EFFECT OF PHYTOCHEMICAL CONSTITUENTS OF MORINGA LEAVES EXTRACT ON THE SHELF LIFE OF GROUND BUFFALO MEAT (KEEMA)

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Effect of Phytochemical Constituents of Moringa Leaves Extract on the Shelf Life of Ground Buffalo Meat (Keema)

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Approval Letter

This ***dissertation*** entitled ***Effects of Phytochemical Constituents of Moringa Leaves Extract on Shelf Life of Ground Buffalo Meat (Keema)*** presented by **Simron Shrestha** has been accepted as the partial fulfillment of the requirements for the **B. Tech. degree in Food Technology**

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Abstract

The aim of the work was to study the effect of phytochemical constituents of moringa leaves extract (MLE) on shelf life of ground buffalo meat. The moringa leaves collected were extracted by decoction, hot infusion and cold infusion method. The extracts were then analyzed for total phenols, total flavonoids and radical scavenging capacity. The extract with highest amount of these parameters was incorporated in the ground meat at the concentration of 1% and 1.5% of total ground meat mass. It was then compared with meat treated with 0.01% BHT (Butylated hydroxytoluene) of sample and control. Then the subsequent changes in peroxide value (PV), TBARS value and total plate count (TPC) of the samples was studied every alternate day stored in the refrigerated system at 4°C. Analysis of variance was done and Tukey's honesty test was performed by JMP version 14 at $p<0.05$.

From ANOVA and PCA result, sample extracted by Decoction method was found to have highest amount of phenol, flavonoid, and DPPH radical scavenging capacity. Therefore, it was incorporated in the ground buffalo meat sample. Peroxide values for control, 0.01% BHT, 1% MLE and 1.5% MLE, peak value reached at 6^{th}, $10^{th,}$ 12^{th} and 14^{th} day of storage respectively. TBARS value for control, 0.01% BHT, 1% MLE and 1.5% MLE added samples, reached the threshold of 1 mg MDA/kg sample at 6^{th}, 10^{th}, 12^{th} and 16^{th} days respectively. For Total plate count, the control sample exceeded the legal threshold (10^7) in 8 days while the samples with 0.01% BHT 1% MLE and 1.5% MLE exceeded the limit in 10, 14 and 16 days respectively. The performance of MLE extract in terms of antioxidative and antimicrobial properties was significantly higher compared to the synthetic antioxidant.

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List of Abbreviations

Abbreviation	Full form
TPC	Total plate count
TBARS	Thiobarbituric acid reactive substances
BHA	Butylated hydroxy anisole
BHT	Butylated hydroxyl toluene
TBHQ	Tert-butyl hydroquinone
LDL	Low density lipid
MLE	Moringa leaves extract
FAO	Food and agricultural organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
GON	Government of Nepal
PV	Peroxide value
AV	Acid value
WOF	Warmed-over flavor
ROS	Reactive oxygen species
Lox	Lipid oxidation
UAE	Ultrasound assisted extraction
MAE	Microwave assisted extraction
PLE	Pressurized liquid extraction
SE	Solvent extraction
CFU	Colony-forming unit

Part I

Introduction

1.1 General introduction

The FAO estimates that there are 204 million buffaloes spread throughout 47 nations. Among these countries, India (109.8 million), China (27.3 million), and Pakistan (40.0 million) inhabit most of the population (Khan, 2022). The awareness of the potential of water buffaloes for meat has increased in recent years throughout the world due to its high content of lean meat. Buffalo meat is 25% higher in protein than beef and 50% lower in cholesterol (Uriyapongson, 2013). Buffalo meat may provide health benefits over beef, due to its composition, nutritional, and functional properties. In particular, meat obtained in good management conditions may have a better nutritional quality than beef with lower total lipids (1.370g), saturated fatty acids (0.460g), and monounsaturated fatty acids (0.420g) in comparison with beef 10.190g, 4.330g, 4.380g respectively (Naveena and Kiran, 2014).

