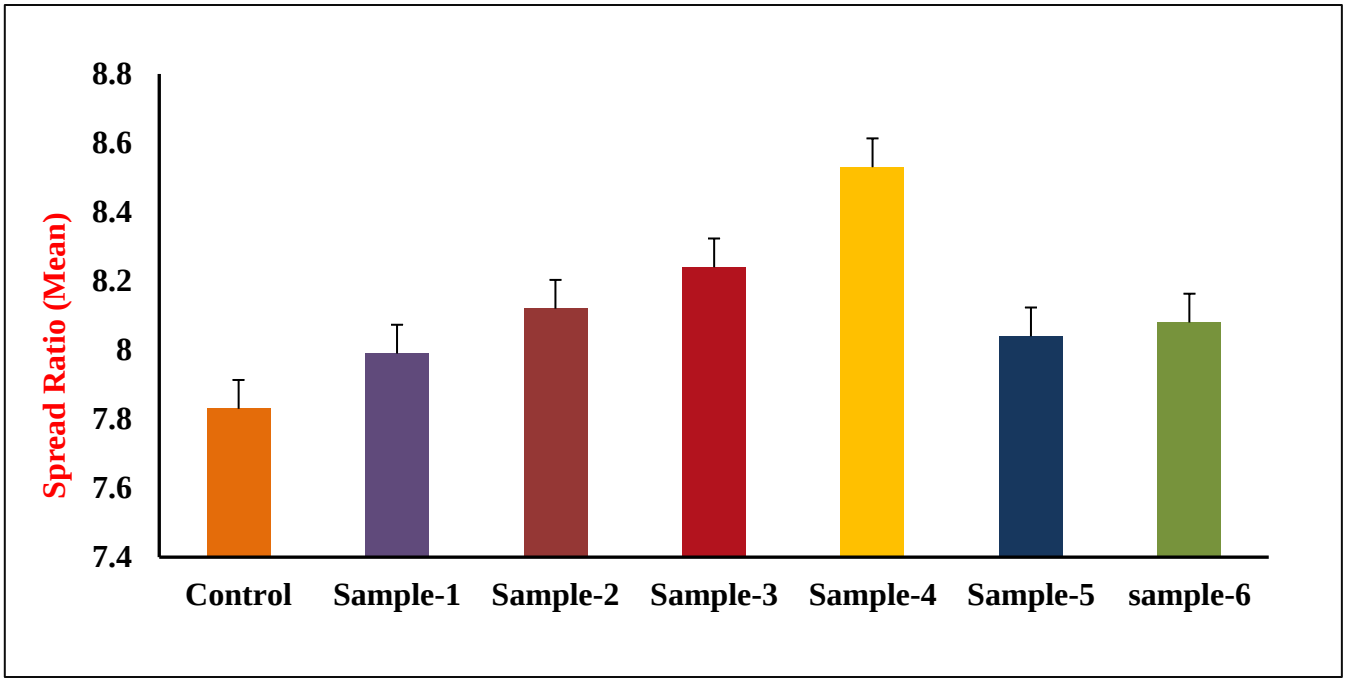


Fig 4.12: Spread ratio values of biscuits

4.1.2.5 Bake Loss

The bake loss of the biscuits ranged from 21.0, 19.82, 18.45, 16.47, 15.19, 18.88, and 19.41 g/100g respectively. The control sample had the highest value (21.0 g), whereas biscuit sample-4 had the lowest value (15.1 g). The bake loss values of biscuit samples significantly ($p \leq 0.05$) decreased as the incorporation level of plants parts increased from 1 to 4%. The results from Table 4.2 clearly represent decreasing similar tend as observed by Chauhan et al., (2016), for cookies produced from wheat and amaranth flour blends. The decrease in bake loss can be due to higher water holding capacity as well as high-protein content of the flour used in the production of the products (Jerome et al., 2007; Singh et al., 2011)

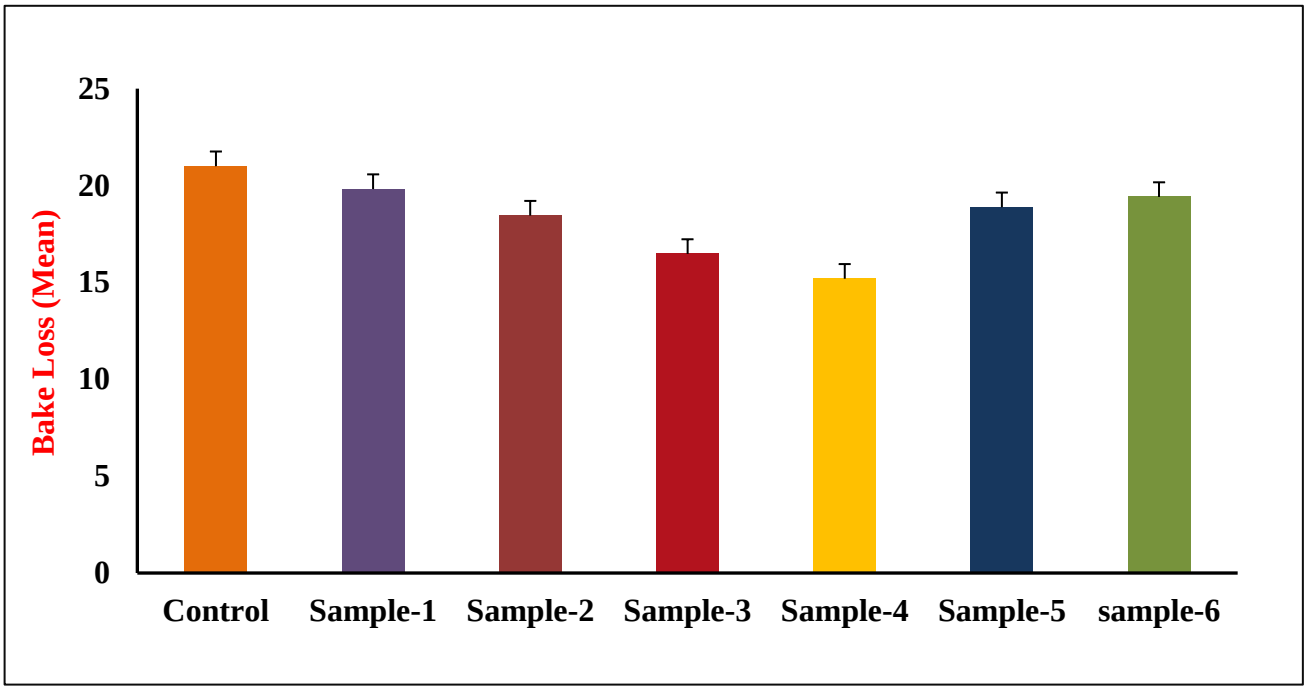


Fig 4.13: Bake loss values of biscuits.

4.1.2.6 Colour analysis of biscuits

Color is an essential element which makes a significant contribution to the initial as well as overall acceptability of baked products by consumers. Despite Cauvain (2017) reported that color development of the baked products usually occurs during the later stages of baking and it can be helpful or used to judge completion of the baking process. *L**, *a**, and *b** are the hunter parameters which measured from the top and bottom surface of baked products. The results of the physical color assessment of biscuits produced from under-utilized plants parts are presented in Table 4.3. The mean score of color *L** value, *a** value, and *b** value for the biscuit samples were significantly ($p \leq 0.05$) different from each other. The control sample had the lowest score for *a** and *b** attributes observed, except for *L** value.

Table 4.3: Color analysis of formulated biscuits

Treatments	*L**-value	*a**-value	*b**-value
Control	63.35± 0.99	6.49± 0.31	25.90± 1.66
Sample-1	63.14± 0.56	6.56± 0.34	26.34± 0.58
Sample-2	62.390± 1.35	7.10± 0.82	27.46± 0.99
Sampl-3	61.75± 2.34	7.58± 0.44	28.79± 0.56
Sampl-4	60.82± 0.72	7.77± 0.43	29.96± 0.64
Sample-5	59.56± 0.68	8.31± 0.16	30.12± 0.82
Sample-6	59.54± 0.44	8.55± 0.23	33.32± 1.34

Note: Sample-1 (1%), sample-2 (2%), sample-3 (3%), sample-4 (4%), sample-5 (1%), sample-6 (1%) of underutilized plants materials.

Color parameters values for different samples with different significant letters presented on the same column in the table display are statistically significantly different ($p \leq 0.05$); values are represented in means± SD (Standard deviations) of three determinations (n = 3)

a & b* value: +ve indicates red and –ve indicates green color*

4.1.2.6.1 *L** Value

*L** value is a hunter parameter which is used to measure the lightness-darkness (*L** = 0 indicates black and *L** = 100 yields a white fraction of the product. The results from Table 4.3 indicates that the *L** value of the biscuit samples showed a decreasing trend along with the increasing substitution level of under-utilized plants materials. The control sample had the highest lightness (*L**) value for the top along with the bottom surface of the biscuit samples. The data showed that there was a significant difference ($p < 0.05$) between one another samples. The findings agree with the study observed by Mamat et al., (2010) produced semi-sweet biscuits from wheat flour; wheat and amaranth flour blends (Chauhan et al., 2016). Gallagher et al., (2003) reported that high protein content of the product has a reverse correlation with the lightness of biscuits (*L*-value), represents that an increased protein content in biscuits formulation decreased the *L** value). Moreover, the decrease in *L** value reveals that the under-utilized plants' incorporated biscuits are darker in color at higher levels of substitution are shown in Fig 4.14.

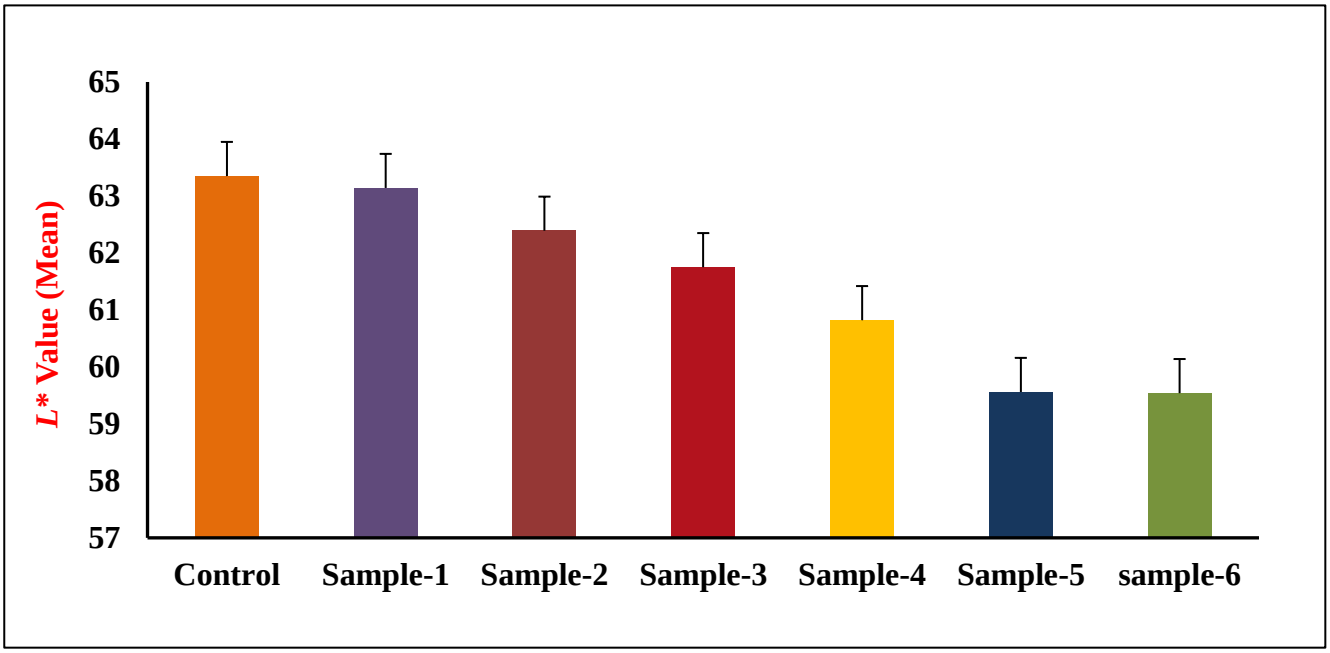
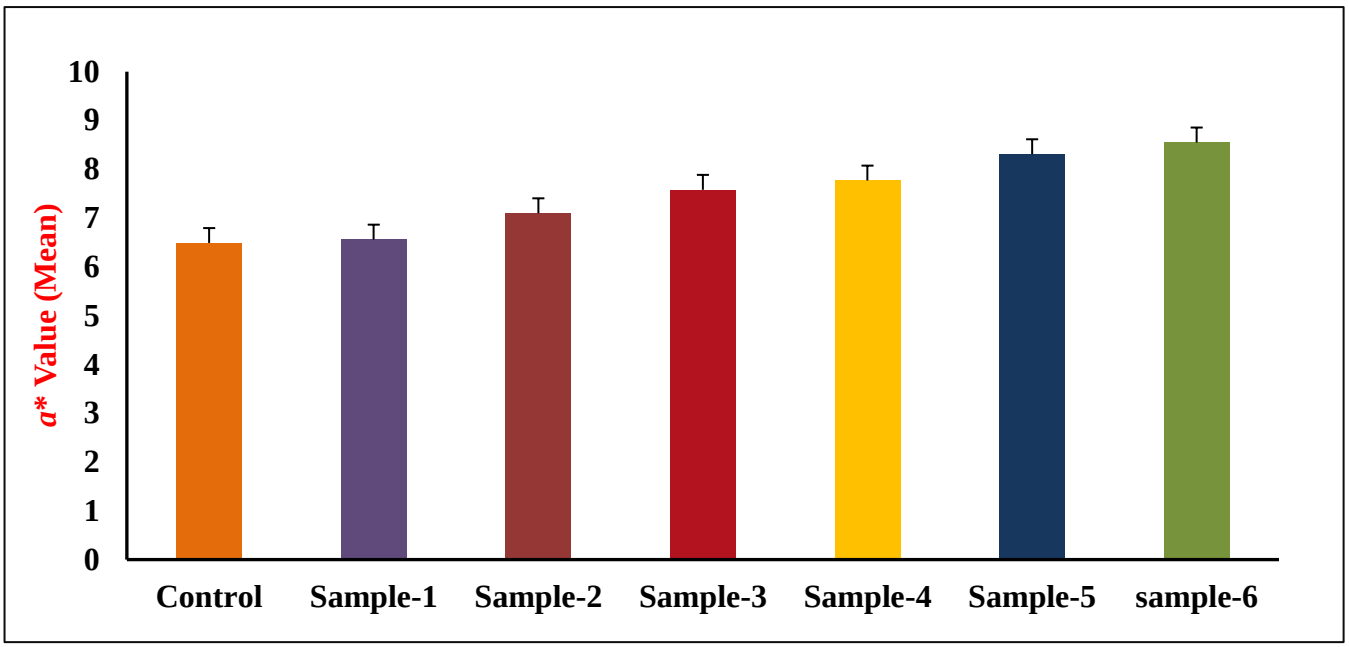