However, one of the most important causes of meat deterioration and deterioration of organoleptic characteristic, the lipid peroxidation, should be faced (Cascone *et al.*, 2007). The degradation begins with the sacrifice of the animal and continues progressively until the final product is consumed (Chaijan and Panpipat, 2017). Several authors conclude that one of the most important problems of lipid oxidation is the generation of harmful compounds that implicate several human pathologies, including atherosclerosis, cancer, inflammation and aging processes, among others. The main objective of the industry and researchers is to understand the lipid oxidation mechanisms and identify the most effective methods to control this process (Domínguez *et al.*, 2019).

As the solution, different synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertbutyl hydroquinone (TBHQ), propyl gallate (PG), and nitrite are used in the meat industry. However, because of the potential health hazards and toxicity of these synthetic chemicals on humans, as well as the growing trend to eat natural ingredients, their usage has been restricted. A variety of studies have been carried out in order to identify a new and natural source of antioxidants to replace synthetic antioxidants (Bianchin *et al.*, 2017).

Some natural antioxidants inhibit the development of free radicals and promotion of ROS. Ascorbic acid (vitamin C), alpha tocopherol (vitamin E), beta-carotene (vitamin A precursor), many flavonoids, and other phenolic components are all linked to the total antioxidant capacity of plant materials such as culinary herbs, spices, vegetables, fruits, and oilseed products (Brewer, 2011).

Moringa oleifera (*M. oleifera*) or drumstick plant is a member of Moringaceae (Gopalakrishnan *et al.*, 2016). Their leaves contain nutrients especially essential amino acids, vitamins, minerals and β-carotene. For this reason, it is used as an alternative source for nutritional supplements and growth promoters in some countries. Polyphenol is recognized as a potent antioxidant is found in *M. oleifera* extracts. Recently, Charoensin (2014) reported that the aqueous extract of *M. oleifera* leaves contained polyphenols and had DPPH radical scavenging activity and have antioxidant activity. In accordance with the previous works, *M. oleifera* leaves extracted with methanol and dichloromethane also showed antioxidant activity. The chemical analysis of *M. oleifera* extracted with methanol had shown that the major polyphenols comprised of gallic acid, quercetin and kaempferol (Sreelatha and Padma, 2009).

Moringa oleifera commonly known as *sitalchini* or *sajiwan* in Nepal has both nutritional and medicinal importance with some useful minerals, vitamins, amino acids, etc. Although there are 12 varieties of Moringa species *Moringa oleifera* is the best known of all species of the genus Moringaceae (Padayachee and Baijnath, 2012).

1.2 Statement of the problem

The use of synthetic antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy-toluene (BHT) and tert-butyl hydroquinone (TBHQ) is prevalent in the food industry. Safety issues regarding those have been raised over time. There are several published studies indicating a relationship between the long-term intake of synthetic antioxidants and some health issues, such as skin allergies, disorders in pregnant women and children, gastrointestinal tract problems and in some cases increased the risk of cancer induce premature senescence (Lourenço *et al.*, 2019). TBHQ is banned in Japan and certain European countries, BHA and BHT have already been found to be responsible for adverse effects on the liver and for carcinogenesis in animal studies. This has promoted consumer-driven demands supporting the use of natural antioxidant compounds. Hence, research into

safer and more effective natural antioxidants is under way, and several natural sources are being examined (Das *et al.*, 2011).

Moringa is a severely underutilized plant. It has a considerable role in nutritional, industrial and medicinal application (Dawit *et al.*, 2016). They are the potent source of phenolics and have immense nutraceutical value (Gopalakrishnan *et al.*, 2016). But the potential and prospective of the plant has not been quite explored.

1.3 Objectives

1.3.1 General objective

General objective was to study the effect of phytochemical constituents of Moringa leaves extract on shelf life of ground buffalo meat.