Fig 4.14: *L value of biscuits**

4.1.2.6.2 *a** Value

*a** value in color measurements depicts an indication of the redness of the product if positive and negative indicates green. From Table 4.3 results displayed an increasing trend in a* value of biscuit samples along with the increased substitution level of under-utilized plants materials in biscuits preparation. The top surface results revealed that sample-6 had the highest *a** value, whereas control sample had least. All the biscuit samples were significantly different ($p \leq 0.05$) from one another. The results showed the similar an increasing trend with the findings observed by Mamat et al., (2010) produced fat-reduced tea biscuits from wheat flour; wheat and amaranth flour blends (Chauhan et al., 2016). Moreover, an increasing pattern of *a** value was found for both the top and the bottom surface of biscuit samples that were dark in color (*L** value) shown

in Figure 4.8. In addition, Agrahar-Murugkar et al., (2015) stated that color formation of the baked products is due to Millard reactions between sugar and proteins. The Millard reaction is a non-enzymatic reaction which causes the browning and caramelization of sugar which produce brown pigments during baking. Cronin & Preis (2000) reported that there are several factors (such as; composition of ingredients, baking time, humidity in the oven atmosphere in the initial stages of baking) may be contributing to the color development of final products. Inconsistent



with these studies, Mahdi et al., (2008) observed that Malt extract is one of the main sugars and protein contributing flavor and color to the final baked products.

Fig 4.15: *a value of biscuits**

4.1.2.6.3 *b** Value

The *b** value is a measure of the yellowness of the biscuits surface color. The control sample top surfaces had the lowest *b** value (25.9), while sample-6 had the highest (33.32) respectively. The

results were significantly different ($p \leq 0.05$) for the biscuit samples from each other. From the results, it was noticed that yellowness (*b** value) in the biscuits top surface had a similar trend as redness (a* value) along with the increased under-utilized plants' parts incorporation level in biscuit preparation. Moreover, the results showed a similar trend with the findings reported by Mamat et al., (2010) produced fat-reduced commercial tea biscuits from different brands of wheat flour blends; wheat and amaranth flour blends (Chauhan et al., 2016).

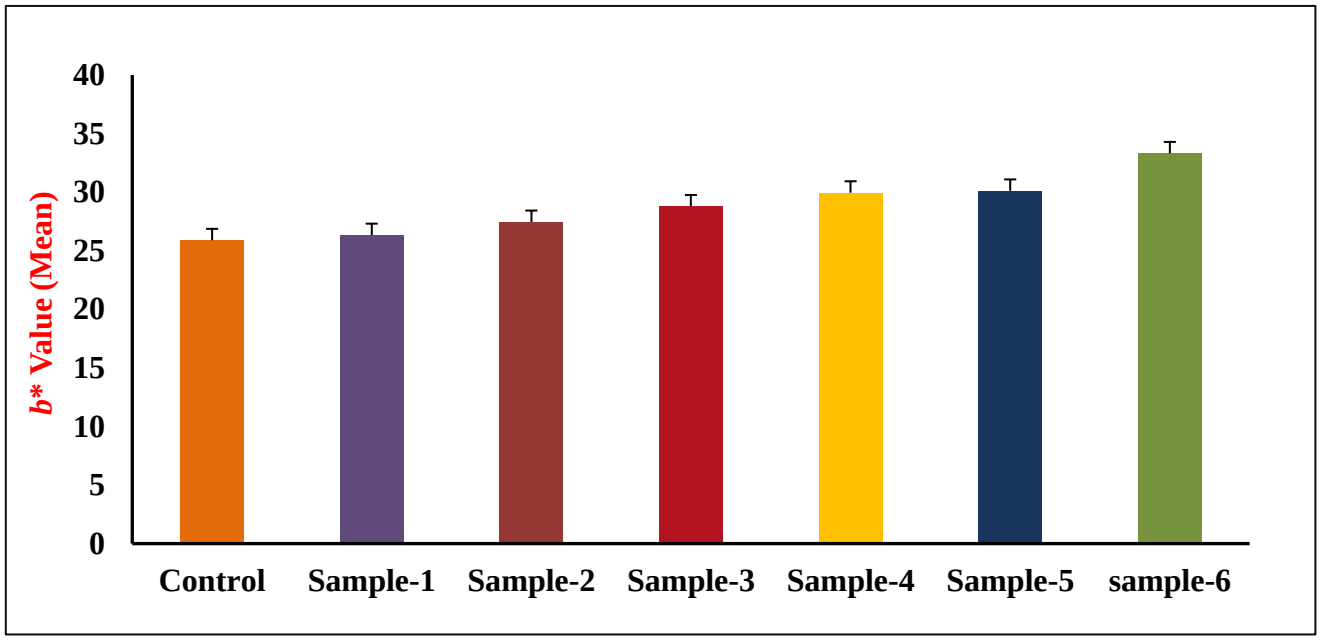


Fig 4.16: *b value of biscuits**

4.2 Antioxidant activities of plant extracts and their correlation with antioxidant contents

Four traditionally well-known and scientifically evaluated plants' parts were used in this study to develop antioxidant rich biscuits. These are Moringa leaves, Goji berries, Schisandra berries, and

Gotu kola leaves (Chapter 2/ Section 2.6). A brief summary of the traditional use of these plants together with the scientific evaluation of their health beneficial properties are presented in Table 4.4.

With a view to evaluating the antioxidant activities and polyphenol contents of these plants, dry powders of each of the plant were subjected to hot water extraction procedure (Chapter 3/ Section 3.6).

Name of plants	Traditional uses and scientific findings	References
Moringa	Anticancer, anti-inflammatory, antimicrobial, antihypertensive, anti-obese, and antidote to poisonous bites of snakes and scorpion	Abdull et al., 2014
Gotu Kola	Antidiabetic, wound healing, anti-aging, therapeutic agent for Alzheimer's diseases, and memory enhancing Stimulate cell rejuvenation, nerve tonic, neuroprotective, and improve physical work capacity	Hamidpour, 2015
Schisandra Berry	Anti-inflammatory, anti-stress effects, blood pressure, and stimulate physical work capacity	Hashim, 2011
Goji Berry	Anti-cardiovascular, antidiabetic, immunostimulatory, neuroprotective, anti-fungal, treatment of cervical cancer, and alleviate menstrual discomfort	Kulczyński et al., 2016

Table 4.4: Biological activities and scientific uses of the plants used in this study

4.2.1 Hot water extraction yield

About 5 g of dry plant powder was used to perform hot water extraction and the extraction yields are presented in Table 4.5 below. These extracts contain all water-soluble compounds that include polyphenols (phenolics and flavonoids), essential oils, vitamins, polysaccharides, proteins, and other phytonutrients such as trace metals. In this study, special focus was given to phenolic and flavonoid contents due to their high antioxidant and many other health beneficial activities.

Table 4.5: Hot water extraction yield of the plants used in this study

Plants samples	Quantity of plants Powder used (g)	Extraction yield (g)
Moringa Leaves	5.2271	1.1084
Gotu Kola Leaves	5.2072	1.0412
Schisandra Berries	5.2428	2.1613
Goji Berries	5.2576	2.9829

4.2.2 Total phenolic contents (TPC) and total flavonoid contents (TFC)

Folin-Coicalteu assay and aluminium chloride tests were used to determine the total flavonoid and phenolic contents respectively, of hot water extracts of the selected four plants and the results are presented in Table 4.6 and Fig 4.17. The phenolic contents were expressed in Gallic acid equivalent (GAE mg/g) units and the flavonoid contents were expressed in Quercetin equivalent (QE mg/g) units of the extracts per gram of the dried plant material. The total

phenolic contents of the selected four plant extracts were in the range of 30.11 to 70.07 mg/g. The highest phenolic contents were observed in the extracts of Schisandra berries (70.07± 0.10 mg/g) and this observation is consistent with the literature that these fruits are being used to improve the functional benefits of certain food products (Chen et al., 2012, Lin et al., 2009).

The findings also revealed that the phenolic contents were relatively larger in all the extracts compared to their flavonoid contents (Table 4.6 and Fig 4.17). Extremely high quantities of phenolic and flavonoid contents were observed in Schisandra berry which is followed by Goji berry (Table 4.6 and Fig 4.17). As can be seen from the results, Moringa and Gotu kola leaves also had high levels of phenolic and flavonoid contents.

These are important findings and the results are consistent with the literature on all the four plants revealing their tremendous health beneficial effects (Chen et al., 2012, Coppin et al., 2013, Lin et al., 2009, Wang et al., 2010, Yan et al., 2014). These findings indicate that high antioxidant contents of the four plants and their edible nature with least side effects make them extremely suitable candidates to be used for functionally enriched food products.

Table 4.6: Total phenolic and flavonoid contents of hot water extracts of plants used in this study along with their radical scavenging activities

Plant Samples	Phenolic content (GAE mg/g)*	Flavonoid content (QE mg/g)*	$ABTS^{\cdot+}$ scavenging activity (Ascorbate equivalent µM/)#
Moringa Leave	30.77± 0.18	10.9± 0.26	822.62± 1.65
Gotu Kola Leave	30.11± 0.22	11.28± 0.67	844.05± 2.97
Schisandra Berry	70.07± 0.10	35.43± 0.95	860.24± 1.65
Goji Berry	46.75± 0.44	15.47± 0.65	846.90± 1.65

**Total phenolic and flavonoid contents are expressed as Gallic acid and Quercetin equivalent mg per gram respectively*

#$ABTS^{\bullet+}$ free radical scavenging activities are expressed as ascorbic acid equivalent (µM) at 1mg/mL of plant extracts

&All values are expressed as mean ± SD, n=3 ($p \leq 0.005$)

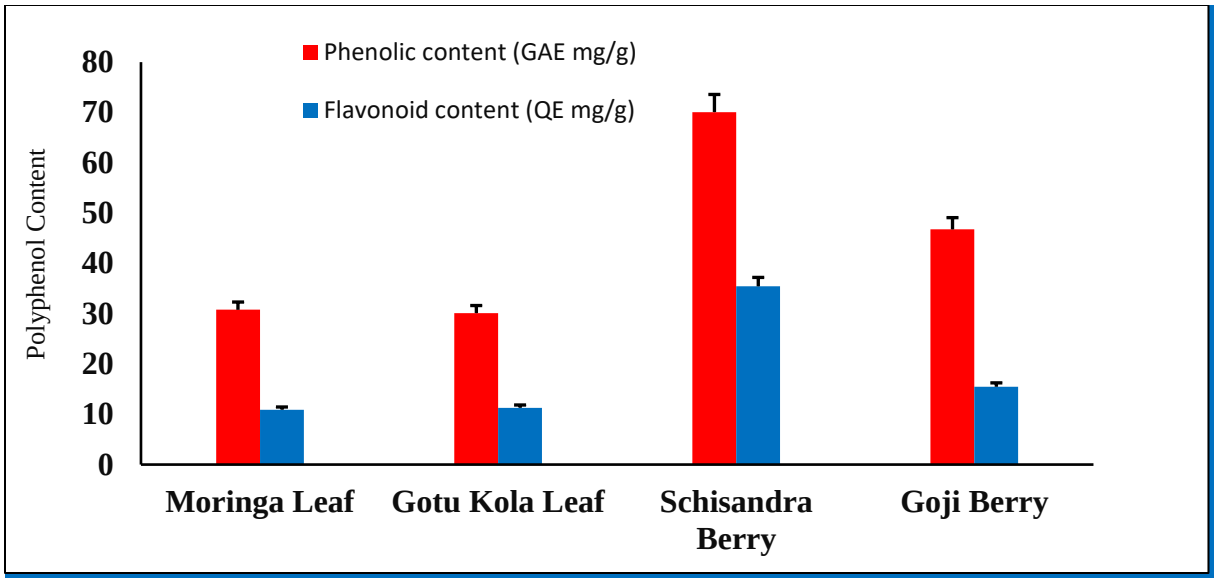


Fig 4.17: Total phenolic and flavonoid contents of hot water extracts of plants:

The RED bar represents total phenolic content and the BLUE bar represents total flavonoid content per 40 grams of biscuits

4.2.3 Antioxidant activities of plant extracts and their correlation with TPC and TFC

As mentioned before, four traditionally known and underutilized plant parts, namely Moringa leaves, Gotu kola leaves, Goji berries, and Schisandra berries were used in this study to develop antioxidant rich biscuits (Chapter 2/ Section 2.6). Radical scavenging activities of their water extracts were evaluated against $ABTS^{\bullet+}$ radicals. These results are presented in Table 4.6 and Fig 4.18. All the four plant extracts exhibited highly significant $ABTS^{\bullet+}$ scavenging activities and these activities are superior when compared with the scavenging activities of several medicinal herbs studied previously in our Laboratory (Zhang et al., 2017). As can be seen from Table 4.6, the activities of the extracts ranged from 822.62 µmol to 860.24 µmol ascorbic acid equivalent. Extremely high scavenging activities were displayed by the extracts of Schisandra

berry which is followed by Goji berry (Table 4.6 and Fig 4.18). Extremely high activity of Schisandra berries is in agreement with the literature findings that they contain large quantities of polyphenols as well as essential oils, vitamins and other antioxidant constituents (Chen et al., 2012, Wang et al., 2010).