1.3.2 Specific objectives

The specific objectives were as follows:

i. To prepare aqueous extract of Moringa leaves by three different methods namely Decoction, Hot Infusion and Cold Infusion.
ii. To determine total phenols, total flavonoids and antioxidant activity of the extracts and make a comparison.
iii. To incorporate the best extract in the minced buffalo meat in concentrations of 1% and 1.5% of total mass.
iv. To study the changes in peroxide value (PV), Thiobarbituric acid reactive substances (TBARS) number and Total plate count (TPC) of the extract incorporated sample overtime and compare those with a synthetic antioxidant i.e., BHT (Butylated hydroxytoluene).

1.4 Significance of the work

Nanda and Nakao (2003) addresses buffaloes as an important but little-studied species. The little to no study on buffalo meat has been a major factor in overshadowing the benefits of buffalo meat. This study aims to widen the facets for production and distribution of buffalo meat.

It is a relevant topic in hindu prevalent countries like ours where religious beliefs do not allow consumption of beef. Buffalo meat is well comparable to beef in many of the physicochemical, nutritional, functional properties and palatability attributes (Anjaneyulu *et al.*, 1990). Furthermore, its utility in meat processing is on increase because of higher content of lean meat and less fat.

Furthermore, using antioxidants from natural sources not only eliminates the harmful effects of synthetic antioxidants, but also aids in the incorporation of nutritional and therapeutic advantages. Moringa leaves being another underutilized component, when used as a source of valuable compounds it not only will provide health benefits but also have a substantial financial value. This study will facilitate the comparison of approaches for obtaining efficient extraction and improved antioxidant properties in ground buffalo meat.

1.5 Limitations and delimitations

i. Variation in storage temperature was not maintained.
ii. Traditional methods were used for extraction of phytochemicals from moringa leaves.
iii. Only aqueous method of extraction was used for the determination of phytochemicals.

Part II

Literature review

2.1 Buffalo Meat

Buffalo meat is the healthiest meat among red meats known for human consumption. Buffalo meat has grown in popularity in recent years due to domestic demand and export potential. In 2019, the world buffalo meat production was 4,290,212 tons, based on 27,692,388 slaughtered head, with Asia accounting for more than 90% of the total (Stasio and Brugiapaglia, 2021).

Red meat comprises all of the necessary amino acids, as well as heme iron, zinc, calcium, lipids, and B-group (Tamburrano *et al.*, 2019). The total protein composition of buffalo meat varies between 19 and 24 g/100 g, with lower values found in meat from young animals and fat content varying approximately between 1 and 4 g/100 g of meat hence, it is commonly believed that buffalo meat is healthier than beef. In addition to that diets including buffalo meat could be recommended in case of nutritional iron deficiency. The most prevalent amino acid is glutamic acid, which is followed by aspartic acid and lysine, while the least abundant amino acids are threonine and methionine (Stasio and Brugiapaglia, 2021). In addition, buffalo meat provides moderate amounts of carnosine and anserine, two dipeptides with antioxidant properties (Landi *et al.*, 2016).

Regular consumers of buffalo meat have shown to have a better lipidic profile in their blood, with lower levels of total cholesterol, high levels of HDL and low levels of triglycerides, compared to recent consumers and non-consumers as per the study performed by Giordano *et al.* (2010). All the above considerations highlight the huge economic potential that might arise from the development of the meat sector in buffalo breeding.

2.2 Oxidation in meat

Many desirable characteristics of meats and meat products are due to lipids. They have an impact on the flavor of meats and help to improve their tenderness and juiciness. However, lipid oxidation is the major non-microbial cause of quality degradation. The degrading process starts with the animal's sacrifice and progresses until the final product is consumed (Domínguez *et al.*, 2019). The intracellular lipids exist in close association with proteins and contain a large percentage of the total phospholipids. Though phospholipid content of meat

is relatively small, the susceptibility of the phospholipids to oxidation makes them important in determining meat quality (Love and Pearson, 1971). The degree of unsaturation in fatty acids determines the susceptibility of muscle lipids to oxidation. The majority of polyunsaturated fatty acids (PUFA) are linked to phospholipids, which are thought to play a larger role in the development of rancidity and warmed-over flavor (WOF) in raw and cooked meat than neutral lipids (Min, 2006).