All the four plants showed concentration dependent scavenging activities in the range of 0 to 1000 µg/mL and these results are presented in Appendix A1 (Table A1.1 and Figures A1.1 to A1.4). The results presented in this section strongly indicate that the four plants studied in this research display highly significant antioxidant activities. It is therefore concluded that these plants are extremely suitable to enrich the antioxidant potential and functional value of bakery products and these observations are consistent with the literature (Cheng et al., 2014, Hashim, 2011, Kulczynski and Gramza-Michalowska, 2016).

An overall conclusion from the antioxidant contents and activities presented in this section reveal that a systematic investigation, of edible plants with traditionally known health benefits, employing contemporary scientific tools are expected to lead to the discoveries of functional foods with major health benefits.

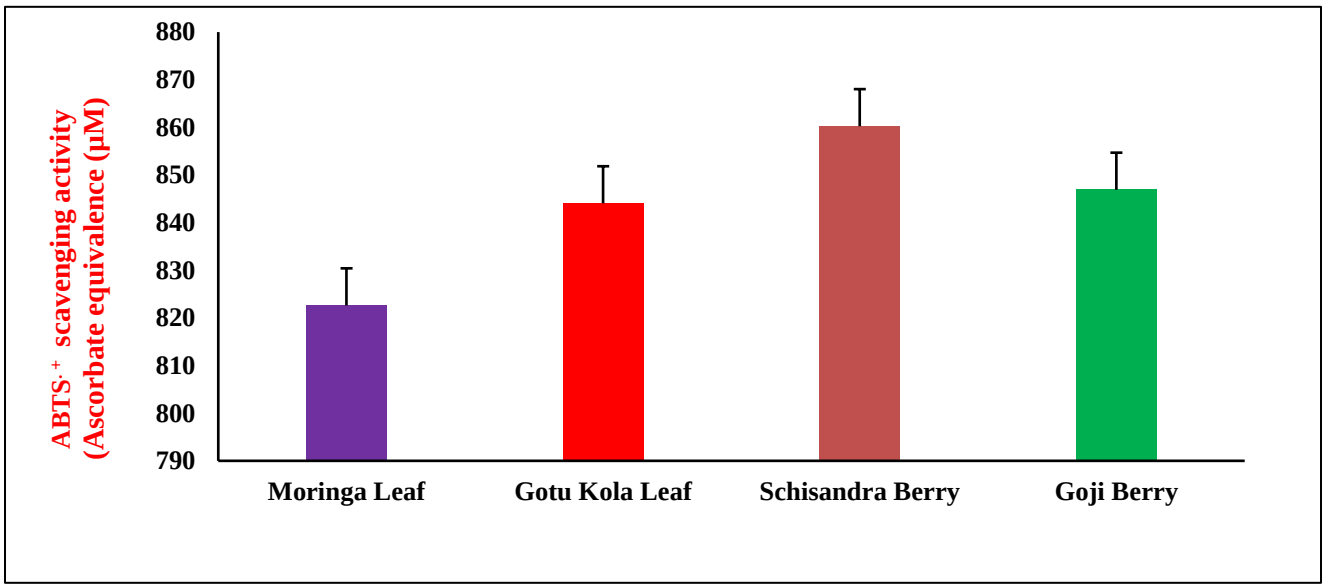


Fig 4.18: ABTS $^{\bullet+}$ scavenging activity of hot water extracts of plants: Scavenging activities are expressed as ascorbate equivalent (µM) at 1mg/mL

4.3. Antioxidant activities of functionally enriched biscuits

Results presented in sections 4.1 to 4.3, clearly demonstrate that biscuits developed and evaluated in this project with high protein flour in combination with nutritionally rich plants' materials have produced high quality products. They indeed had appealing colour and physical characteristics, improved protein contents and lowered carbohydrate (lower energy) contents. Their fibre contents have also improved as the percentage of plant material is increased (Sections 4.1).

With a view to evaluating the antioxidant activities and polyphenol contents of these enriched biscuits, dry powders of each of the six enriched biscuit samples along with the control sample were subjected to hot water extraction procedure (Chapter 3/ Section 3.6). The polyphenol

contents and the antioxidant activities of these extracts are presented and discussed in this section.

4.3.1 Hot water extraction yield

About 50 g of dry biscuit powder was used to perform hot water extraction and the extraction yields are presented in Table 4.7 below. These extracts contain all the waterr-soluble compounds that are part of the plants used as well as the flour. They also include the water-soluble compounds from the other ingredients used in the preparation of biscuits.

Table 4.7: Hot water extraction yield of the functionally enriched biscuits developed in this study

Biscuit samples	Quantity of hot water extracts of biscuits used (g)	Extraction yield (g)
Control	50.0028	3.3657
Sample-1	50.0022	3.3634
Sample-2	50.0018	3.3113
Sample-3	50.0020	3.3880
Sample-4	50.0024	3.3387
Sample-5	50.0029	3.3811
Sample-6	50.0019	3.4123

4.3.2 Total phenolic contents (TPC) and total flavonoid contents (TFC) of enriched biscuits

In this study, phenolic and flavonoid contents of the biscuit extracts were determined as their antioxidant enrichment is expected to be due to the increasing levels of plant materials for different biscuit samples (Chapter 3/ Table 3.2). These results are presented in Table 4.8 and Fig 4.19. In should be noted that the quantities of phenolics and flavonoids presented in Table 4.8 are calculated for 40g of biscuit samples. This is because of the fact that the average weights of biscuit samples prepared in this study are about 20g per biscuit (Table 4.2) and it is assumed that two biscuits are considered as one serving (40g per serving).

The total phenolic contents of the seven biscuit samples prepared in this study were in the range of 12.80 to 28.80 mg per 40g of biscuits. As expected, the highest phenolic content was observed in sample 4 (28.80± 0.52 mg per 40g). Similarly, the total flavonoid contents of the seven biscuit samples were in the range of 23.20 to 61.60 mg per 40g of biscuits. Again, the highest flavonoid content was observed in sample 4 (61.60± 1.20 mg per 40g).

It should be noted here that the major constituents of the biscuit extracts are polysaccharides (carbohydrates) and protein. The plant polyphenols are only minor constituents as the plant material added varied from 1 to 4% of the total flour.

Table 4.8: Total phenolic and flavonoid contents of hot water extracts of biscuit samples along with their radical scavenging activities.

Biscuit treatments	Phenolic content (GAE mg/40g)*	Flavonoid content (QE mg/40g)*	$ABTS^{\bullet+}$ scavenging activity (Ascorbate equivalent µM) #
Control	12.8± 0.44	23.2± 1.04	291.66± 0.10
Sample-1	20.8± 0.76	36.8± 1.08	370.71± 0.009
Sample -2	23.2± 0.60	43.2± 1.2	264.04± 0.004
Sample -3	25.6± 0.48	55.2± 1.08	163.57± 0.001
Sample -4	28.8± 0.52	61.6± 1.20	314.52± 0.012
Sample -5	17.2± 0.36	28.1± 1.24	193.10± 0.002
Sample -6	18.8± 0.48	29.2± 1.68	310.24± 0.004

**Total phenolic and flavonoid contents are expressed as Gallic acid and Quercetin equivalent units respectively. The quantities are calculated for 40 grams of biscuit samples*

#$ABTS^{\bullet+}$ free radical scavenging activities are expressed as ascorbate equivalent (µM) at 5mg/mL of biscuit extracts

&All values are expressed as mean ± SD, n=3 ($p \leq 0.005$)

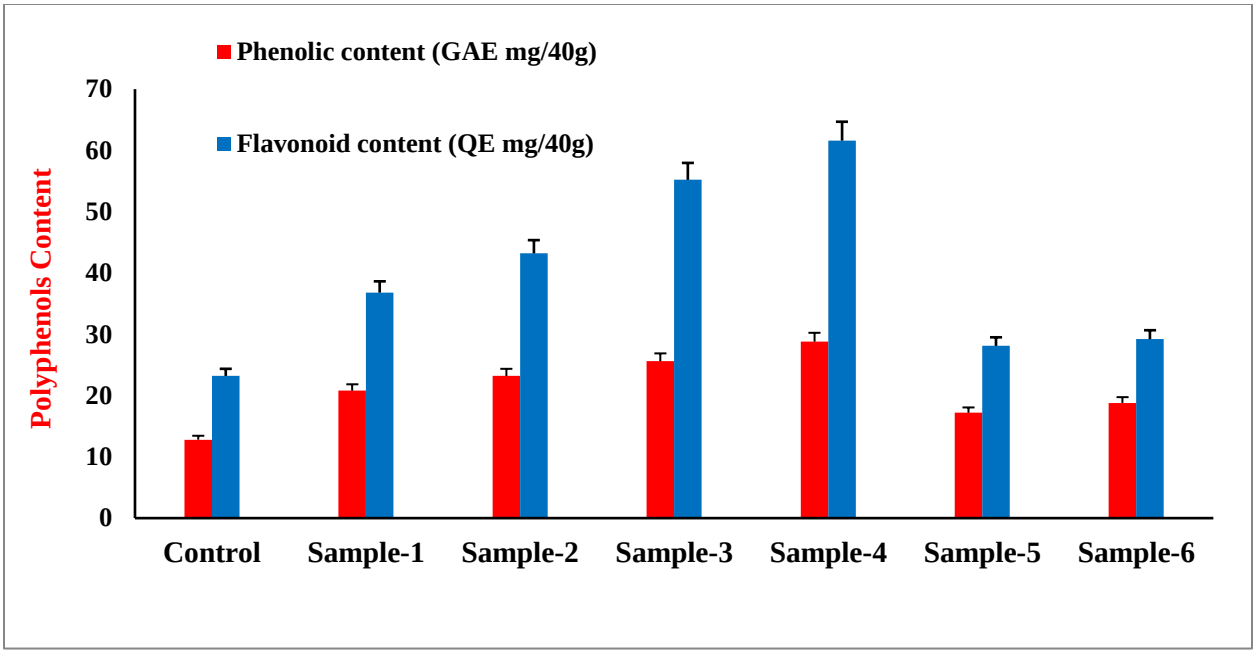


Fig 4.19: Total phenolic and flavonoid contents of hot water extracts of biscuit samples: RED bars represent total phenolic content and BLUE bars represent total flavonoid content per 40 grams of biscuits

4.3.3 Antioxidant activities of biscuit extracts and their correlation with TPC and TFC

As can be seen from the scavenging activities of the biscuit extracts presented in Table 4.8, an increased percentage of plants' material did not significantly improve their activities. In fact, the activities have remained very similar for all the samples (Table 4.8). This may be due to the fact that the major constituents of all the hot water biscuit extracts are polysaccharides (carbohydrates) and protein. The polyphenols are only minor constituents (only 1 to 4% of the plant material was added). It should be noted from the literature that plant polysaccharides and protein display significant antioxidant activities (Zhang et al., 2018). Hence, the contribution of plant polyphenols for water extracts of biscuit samples is small when compared to the contribution from polysaccharides.

It is therefore planned to isolate the plant polyphenols from the hot water extracts of biscuits. This can be achieved by ethanol treatment of hot water extracts (Zhang et al., 2017). Results on the isolation of ethanol soluble small molecular phytonutrients (polyphenols) and their antioxidant activities are presented in the following sub-sections (sections 4.3.4 to 4.3.6).

4.3.4 Isolation of ethanol solubles from hot water extracts of enriched biscuits

As discussed before, the hot water extracts of biscuit samples contain biopolymers (polysaccharides and proteins as major constituents). In order to remove these biopolymers and to isolate the ethanol soluble small organics (including polyphenols, vitamins, small sugars, minerals etc.) from water extracts, the samples were treated with ethanol as described in section 3.7 (Chapter 3; Fig 3.3). The results are presented in Table 4.9. In should be noted that the quantities of polyphenols presented in Table 4.9 are calculated for 40g of biscuit samples (one serve of biscuits).