The first shift leads to a steady decrease in sensory quality. Consumer approval is influenced by changes in color, texture, and appearance of rancid odor and flavor. In addition, lipid oxidation produces a number of hazardous chemicals. Several scientists argue that one of the most serious repercussions of lipid oxidation is the production of toxic substances that are linked to a variety of human diseases (Domínguez *et al.*, 2019). Although raw frozen meat is typically resistant to oxidation, rancidity can occur during freezing and thawing. Temperature variations and a lack of protection from oxygen can hasten the development of rancidity (Love and Pearson, 1971).

Different factors have an impact on these reactions. Extrinsic (meat composition) and intrinsic (processing and storage conditions) factors can both accelerate and prevent oxidative responses. As a result, the balance of anti- and prooxidant compounds determines the oxidative stability of meat. Regarding the intrinsic parameters, the content of heme-proteins, metals, prooxidant enzymes or antioxidant compounds such as vitamins, antioxidant enzymes or peptides are determinants of oxidative processes, the fatty acid composition being the major substrate. Storage conditions, on the other hand, are extremely important in promoting lipid oxidation These reactions are accelerated by many parameters such as temperature, light, and oxygen. Similarly, some processing techniques encourage oxidation. In this context, processes such as cutting, deboning, grinding, or cooking accelerate the oxidation of lipids (Domínguez *et al.*, 2019).

2.2.1 Mechanism of lipid oxidation

Lipid peroxidation is a free radical-driven process of fatty acid oxidation producing fatty acid peroxides. It occurs in three steps: initiation, propagation and termination (Wójciak and Dolatowski, 2012). During the process, a free radical combine with the fatty acid's hydrocarbon chain, generating peroxides, which then react with other hydrocarbon chains, abstracting hydrogens and forming hydroperoxides. The hydrogens have been extracted

from the carbon chain, which will function as new peroxide, continuing the cycle (Estévez, 2015).

Free radicals are highly reactive species that have one or more free electrons, which can exist independently for a short period. Some examples of these reactive oxygen molecules are: hydroxyl radical (HO), organic compound oxygen radicals, peroxyl (ROO) and alkoxyl (RO) radicals, superoxide radical (O^{-}_{2}) and its radical conjugate hydroperoxide acid (HO_2), and singlet oxygen (O^{1}_{2}). These reactive oxygen molecules can be produced intentionally or accidentally. In biological systems, they are produced during the normal aerobic metabolism. Mitochondria consume molecular oxygen reducing it by sequential steps to produce ATP and H_2O. During this process, O^{1}_{2}, H_2O_2, HO are formed as unwanted by-products. Meanwhile, the cells that protect the body (phagocytes) deliberately generate $O^{1}.{}_{2}$ and H_2O_2 to inactivate bacteria and viruses (Lima *et al.*, 2013).

During the initiation stage of LOx, a hydrogen atom (H) is abstracted from a neighbor carbon to a double bond in an unsaturated fatty acid (RH) forming the alkyl R (E1) radical (Sumah and Joseph, 2013).

$$R : H + O :: O + \text{Initial} \rightarrow R\ (\text{Alkyl radical}) + HOO \quad (1)$$

This alkyl radical can react with a molecular oxygen and generate various radical species, such as the peroxyl (ROO) radical (E2). These radicals, in turn, may find stability in the subsequent propagation stage by abstracting a hydrogen atom from another susceptible molecule, such as an adjacent RH forming a lipid hydroperoxide (ROOH) and a new R (E3) (Min and Ahn, 2005) .

$$R + O_2 \neq ROO \quad (2)$$

$$ROO + RH \rightarrow ROOH + R \quad (3)$$

These propagation mechanisms may occur up to 100 times before two R combine and terminate the process. Radical species formed during the process may be stabilized into non-radical compounds. The peroxides that are commonly formed as LOX primary products can subsequently undergo scission to form lower molecular weight volatile and non-volatile compounds (secondary LOX products) such as carbonyls, alcohols, hydrocarbons, and

furans. Among these, aldehydes are one of the most abundant products found in meat, such as hexanal malondialdehyde (MDA) and 4-hydroxy-2-trans-nonenal (Estévez, 2015).