Table 4.9: Yield of polyphenols in hot water extracts of functionally enriched biscuits

Biscuit samples	Quantity of hot water extracts used for isolation (g)	Yield of polyphenols (g)	Yield of polyphenols in 40g of biscuits*
Control	1.5052	0.5708	1.021
Sample-1	1.5055	0.5789	1.034
Sample-2	1.5053	0.5750	1.011
Sample-3	1.5056	0.5734	1.032
Sample-4	1.5058	0.5778	1.040
Sample-5	1.5058	0.5781	1.038
Sample-6	1.5059	0.5787	1.049

#This is total yield after ethanol isolation and contains small sugars and other ethanol solubles organic molecules in addition to polyphenols.

**Yield of isolated polyphenols in 40 g of biscuit samples is calculated from the total quantity of water extracts (shown in Table 4.7)*

4.3.5 Total phenolic contents (TPC) and total flavonoid contents (TFC) of ethanol solubles in the extracts of enriched biscuits

The total phenolic and flavonoid contents of ethanol soluble water extracts of the biscuit samples are presented in Table 4.10 and Fig 4.20 and these values are expressed per 40g of biscuit samples (per one serving). Results indicate that the total phenolic contents of the seven biscuit samples prepared in this study were in the range of 25.60 to 57.6 mg per 40g of biscuits. As expected, the highest phenolic content was observed in sample 4 (57.60± 1.6 mg per 40g). Similarly, the total flavonoid contents of the seven biscuit samples were in the range of 21.36 to

171.50 mg per 40g of biscuits. Again, the highest flavonoid content was observed in sample 4 (61.60± 1.20 mg per 40g).

Clearly, the sample 4 with the largest percentage of plant material (Chapter 3; Table 3.2) displayed the highest quantities of total phenolics and flavonoid compounds. Results presented in Figure 4.20, clearly demonstrates that the antioxidant contents of seven biscuit samples increased as a function of the quantity of plant material added. It is therefore concluded that the polyphenol contents of the plant material have been retained in the developed biscuits (no loss of these antioxidant compounds occurred during the baking process of the biscuits).

Table 4.10: Total phenolic and flavonoid contents of ethanol solubles isolated from hot water extracts of biscuits along with their radical scavenging activities

Biscuit Treatments	Phenolic content (GAE mg/40g)*	Flavonoid content (QE mg/ 40g)*	$ABTS^{\cdot+}$ scavenging activity (Ascorbate equivalent µM)[#]
Control	25.6± 0.4	21.36± 6.21	116.40± 6.9
Sample-1	31.6± 1.2	80.53± 8.22	143.10± 7.6
Sample-2	36± 2.4	98.72± 11.0	188.90± 2.57
Sample-3	50± 2.8	133.53± 6.25	308.10± 2.83
Sample-4	57.6± 1.6	171.50± 8.28	348.60± 5.49
Sample-5	28.8± 2.0	62.93± 11.26	134.20± 2.88
Sample-6	30± 1.2	68.97± 8.33	141.40± 3.46

**Total phenolic and flavonoid contents are expressed as Gallic acid and Quercetin equivalent units respectively. The quantities are calculated for 40 grams of biscuit samples*

[#]$ABTS^{\bullet+}$ free radical scavenging activities are expressed as ascorbate equivalent (µM) at 1mg/mL of biscuit extracts

[&]All values are expressed as mean ± SD, n=3 ($p < 0.005$)

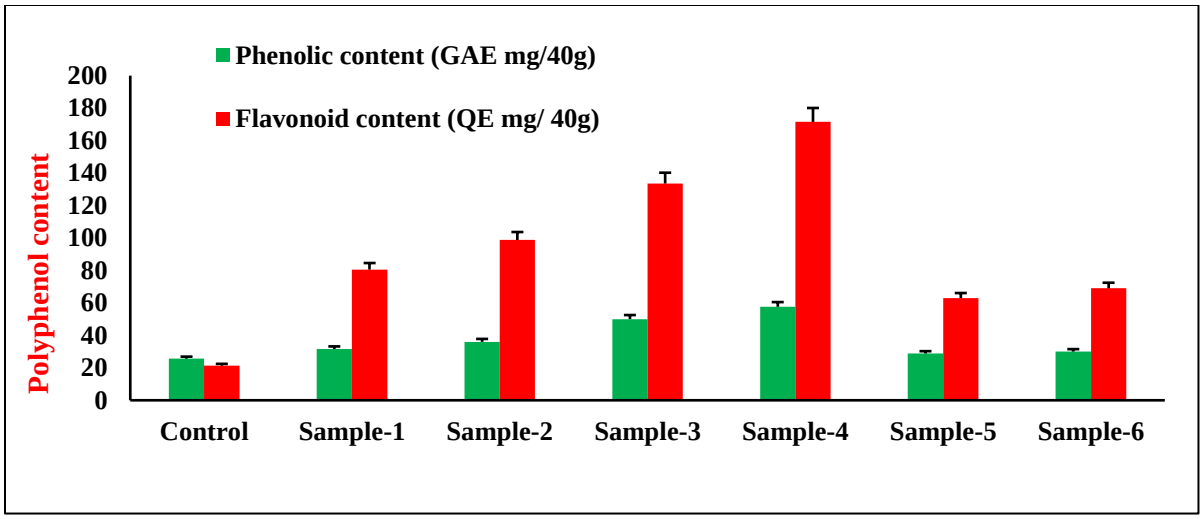


Fig 4.20: Total phenolic and flavonoid contents of ethanol solubles isolated from hot water extracts of biscuit samples: GREEN bars represent total phenolic content and RED bars represent total flavonoid content per 40 grams of biscuits

4.3.6 Antioxidant activities and their correlation with TPC and TFC of enriched biscuits

The $ABTS^{\bullet+}$ radical scavenging activities of functionally enriched biscuits (as measured from the isolated ethanol solubles from each biscuit sample) are presented in Table 4.10 and Fig 4.21 and 4.22. These Figures present the activities at three different concentrations for each biscuit sample. It should be noted that the biscuit samples 1 to 4 (Table 3.2) contain an increasing proportion of plant material and the trend of scavenging activities reflect such an increase. For instance, biscuit sample 4 with the largest proportion of plant material displayed the highest scavenging activity. Also, the correlation plots (Fig4.23 and 4.24) clearly demonstrate the relationship between antioxidant activities of various biscuit samples with their polyphenol contents. The polyphenol contents of the biscuits are in turn depended on the amount of plant material substituted into different biscuit samples.

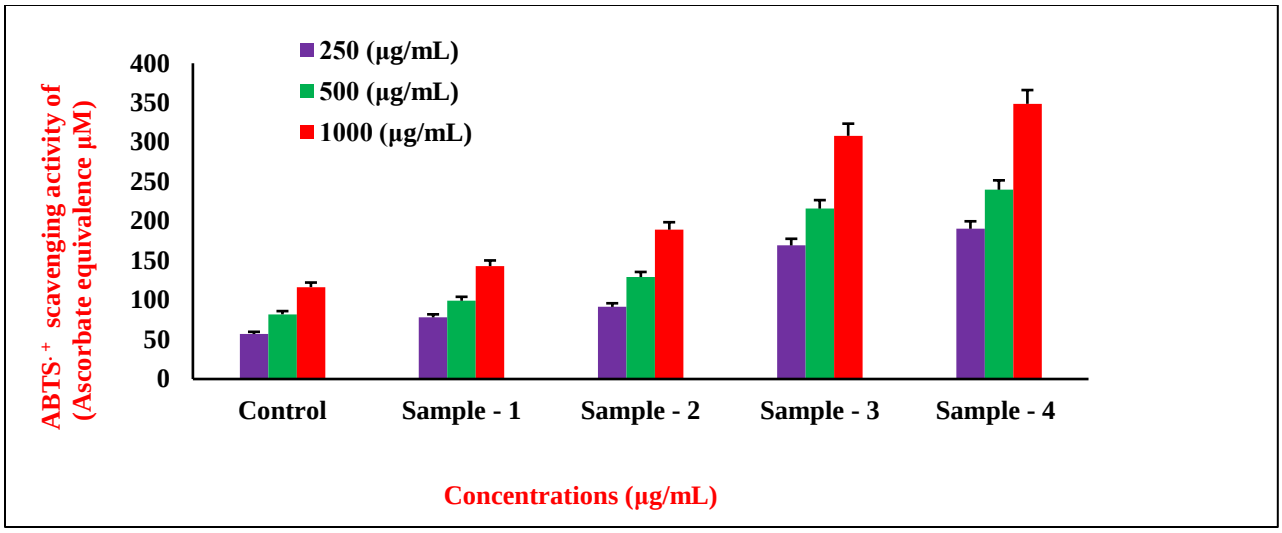


Fig 4.21: ABTS$^{\bullet+}$ scavenging activity of ethanol soluble isolated from hot water extracts of biscuit samples from Control to Sample-4 (with different proportions of plants material). Activities are expressed as ascorbic acid equivalent (µM) at three different concentrations: PURPLE bars represent at 250µg/mL, GREEN bars represent at 500 µg/mL and RED bars represent at 1000 µg/mL

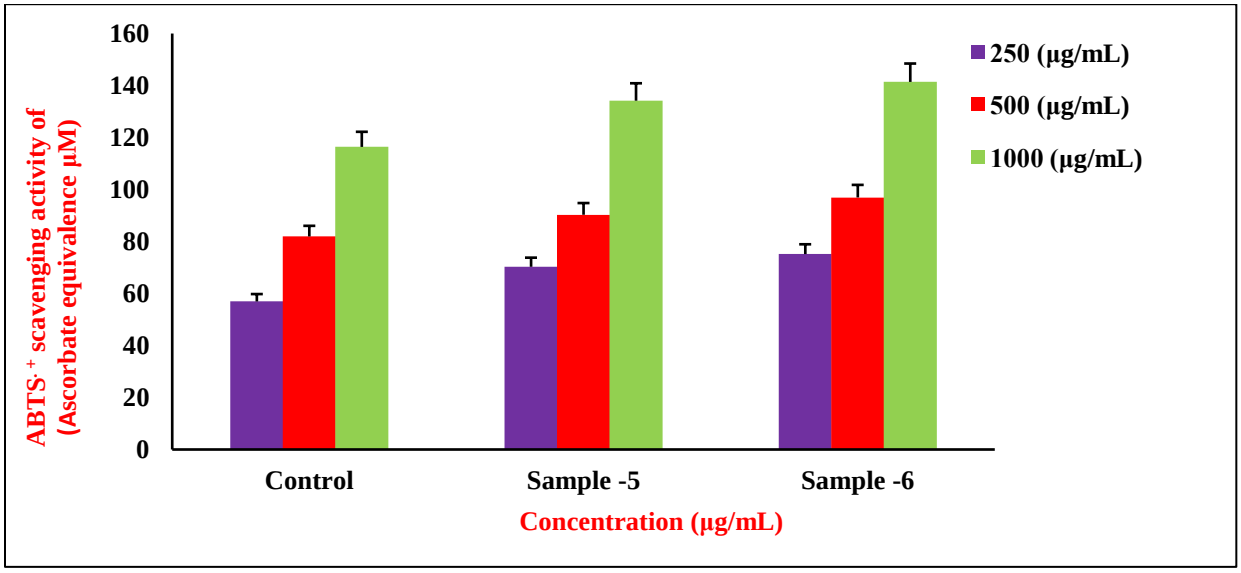


Fig 4.22: ABTS$^{\bullet+}$ scavenging activity of ethanol soluble isolated from hot water extracts of biscuit samples: Control, Sample-5 (with two plants leaves), Sample-6 (with two berries). Activities are expressed as ascorbic acid equivalent (µM) at three different concentrations: PURPLE bars represent at 250µg/mL, GREEN bars represent at 500 µg/mL and RED bars represent at 1000 µg/mL

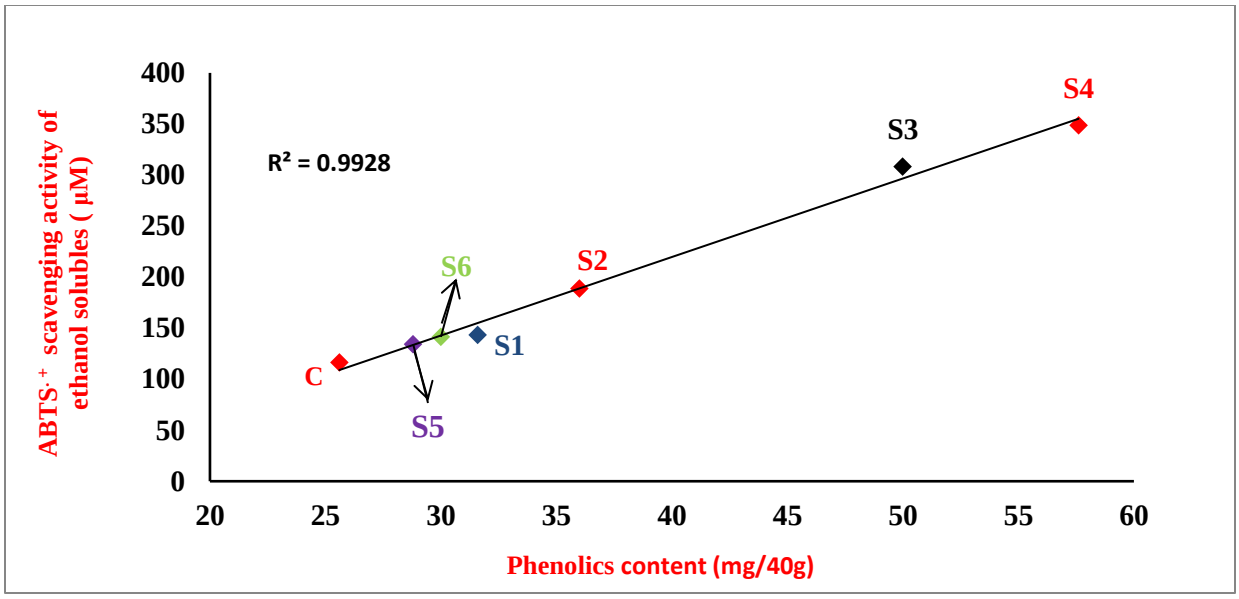


Figure 4.23: Correlation between ABTS$^{\bullet+}$ radical scavenging activity with total phenolic content of antioxidant rich biscuit samples with varying proportions of plant material: C represents control; S1 to S6 represent samples with different proportions of plants material

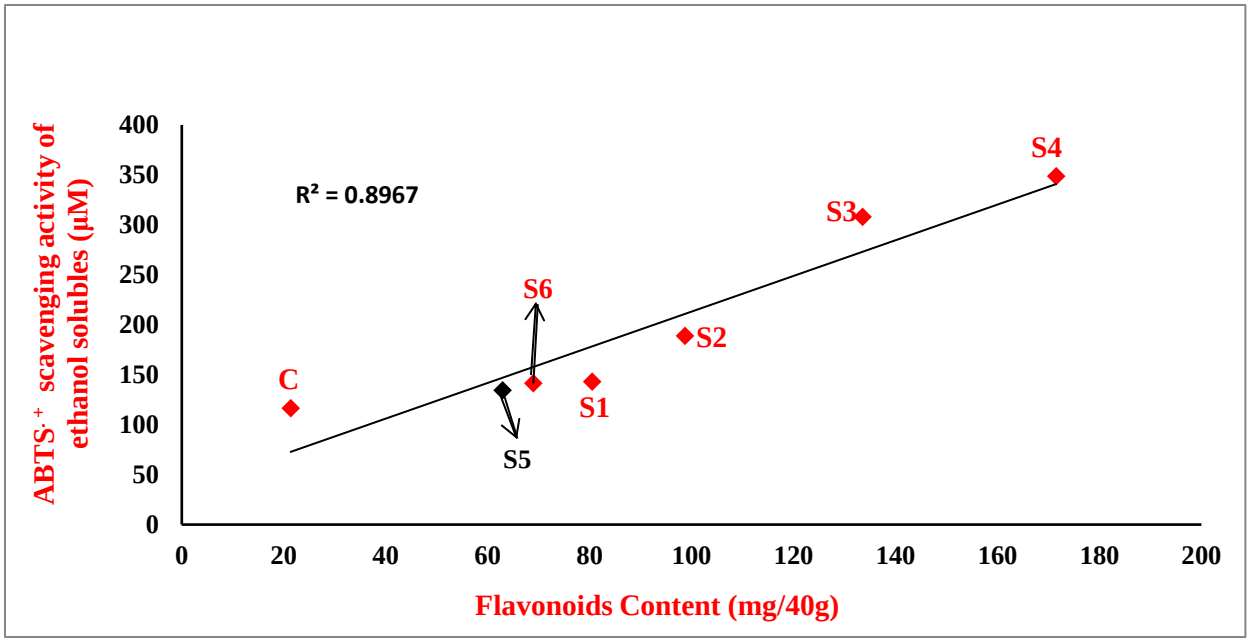


Figure 4.24: Correlation between ABTS$^{\bullet+}$ radical scavenging activity with total flavonoids content of antioxidant rich biscuit samples with varying proportions of plant material: C represents control; S1 to S6 represent samples with different proportions of plants material

As discussed in section 4.1, all the biscuit samples prepared in this project have high nutritional value. It is therefore concluded that the biscuits with 4% plant material (sample 4) is the best substitution with the highest functional benefit and expected to provide an overall health benefit to the consumers of all age groups.

Overall, it may be concluded based on the health benefits of the plants used in this project (Table 4.4) that the antioxidant rich biscuits are expected to provide tremendous health benefits including alleviation of oxidative stress and associated diseases; prevention of high blood sugar, cardiovascular diseases, cancer; have the capacity to heal wounds, enhance memory, and exhibit neuroprotective benefits.

CHAPTER 5
CONCLUSION &FUTURE RECOMMENDATIONS

Chapter 5

Conclusion & Future Recommendations

5.1 Conclusion and recommendations for future research

Interest in naturally occurring under-utilized plants rich in bioactive compounds has considerably increased and natural food products have regained prominence in recent years. It has become necessary to search new sources of natural antioxidants and proteins to develop novel foods which are low cost, safe, and affordable by all population. Thus, under-utilized plants incorporated products have great future potential for the discovery of new and highly nutritious food products with overall health benefits.

The main objective of this study was to develop antioxidant and protein rich food products using under-utilized plants parts. In order to fulfill the main objectives, different under-utilized plants such as Moringa leaves, Gotu kola leaves, Schisandra berry, and Goji berry have been selected. Parts of these plants with high nutritional and antioxidant activities were incorporated into flour to make different biscuit samples at different substitution levels of plants and were evaluated for their physicochemical, nutritional, and antioxidant properties. Quantification of proximate nutrients (Moisture, ash, protein, fat, fibre, carbohydrates, and energy), antioxidant and bioactive compounds (phenolics and flavonoids) have been carried out to determine the nutritional and bioactive properties of plants parts incorporated biscuit samples. Different under-utilized plants substituted biscuit samples displayed a range of nutritional and biological activities especially, antioxidant activities.

The results of this study revealed that under-utilized plants are good sources of protein, fibre, carbohydrate, and energy. The physical properties (weight, thickness, diameter, color, spread ratio, and bake loss) of biscuit samples were affected by the incorporation of plant parts in a positive way. The color characteristics of the biscuit samples were significantly influenced by the addition of plants materials.

In order to evaluate the antioxidant activities of the biscuit samples, their radical scavenging activities, as well as their polyphenol contents, were determined. These results clearly suggest that the biscuit samples prepared with increased levels of plant material display increased antioxidant activities. It is also concluded from the present research that four traditionally known edible plant parts have been successfully used to improve the antioxidant potentials of the biscuits. Owing to the fact that the plant parts used in this study have displayed extremely high antioxidant activities and these effects have been incorporated into the biscuits. It is therefore expected that the antioxidant rich biscuits developed in this project are good sources to provide overall health benefits to the consumers. The enhanced nutritional and antioxidant properties of biscuits observed in this research are desirable characteristics in the modern food industry. The developed biscuit products are expected to be a good source of the required nutrition and functional bioactive compounds which benefits overall human health including addressing obesity, diabetes, cancer, and other age-related diseases.

Baked products have become very popular among all age groups. Among baked products, biscuits are one of the most popular snacks and can be substituted with functional and bioactive nutrients. These can be used as a very easy vehicle for providing a sufficient amount of nutrients needed by the population. Some of the under-utilized plants are a good source of bioactive nutrients and can be used to fortify or develop new food products. Further studies on the development of under-utilized plant based products are recommended for overall sensory acceptability by consumers.

Future research possibilities include the use of the four plants studied in this project to other bakery products such as cookies and also the use of other well-known herbal plants into bakery products. Further research employing modern food technology methods combined with contemporary scientific tools is expected to lead to the discoveries of novel functional foods with major health benefits.

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APPENDIX

Appendix A1:

Tables with statistical analysis (One-Way ANOVA) of biscuits samples prepared in this research are provided in this Appendix (Table A1.1, Table A1.2, and Table A1.3).

Appendix A2:

Concentration dependant $ABTS^{\bullet+}$ radical scavenging activity of hot water extracts of plants used in this study are provided in this Appendix (Table A2.1, Fig A2.1, Fig A2.2, and Fig A2.3).

Appendix A3:

Concentration dependant $ABTS^{\bullet+}$ radical scavenging activity of ethanol solubles from hot water extracts of biscuits developed in this study are provided in this Appendix (Table A3.1,Table A3.2, Fig A3.1, Fig A3.2, Fig A3.3, Fig A3.4, Fig A3.5, Fig A3.6, and Fig A3.7).

A1.1: Proximate analysis of biscuit samples (One-Way ANOVA)

Descriptive										ANOVA		
		Mean	Std. Deviation	N	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	F	P Value	Result
						Lower Bound	Upper Bound					
Moisture Content	Control	4.797	0.057	3	0.033	4.656	4.937	4.73	4.84	6.370	0.002	Significant
	Sample 1	4.638	0.057	3	0.033	4.497	4.778	4.60	4.70			
	Sample 2	4.594	0.116	3	0.067	4.305	4.882	4.50	4.72			
	Sample 3	4.771	0.062	3	0.036	4.616	4.925	4.70	4.81			
	Sample 4	4.890	0.111	3	0.064	4.614	5.166	4.76	4.97			
	Sample 5	4.563	0.079	3	0.045	4.368	4.759	4.51	4.65			
	Sample 6	4.621	0.085	3	0.049	4.409	4.833	4.53	4.70			
Ash content	Control	2.129	0.036	3	0.021	2.038	2.219	2.10	2.17	95.259	<0.001	Significant
	Sample 1	2.301	0.056	3	0.032	2.162	2.441	2.24	2.35			
	Sample 2	2.453	0.032	3	0.019	2.373	2.533	2.43	2.49			
	Sample 3	2.570	0.013	3	0.008	2.537	2.603	2.56	2.58			
	Sample 4	2.713	0.037	3	0.021	2.620	2.805	2.67	2.75			
	Sample 5	2.254	0.037	3	0.021	2.162	2.345	2.21	2.29			
	Sample 6	2.276	0.026	3	0.015	2.211	2.341	2.26	2.31			
Protein Content	Control	13.411	0.025	3	0.014	13.349	13.473	13.39	13.44	113.769	<0.001	Significant
	Sample 1	14.838	0.057	3	0.033	14.697	14.978	14.80	14.90			
	Sample 2	14.860	0.121	3	0.070	14.559	15.161	14.72	14.96			
	Sample 3	14.971	0.140	3	0.081	14.623	15.320	14.85	15.12			
	Sample 4	15.015	0.084	3	0.048	14.808	15.223	14.94	15.10			
	Sample 5	14.763	0.079	3	0.045	14.568	14.959	14.71	14.85			
	Sample 6	14.821	0.082	3	0.048	14.617	15.026	14.74	14.90			
Fat Content	Control	15.186	0.106	3	0.061	14.923	15.449	15.08	15.30	0.618	> 0.006	Not Significant
	Sample 1	15.582	0.455	3	0.263	14.450	16.713	15.07	15.94			
	Sample	15.377	0.061	3	0.035	15.226	15.528	15.31	15.43			

	2											
	Sample 3	15.312	0.280	3	0.162	14.617	16.007	14.99	15.51			
	Sample 4	15.139	0.096	3	0.056	14.900	15.378	15.05	15.24			
	Sample 5	15.254	0.501	3	0.289	14.009	16.500	14.89	15.83			
	Sample 6	15.326	0.392	3	0.226	14.353	16.299	14.89	15.66			
Fibre content	Control	2.507	0.012	3	0.007	2.478	2.535	2.50	2.52	8505.063	<0.001	Significant
	Sample 1	3.150	0.030	3	0.017	3.075	3.225	3.12	3.17			
	Sample 2	3.736	0.004	3	0.003	3.725	3.747	3.73	3.74			
	Sample 3	4.232	0.028	3	0.016	4.162	4.302	4.20	4.25			
	Sample 4	4.956	0.013	3	0.007	4.925	4.987	4.94	4.97			
	Sample 5	2.788	0.004	3	0.002	2.778	2.798	2.78	2.79			
	Sample 6	2.695	0.003	3	0.002	2.687	2.704	2.69	2.70			
Carbohydrates	Control	66.767	0.120	3	0.069	66.470	67.064	66.63	66.86	55.644	<0.001	Significant
	Sample 1	64.129	0.433	3	0.250	63.053	65.205	63.80	64.62			
	Sample 2	63.573	0.085	3	0.049	63.362	63.784	63.51	63.67			
	Sample 3	62.915	0.404	3	0.233	61.910	63.919	62.63	63.38			
	Sample 4	62.176	0.226	3	0.130	61.616	62.737	61.95	62.40			
	Sample 5	64.941	0.544	3	0.314	63.590	66.291	64.32	65.32			
	Sample 6	64.882	0.382	3	0.220	63.934	65.830	64.61	65.32			
Energy	Control	457.390	0.603	3	0.348	455.893	458.888	456.74	457.93	25.829	<0.001	Significant
	Sample 1	456.104	2.143	3	1.237	450.780	461.429	453.70	457.81			
	Sample 2	452.129	0.407	3	0.235	451.117	453.140	451.66	452.41			
	Sample 3	449.351	1.331	3	0.768	446.045	452.657	447.82	450.25			
	Sample 4	445.021	0.304	3	0.175	444.266	445.777	444.77	445.36			
	Sample 5	456.104	2.650	3	1.530	449.520	462.689	454.19	459.13			
	Sample 6	456.745	1.892	3	1.092	452.045	461.446	454.64	458.30			

A1.2: Physical analysis of biscuit samples (One-Way ANOVA)