Oxidative deterioration takes place according to the mechanisms described above as soon as the antioxidant capacity of proteins and other redox-active components in the environment is exceeded (Estévez, 2015). After slaughter, in vivo the antioxidant mechanisms collapse while the biochemical changes that occurred during conversion of muscle to meat favor oxidation (Min and Ahn, 2005). The pH decline facilitates the oxidation of the muscle components as H^+ may promote the redox cycle of myoglobin and its pro-oxidant action. In addition to the pH decline, other post-mortem biochemical changes, such as changes in the cellular compartmentalization and the release of free catalytic iron and oxidizing enzymes also contribute to the promotion of Lox (Zhang *et al.*, 2011). The extent of LOx in post-mortem meat is highly dependent on the origin of the meat, type of muscle, species, and storage conditions (Estévez, 2015).

2.2.2 Peroxide value

Peroxide value is considered as a useful index to control food safety and quality. It helps in quantifying the amount of hydroperoxide in fat and oil (Gotoh and Wada, 2006). Autoxidation is aided by the double bonds found in fats and oils. Autoxidation is particularly common in oils with a high degree of unsaturation. Determining the peroxide value is the best test for autoxidation (oxidative rancidity). In the autoxidation reaction, peroxides are intermediates. Autoxidation is a free radical reaction involving oxygen that causes fats and oils to deteriorate, resulting in off-flavors and aromas. The peroxide value, or the amount of peroxide in an oil or fat, is important for determining how far spoiling has progressed (Chakrabarty, 2003).

In a study where moringa leaves extract was used as natural antioxidant, it was found that peroxide value was increased slowly over the observation period in Moringa leaf extract treated nuggets than BHA treated nuggets. 0.3% Moringa leaf extract treated nuggets having more antioxidant functions than other lower treatments. Peroxide levels in control samples were consistently greater than in other samples throughout the storage period. *M. oleifera* leaf extract treated nuggets had a greater antioxidative effect on peroxide value than control and BHA treated samples. This could be attributed to the high total phenolic component content as found by (Das *et al.*, 2011). These results suggested that there were significant

differences ($p<0.5$) based on antioxidant treatment and longer storage time was associated with increased peroxide value (Rahman *et al.*, 2020).

2.2.3 Acid Value

The acid value is a measurement of the amount of potassium hydroxide (mg) needed to neutralize the free fatty acid in a unit mass (g) of chemical material. It is a biofuel property that shows the quality of lubricity or rate of degradation while stored for a period of time, having implications for stability and shelf life (Onu and Mbohwa, 2021). When newly derived from the source, natural fats and oils are mostly in the triglyceride form. Long-term storage, on the other hand, causes the triglycerides to break down, releasing free fatty acids (FFA). Hydrolysis is caused by a number of factors, including the presence of moisture in the oil, high temperatures, and, most importantly, lipases (enzymes) from the source or contaminating microbes. As a result, triglycerides, diglycerides, monoglycerides, free fatty acids, and glycerol are formed from the neutral oil. The presence of extra free fatty acids in oil is a strong sign that it is out of its normal form. The number of milligrams of KOH required to neutralize the FFA present in 1 g of oil is known as the acid value (KC and Rai, 2007)

2.2.4 Thiobarbituric acid reactive substances (TBARS)

Another approach for detecting lipid oxidation is the thiobarbituric acid reactive substance (TBARS) assay. This assay detects malondialdehyde (MDA), a split product of an endoperoxide of unsaturated fatty acids produced by lipid substrate oxidation (Kumar *et al.*, 2018). Malondialdehyde (MDA), which is regarded as one of the important oxidation products is key marker in lipid peroxidation (Zeb and Ullah, 2016). This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA at 25°C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm TBARS is now considered as a standard marker for the lipid peroxidation induced oxidative stress (Zeb and Ullah, 2016)

2.2.5 Antioxidant additives

As per Britannia Encyclopedia, antioxidant is any of various chemical compounds added to certain foods, natural and synthetic rubbers, gasolines, and other substances to retard autoxidation, the process by which these substances combine with oxygen in the air at room temperature. Retarding autoxidation delays the appearance of such undesirable qualities as rancidity in foods, loss of elasticity in rubbers, and formation of gums in gasolines. Antioxidants most commonly used are such organic compounds as aromatic amines, phenols, and amino phenols (Franco *et al.*, 2019). Antioxidants can act by: (a) removing oxygen or decreasing local oxygen concentrations; (b) removing catalytic metal ions; (c) removing key reactive oxygen species such as superoxide and hydrogen peroxide; (d) scavenging initiating free radicals such as hydroxyl, alkoxyl, and peroxyl species; (e) breaking the chain of an initiated sequence; or (f) quenching or scavenging singlet oxygen (Gutteridge, 1995).

Antioxidants prevent lipid oxidation and rancidity without compromising sensory or nutritional characteristics, preserving meat and meat products' quality and extending their shelf life. Although living muscles contain inherent components that inhibit lipid oxidation, they are frequently lost after slaughtering, during muscle conversion to meat, primary and secondary processing, handling, and storage, necessitating the use of extrinsic antioxidants. As a result, synthetic antioxidants like BHT and BHA have become popular for delaying or preventing lipid oxidation. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) have been used as antioxidants in meat and poultry products. Synthetic antioxidants have fallen under scrutiny due to potential toxicological effects (Karre *et al.*, 2013).

Therefore, because of the concern over the safety of these synthetic compounds, the use of natural antioxidants in meat has been widely studied. Natural antioxidants have great application potential in the meat industry. It is known that plant extracts, herbs, spices, and essential oils have significant antioxidant capacity, but their application in the industry is still limited due to the lack of sufficient data about their efficiency and safety in different amounts and products (Kumar *et al.*, 2015).

Antioxidants, which include both natural (such as tocopherols) and synthetic antioxidants (for example phenolic antioxidants), stop the detrimental effects of oxygen generation and prevent free radicals from damaging the body by trapping and neutralizing them. Vitamin

A, C, E, and polyphenol chemicals are natural antioxidants that are mostly produced from fruits and vegetables and are employed as antioxidants, anticancer agents, and disease prevention (Xu *et al.*, 2021).

2.2.5.1 Synthetic Antioxidants

Synthetic antioxidants are widely used in the food industry; they are characterized by a diverse source of raw materials, mature technology, low price, good scavenging activity and ease of procurement (Kim *et al.*, 2016). Phenolic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG), are currently the most widely utilized synthetic antioxidants in food. Synthetic phenolic antioxidants (SPAs) can interact with food peroxides to prevent the process of food deterioration under specific conditions; hence they play an essential role in enhancing food stability and shelf life. BHA, BHT, TBHQ and PG are probably the most ubiquitous of food additives, being present in animal fats, vegetable oils, cured meats and so on (Xu *et al.*, 2021).

The toxicity of BHA, BHT and TBHQ has been investigated extensively using a variety of experimental conditions. Their excessive addition or incorrect use will be detrimental to human health (Kim *et al.*, 2016), and a mix of antioxidants may increase their toxic effects and cause side effects.

BHA is cytotoxic and carcinogenic. BHT has a number of negative consequences, including tumor promotion, DNA damage, oxidative stress, and the generation of reactive oxygen species (ROS) (Castro *et al.*, 2017). Furthermore, its metabolites may mediate BHT's mechanism of action, resulting in even more hazardous effects than BHT itself. At normal levels, TBHQ is commonly utilized in oils and fats for its antioxidant qualities. When TBHQ is given in excessive dosages to animals in long-term feeding trials, it can produce stomach tumors, DNA damage, and other side effects. Therefore, more and more attention has been paid to their safety (Xu *et al.*, 2021).