Descriptive	ANOVA

		Mean	Std. Deviation	N	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	F	P Value	Result
						Lower Bound	Upper Bound					
Weight (g)	Control	16.28	.25	6	.10	16.02	16.54	16.0	16.5	127.836	<0.001	Significant
	Sample 1	17.19	.19	6	.08	16.99	17.39	17.0	17.5			
	Sample 2	19.70	.54	6	.22	19.13	20.27	19.2	20.6			
	Sample 3	20.45	.30	6	.12	20.13	20.77	20.0	20.9			
	Sample 4	20.81	.51	6	.21	20.28	21.35	20.0	21.5			
	Sample 5	17.53	.30	6	.12	17.22	17.84	17.2	17.9			
	Sample 6	18.22	.40	6	.16	17.80	18.65	17.9	18.9			
Diameter	Control	51.33	.50	6	.21	50.60	51.66	50.5	51.8	4.326	0.002	Significant
	Sample 1	51.39	.35	6	.14	50.96	51.69	50.9	51.9			
	Sample 2	51.48	.30	6	.12	51.16	51.80	51.0	51.8			
	Sample 3	51.78	.43	6	.18	51.33	52.24	51.2	52.4			
	Sample 4	52.23	.47	6	.19	51.74	52.72	51.4	52.7			
	Sample 5	51.35	.30	6	.12	51.44	52.07	51.4	52.2			
	Sample 6	51.46	.57	6	.23	51.20	52.39	51.3	52.8			
Thickness	Control	6.54	.26	6	.11	6.26	6.81	6.3	6.9	3.904	0.004	Significant
	Sample 1	6.50	.25	6	.10	6.33	6.86	6.4	7.1			
	Sample 2	6.37	.23	6	.09	6.12	6.61	6.1	6.7			
	Sample 3	6.26	.27	6	.11	5.97	6.54	5.9	6.7			
	Sample 4	6.25	.31	6	.13	6.02	6.68	5.9	6.7			
	Sample 5	6.10	.24	6	.10	5.85	6.35	5.7	6.4			
	Sample 6	6.05	.19	6	.08	5.85	6.25	5.9	6.4			
Bake loss	Control	21.00	.87	6	.35	20.09	21.91	20.1	22.0	54.948	<0.001	Significant
	Sample 1	19.82	.69	6	.28	19.10	20.55	19.1	20.9			
	Sample 2	18.45	.68	6	.28	17.74	19.16	17.6	19.4			
	Sample 3	16.74	.62	6	.25	16.09	17.40	16.1	17.5			
	Sample 4	15.19	.45	6	.18	14.72	15.67	14.4	15.6			
	Sample 5	18.88	.42	6	.17	18.43	19.32	18.3	19.3			

A1.3: Color analysis of biscuit samples (One-Way ANOVA)

Descriptive										ANOVA		
		Mean	Std. Deviation	N	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	F	P Value	Result
						Lower Bound	Upper Bound					
Color Value L*	Control	63.145	0.563	6	.230	62.554	63.736	62.50	63.99	10.726	<0.001	Significant
	Sample 1	61.755	2.344	6	.957	59.296	64.214	59.00	65.60			
	Sample 2	62.390	1.352	6	.552	60.971	63.809	59.84	63.91			
	Sample 3	60.823	0.728	6	.297	60.059	61.588	60.04	61.90			
	Sample 4	63.358	0.994	6	.406	62.315	64.401	62.07	65.01			
	Sample 5	59.543	0.441	6	.180	59.081	60.006	59.02	60.14			
	Sample 6	59.563	0.688	6	.281	58.841	60.286	58.65	60.53			
Color Value a*	Control	6.565	0.348	6	.142	6.199	6.931	6.03	6.91	19.878	<0.001	Significant
	Sample 1	6.495	0.310	6	.126	6.170	6.820	6.17	6.93			
	Sample 2	7.105	0.829	6	.339	6.235	7.975	5.49	7.77			
	Sample 3	7.583	0.443	6	.181	7.118	8.048	7.09	8.35			
	Sample 4	7.772	0.437	6	.178	7.313	8.230	7.29	8.42			
	Sample 5	8.318	0.168	6	.069	8.142	8.495	8.11	8.53			
	Sample 6	8.558	0.236	6	.097	8.310	8.806	8.18	8.79			
Color Value b*	Control	25.903	1.666	6	.680	24.155	27.651	22.64	27.27	37.868	<0.0010	Significant
	Sample 1	26.347	0.589	6	.241	25.728	26.965	25.43	27.04			
	Sample 2	27.467	0.994	6	.406	26.423	28.510	25.71	28.70			
	Sample 3	28.792	0.568	6	.232	28.196	29.388	28.15	29.62			
	Sample 4	33.322	1.346	6	.549	31.909	34.734	30.76	34.33			
	Sample 5	30.125	0.824	6	.336	29.260	30.990	29.32	31.63			
	Sample 6	29.960	0.648	6	.264	29.280	30.640	29.17	30.93			

A2.1: $ABTS^{\bullet+}$ free radical scavenging activity of hot water extracts of plants used in this study in terms of ascorbate equivalence.

No.	Concentrations (mg/mL)	Scavenging activities of Moringa (Ascorbate equivalence µM)*	Scavenging activities of Gotu Kola (Ascorbate equivalence µM)*	Scavenging activities of Schisandra Berry (Ascorbate equivalence µM)*	Scavenging activities of Goji Berry (Ascorbate equivalence µM)
1	1000	822.62± 1.65	844.05± 2.97	860.24± 1.65	846.90± 1.65
2	500	705.00± 2.47	724.05± 2.18	749.76± 1.65	747.38± 1.65
3	250	544.04± 2.97	598.80± 2.97	639.28± 2.86	608.80± 2.97
4	125	453.57± 2.86	480.24± 2.97	531.19± 2.97	491.19± 1.65
5	62.5	322.14± 2.47	354.05± 2.18	375.47± 2.97	364.51± 2.47
6	31.25	167.86± 2.37	183.57± 2.86	206.90± 2.18	197.38± 2.97
7	15.63	47.85± 2.47	55.95± 1.65	93.09± 3.3	68.33± 2.97
8	7.81	31.19± 2.18	28.81± 2.18	70.38± 3.6	32.14± 2.86

**$ABTS^{\bullet+}$ free radical scavenging activities are expressed as ascorbic acid equivalent (µM) at 1mg/mL of plant extracts*

&All values are expressed as mean ± SD, n=3 ($p < 0.005$)

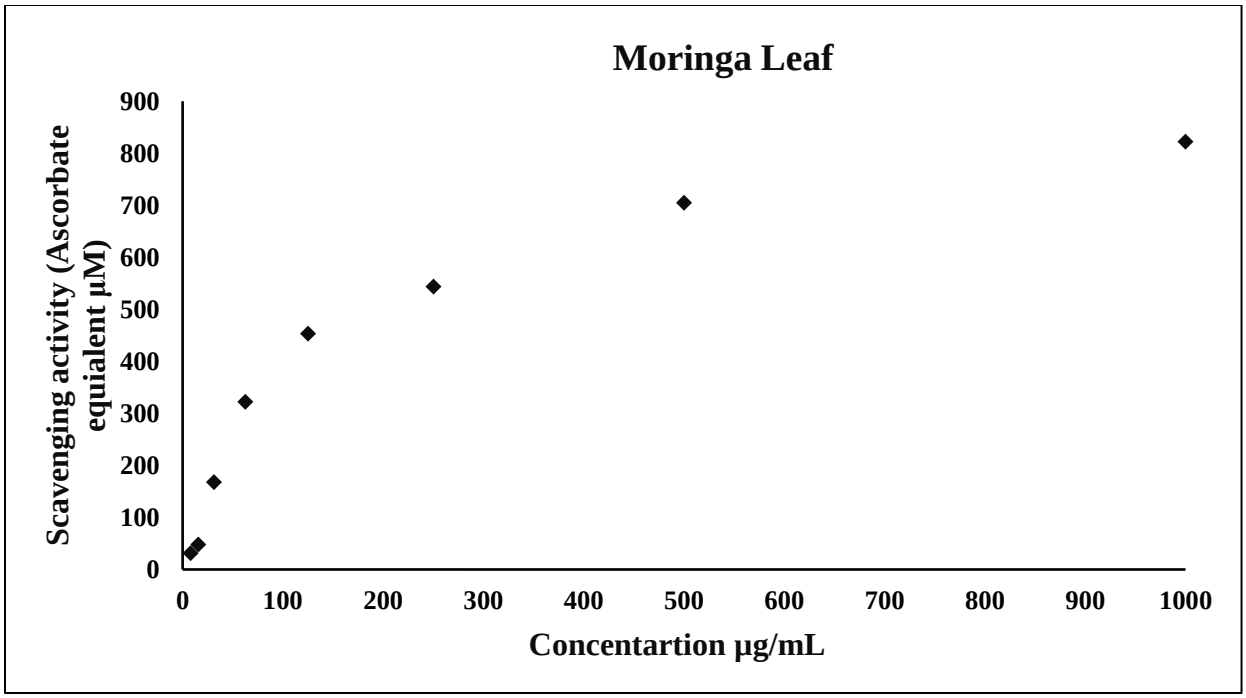


Fig A2.1: ABTS$^{\bullet+}$ free radical scavenging activity of hot water extracts of Moringa leaf used in this study in terms of ascorbate equivalence

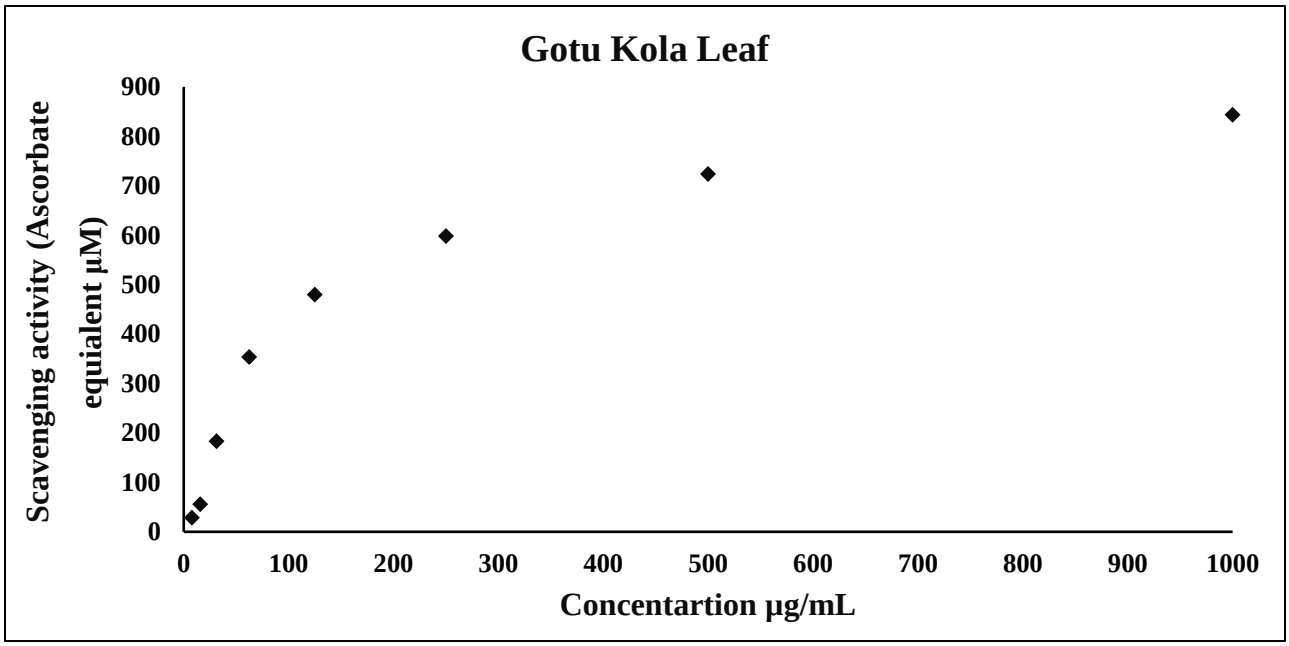
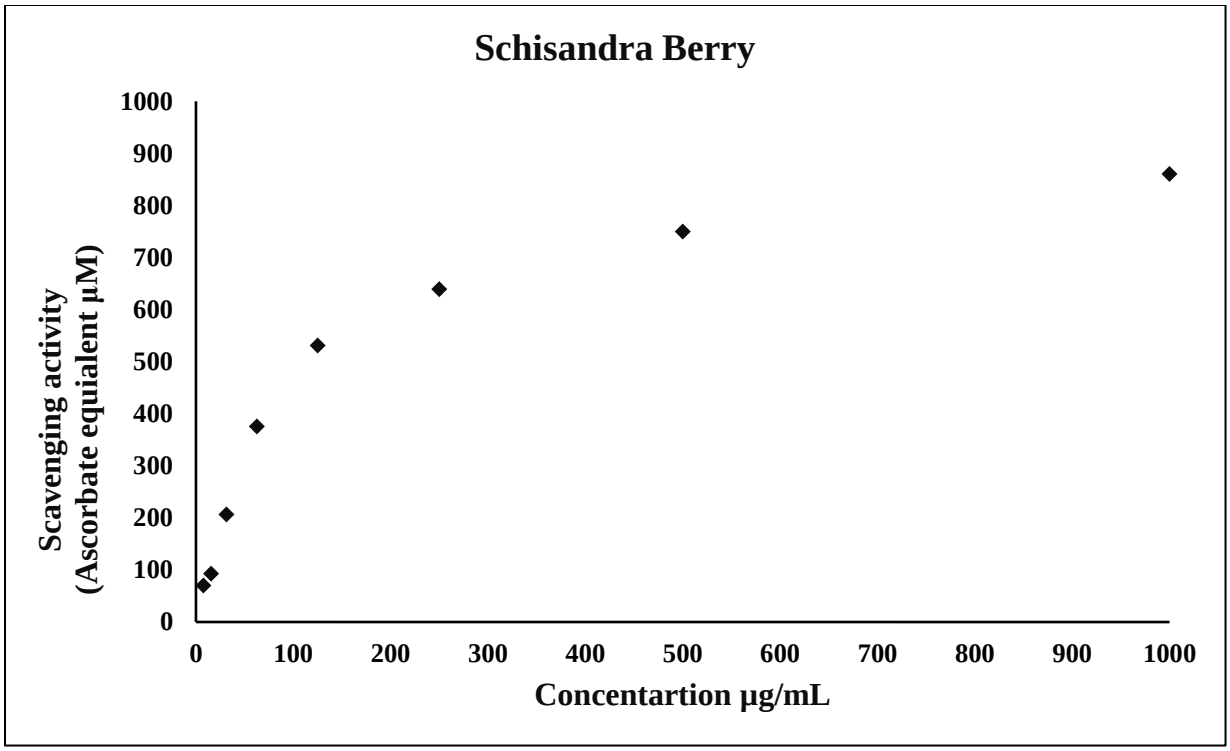
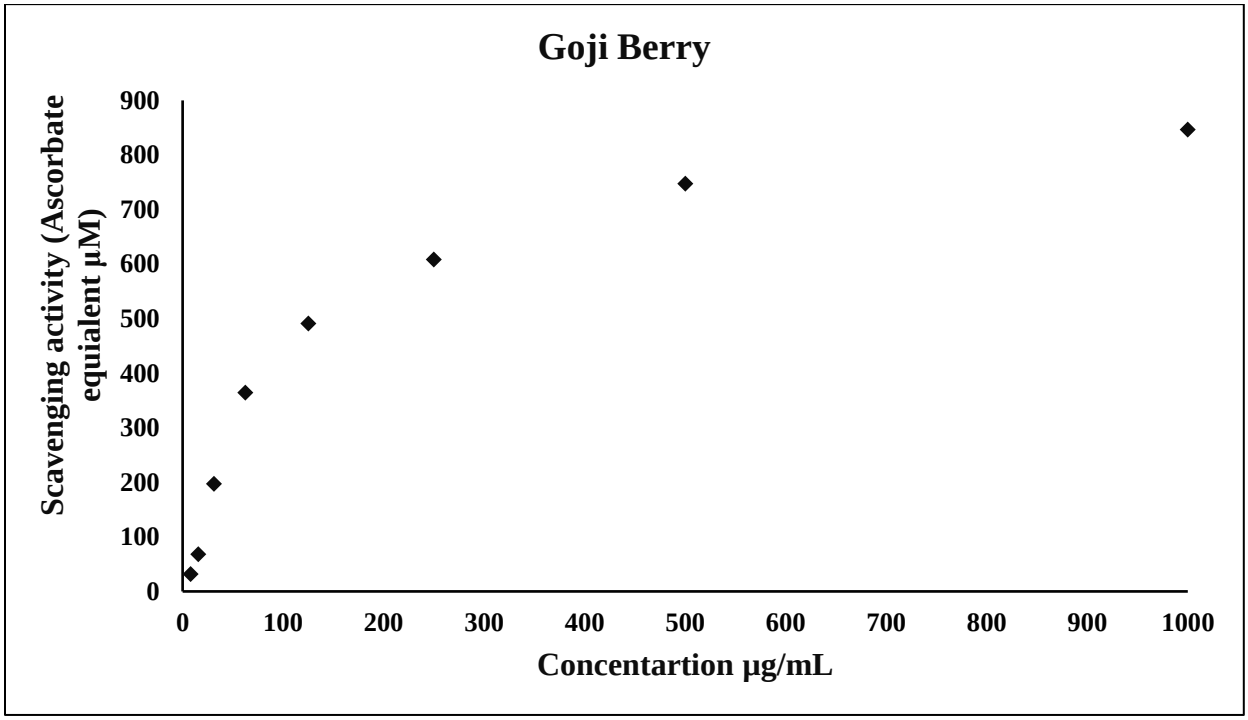