2.2.5.2 Natural antioxidants

The most widely encountered way of antioxidant formation is the synthesis of antioxidants by various microorganisms, fungi, even animals, and most often by plants. They are called

natural antioxidants (Pokorný, 2007). Natural antioxidants are interesting alternatives to conventional antioxidants.

Natural antioxidant system is sorted in two major groups, enzymatic and non- enzymatic. Non-enzymatic antioxidants: Non-enzymatic antioxidants include direct acting antioxidants, which are extremely important in defense against oxidation stress (Gilgun-Sherki *et al.*, 2001). The majority of them, such as ascorbic and lipoic acid, polyphenols, and carotenoids, come from dietary sources. Vitamin E is a generic description for all tocopherol (Toc) and tocotrienol (Toc-3) derivatives. The main source for dietary uptake of vitamin E is plant food (vegetables, fruits, seeds, and seed oils) (Grusak and DellaPenna, 1999).

Vitamin C is an electron donor (reducing agent or antioxidant), and this role accounts for the majority of its biochemical and molecular actions. Citrus fruits and juices are particular important sources of vitamin C (Atta *et al.*, 2017).

Enzymatic antioxidants are capable of stabilizing, or deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. By increasing the intake of antioxidant enzyme rich meals or antioxidant enzyme supplements, our body's potential to reduce the risk of free radical-related health problems is made more palpable (Atta *et al.*, 2017). The repair enzymes that can recreate some antioxidants are SOD, GPx, glutathione reductase (GR), CAT and the other metalloenzymes (Krishnamurthy and Wadhwani, 2012). SOD, CAT, and GPx constitute a mutually supportive team of defense against ROS. While SOD lowers the steady-state level of O_2- , catalase and peroxidases do the same for H_2O_2. Combination of certain antioxidants like glutathione, vitamin C and E, selenium and glutathione peroxidase are very powerful in helping the body fight against the free radicals (Krishnamurthy and Wadhwani, 2012).

Plants (fruits, vegetables, medicinal herbs) may contain a wide variety of free radical scavenging molecules such as phenolic compounds (phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins etc.), nitrogen compounds (alkaloids, amines, etc.), vitamins, terpenoids (including carotenoids) and some other endogenous metabolites which are rich in antioxidant activity (Cai *et al.*, 2003). Rosaceae, Empetraceae, Ericaceae, Grossulariaceae, Juglandaceae, Asteraceae, Punicaceae and Zingiberaceae are families of plants that contain compounds with high antioxidant activities, which include fruits, such as

blackberries, strawberries, blueberries, black currants, walnuts, pomegranates and others. Essential oils from spices and herbs including oregano, thyme, dittany, marjoram, lavender, and rosemary have also been shown to be great sources of natural antioxidant molecules, although their applications are limited due to their strong flavor qualities. Because they contain many components such as catechins, tannins, and other flavonoids, aqueous tea extracts have also been employed as natural antioxidant sources, with the advantage of not having a strong flavor like essential oils (Lourenço *et al.*, 2019).

2.2.6 Phytochemical constituents of plants

Phytochemicals are organic substances that are naturally present in plants and herbs. They have health benefits for people that go beyond those offered by macro- and micronutrients. Phenolics, carotenoids, vitamin E compounds, lignans, beta-glucan, and inulin are some of the most significant categories of phytochemicals present in whole grains (Ghasemzadeh *et al.*, 2015). Phenolics, one of the most abundant groups of phytochemicals in whole grains, are considered natural antioxidants, which act as radical scavengers to decrease the incidence of oxidative stress-induced damage to large biological molecules, such as lipids, proteins, and DNA (Ghasemzadeh *et al.*, 2018).