Fig2.2: ABTS$^{\bullet+}$ free radical scavenging activity of hot water extracts of Gotu kola leaf used in this study in terms of ascorbate equivalence



FigA2.3: ABTS$^{\bullet+}$ free radical scavenging activity of hot water extracts of Schisandra berry used in this study in terms of ascorbate equivalence



FigA2.4: ABTS$^{\bullet+}$ free radical scavenging activity of hot water extracts of Goji berry used in this study in terms of ascorbate equivalence.

A3.1: $ABTS^{\bullet+}$ free radical scavenging activity of ethanol solubles isolated from hot water extracts of biscuits ($p < 0.005$)

Concentrations (mg/mL)	Scavenging activities of Control (Ascorbate equivalence µM	Scavenging activities of Sample-1 (Ascorbate equivalence µM	Scavenging activities of Sample-2 (Ascorbate equivalence µM	Scavenging activities of Sample-3 (Ascorbate equivalence µM	Scavenging activities of Sample-4 (Ascorbate equivalence µM
1000	116.39± 6.93	143.06± 7.69	188.89± 2.57	308.06± 2.83	348.61± 5.49
500	81.94± 4.19	99.16± 3.33	128.89± 3.74	215.83± 0.000	239.72± 3.42
250	56.94± 0.96	78.05± 3.3	91.11± 1.96	169.16± 3.60	190.27± 3.42
125	40.83± 6.00	61.38± 0.96	75.55± 2.74	139.72± 2.07	158.05± 1.57
62.5	34.16± 5.00	53.05± 2.54	62.77± 2.18	125.27± 0.78	140.27± 5.6
31.25	33.05± 1.92	45.27± 0.96	59.44± 5.10	121.94± 0.78	137.5± 2.72
15.63	31.38± 3.46	45.27± 1.92	51.11± 2.38	115.83± 2.35	136.38± 0.78
Blank	8.05± 1.27	6.11± 1.27	6.94± 2.54	7.50± 1.66	6.94± 0.96

A3.2: $ABTS^{\bullet+}$ free radical scavenging activity of ethanol solubles isolated from hot water extracts of biscuits

Concentrations (mg/mL)	Scavenging activities of Control (Ascorbate equivalence µM)	Scavenging activities of Sample-5 (Ascorbate equivalence µM)	Scavenging activities of Sample-6 (Ascorbate equivalence µM)
1000	116.39± 6.93	134.17± 2.88	141.39± 3.33
500	81.94± 4.19	90.27± 7.87	96.94± 3.34
250	56.94± 0.96	70.27± 2.54	75.27± 4.19
125	40.83± 6.00	47.5± 3.33	54.72± 3.84
62.5	34.16± 5.00	30.83± 4.40	37.5± 3.30
31.25	33.05± 1.92	24.16± 1.66	25.27± 0.96
15.63	31.38± 3.46	18.0± 2.54	22.5± 1.66
Blank	8.05± 1.27	7.50± 1.66	6.94± 0.96

*$ABTS^{\bullet+}$ free radical scavenging activities are expressed as ascorbic acid equivalent (µM) at 1mg/mL of ethanol solubles isolated from hot water biscuit extracts

&All values are expressed as mean ± SD, n=3 ($p< 0.005$)

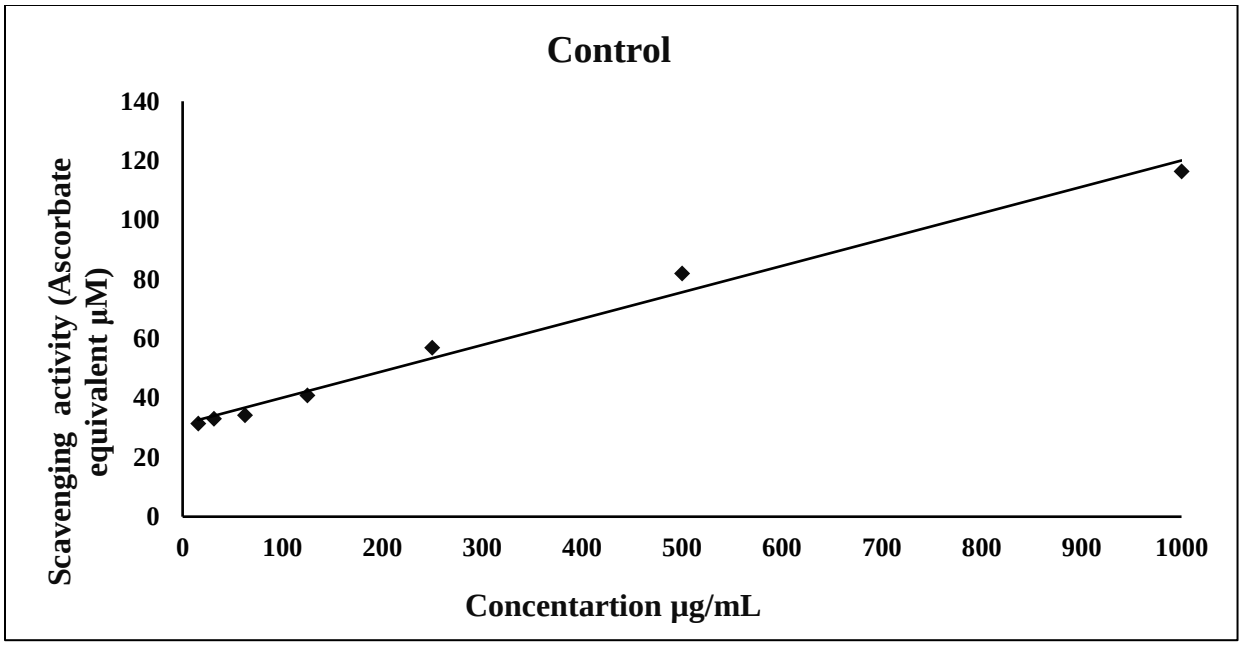


Fig A3.1: ABTS$^{\bullet+}$ free radical scavenging activity of ethanol solubles from hot water extract of control used in this study in terms of ascorbate equivalence

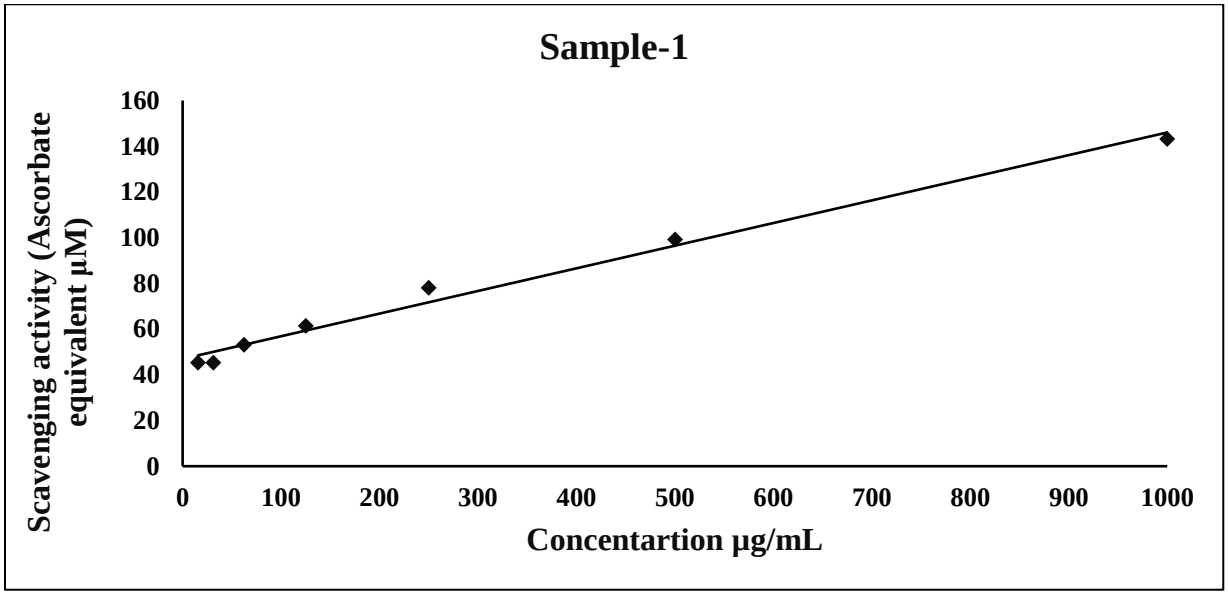


Fig A3.2: ABTS$^{\bullet+}$ free radical scavenging activity of ethanol solubles from hot water extract of sample -1 used in this study in terms of ascorbate equivalence

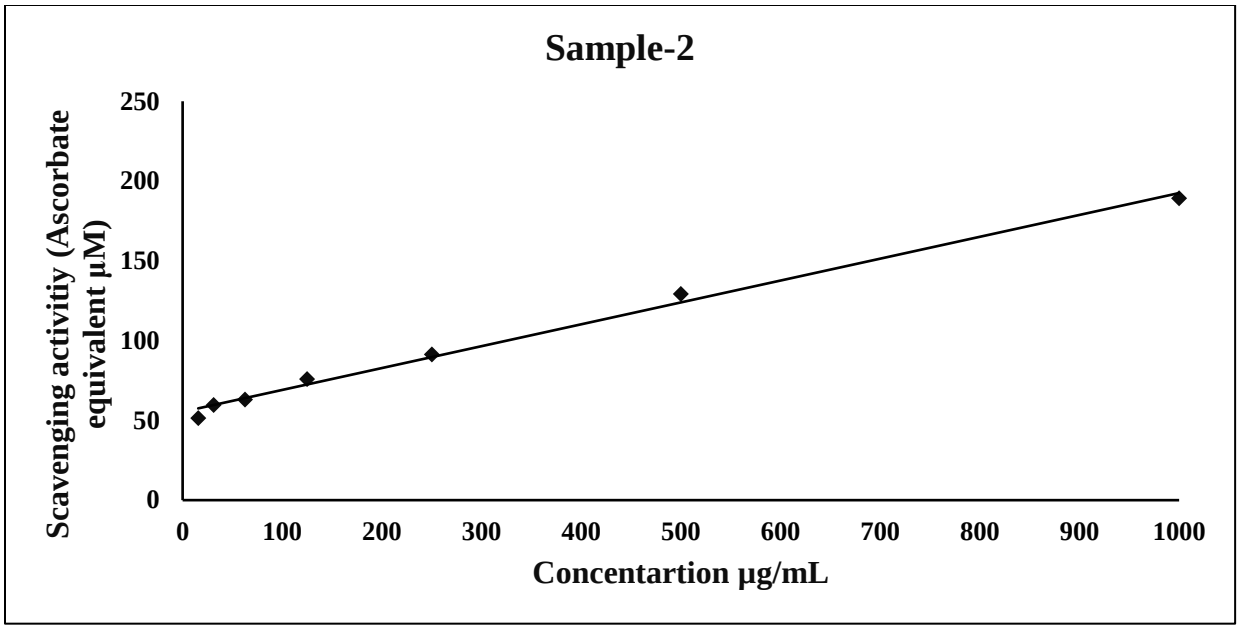


Fig A3.3: ABTS$^{\bullet+}$ free radical scavenging activity of ethanol solubles from hot water extract of sample - 2 used in this study in terms of ascorbate equivalence

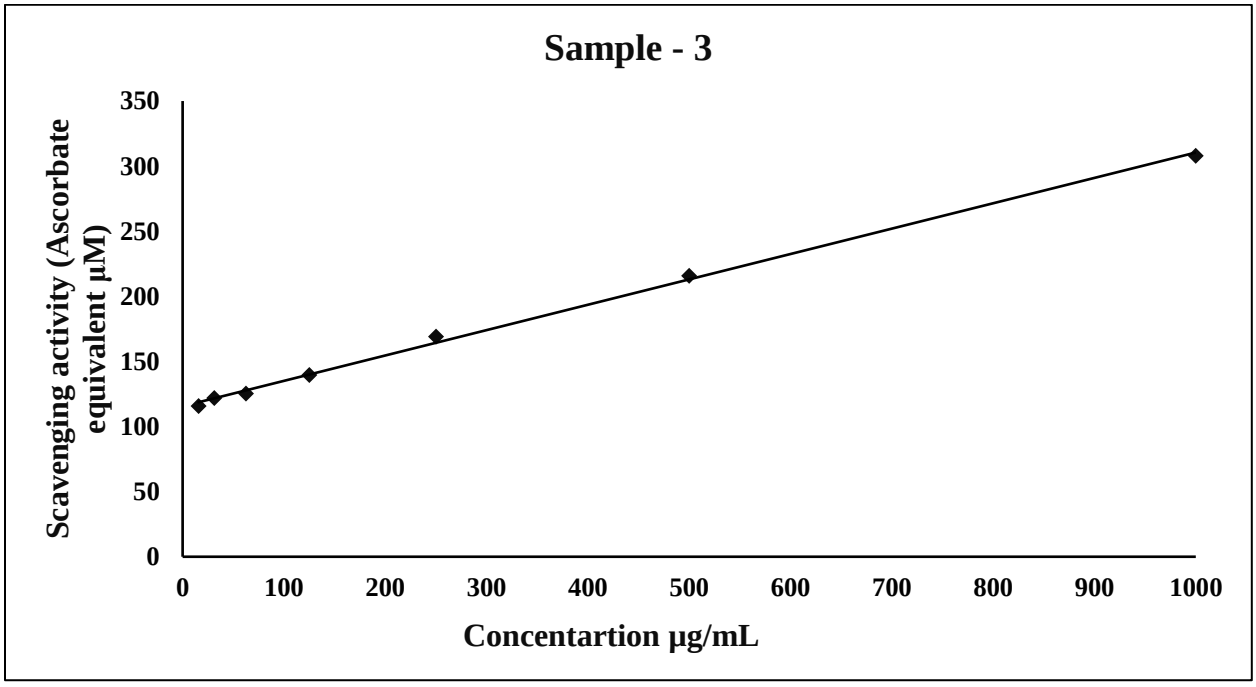


Fig A3.4: ABTS$^{\bullet+}$ free radical scavenging activity of ethanol solubles from hot water extract of sample - 3 used in this study in terms of ascorbate equivalence

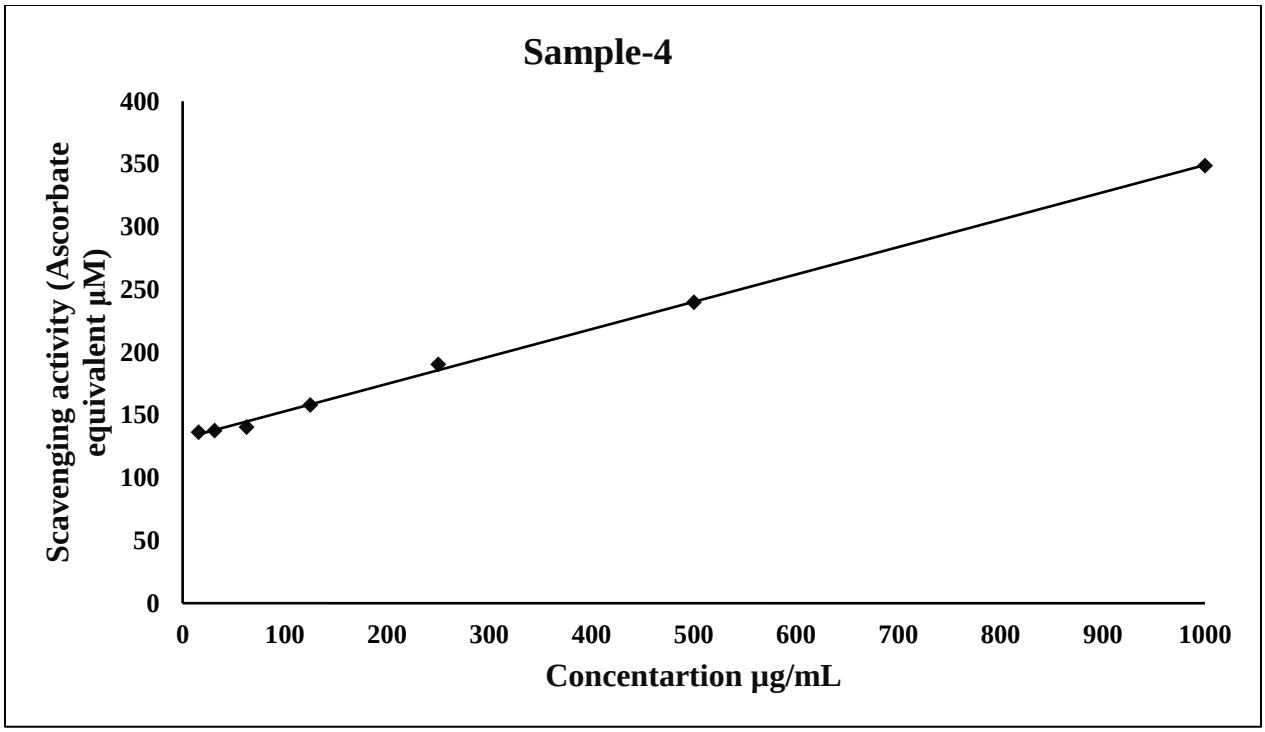


Fig A3.5: $ABTS^{\bullet+}$ free radical scavenging activity of ethanol solubles from hot water extract of sample – 4 used in this study in terms of ascorbate equivalence.

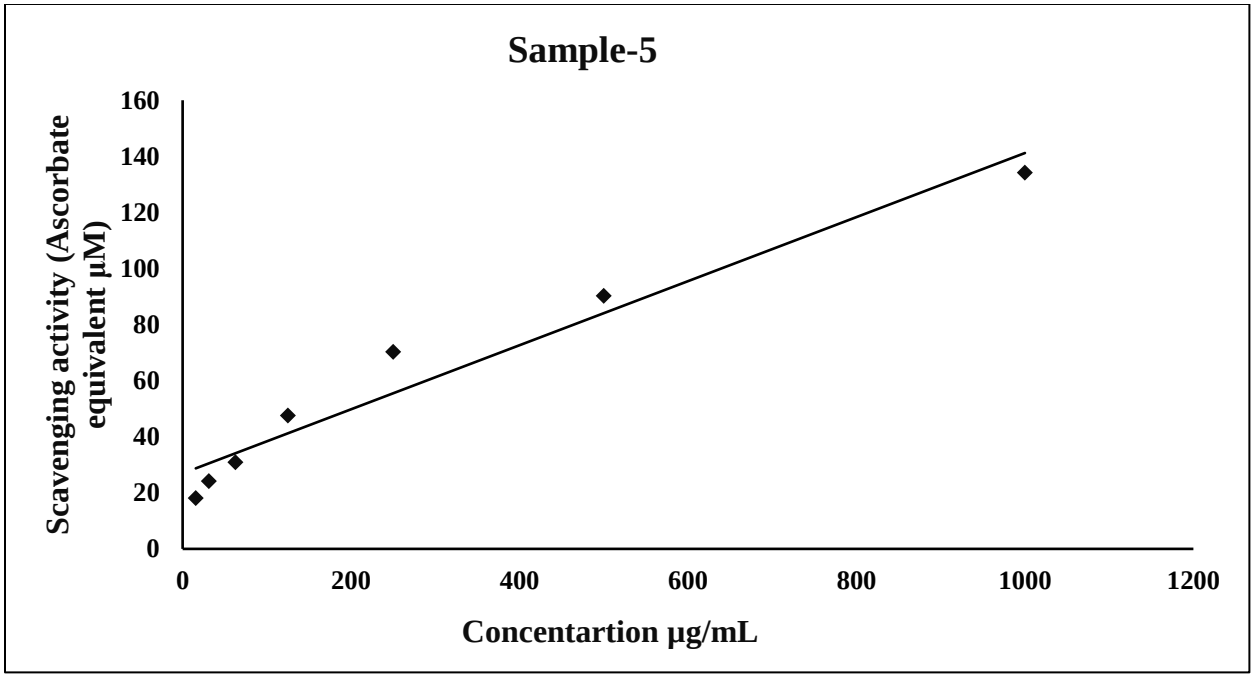


Fig A3.6: $ABTS^{\bullet+}$ free radical scavenging activity of ethanol solubles from hot water extract of sample – 5 used in this study in terms of ascorbate equivalence.

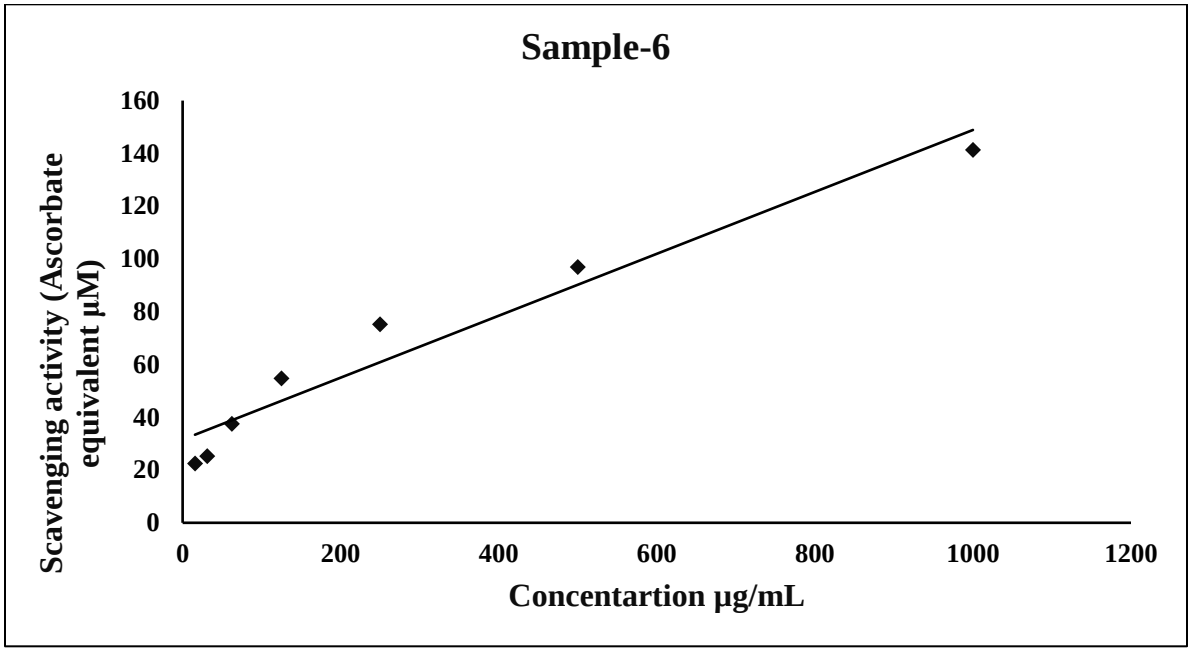


Fig A3.7: ABTS$^{\bullet+}$ free radical scavenging activity of ethanol solubles from hot water extract of sample – 6 used in this study in terms of ascorbate equivalence.