Phenolic compounds are a large group of the secondary metabolites widespread in plant kingdom. Phenolic compounds, especially flavonoids, possess different biological activities, but the most important are antioxidant activity. Phenolic acids generally act as antioxidants by trapping free radicals whereas flavonoids can scavenge free radicals and metal chelates as well (Gheldof and Engeseth, 2002). Phenolics are able to scavenge reactive oxygen species due to their electron donating properties. Their antioxidant effectiveness depends on the stability in different systems, as well as number and location of hydroxyl groups. Many mechanisms have been proposed for polyphenol prevention of oxidative stress and ROS/RNS generation both in vitro and in vivo. Radical scavenging by polyphenols is the most widely published mechanism for their antioxidant activity. In this radical scavenging mechanism, polyphenols sacrificially reduce ROS/RNS, such as OH, O_2 , NO , or OONO after generation, preventing damage to biomolecules or formation of more reactive ROS (Atta *et al.*, 2017).

2.2.7 Extraction procedures of natural antioxidants

As previously stated, many natural antioxidants are found in vegetal matrices and must be segregated for further use. Plant parts such as leaves, roots, stems, fruits, seeds, and peels can all be used to extract antioxidants (Shah *et al.*, 2014).

Many extraction factors play important roles in the extraction efficiency, such as type and concentration of extraction solvent, extraction temperature, extraction time, and extraction pH. Among them, the solvent is one of the most influential factors. Numerous solvents have been used for the extraction of antioxidants from food and medicinal plants. The selection of solvents is based on the chemical nature and polarity of antioxidant compounds to be extracted. Most of the phenolics, flavonoids and anthocyanins are hydro soluble antioxidants. The polar and medium polar solvents, such as water, ethanol, methanol, propanol, acetone and their aqueous mixtures, are widely used for extraction (Xu *et al.*, 2017). Carotenoids are lipid-soluble antioxidants, and common organic solvents, such as the mixtures of hexane with acetone, ethanol, methanol, or mixtures of ethyl acetate with acetone, ethanol, methanol, have been used for extraction. Natural extracts' antioxidant strength is determined not only by the quality of the original source (e.g., geographic origin, nutritional characteristics, and storage), but also by the extraction methods used (Yong and Liu, 2021).

2.2.7.1 Maceration

Maceration is an extraction process that consists of maintaining contact between the plant and a liquid (solvent) for a period of time. It is an extractive technique that is conducted at room temperature. It involves breaking down and softening of various components. This technique is used for the extraction of essential oils and active compounds from plant materials. The maceration procedure consists of multiple steps in extraction. The whole or coarsely powdered crude drug undergoes grinding to increase the surface area for proper mixing of powdered materials with the solvent. This process is done in a closed vessel where an appropriate solvent (menstruum) is added. Next, the solvent is strained off followed by pressing the solid residue of the extraction process known as marc to recover an optimum amount of occluded solution. Both the obtained pressed out liquid and the strained solvent are mixed together and separated from unwanted materials by filtration. Frequent agitation during maceration facilitates extraction by two processes: (1) promotes diffusion, (2)

separates concentrated solution from the sample surface by adding new solvent to the menstruum for increasing the extraction yield (Srivastava *et al.*, 2021).

2.2.7.2 Infusion

Another conventional technique is Infusion. Infusions are dilute solutions containing the readily-soluble constituents of crude drugs. The sample is grinded and placed in a clean container. The hot or cold extraction solvent is then placed on top of the drug material, soaked, and stored for a brief time. This approach is ideal for extracting readily soluble bioactive constituents. Furthermore, it is a suitable procedure for preparing fresh extract prior to usage. Depending on the intended usage, the solvent to sample ratio is commonly 4:1 or 16:1 (Abubakar and Haque, 2020).

Hot or boiling water will swell plant tissues and burst the cells of the herbs. By dissolving the starches and breaking down elements by disintegration, hot water temperatures can extract more of the precious vegetable tissues (Visht and Chaturvedi, 2012).

It is believed that polysaccharides extract well in cold infusion. Volatile oils will be altered slightly in hot infusions, but the extraction is done more quickly. Though cold infusions take longer, the delicate volatile oils will be more safely extracted. Tannins, lactones, and iridoids extract poorly in cold water. Cold water herbal infusions will break down the following plant constituents: sugars, proteins, albuminous bodies, gums, mucilaginous substances, pectin, plant acids, coloring matter, many mineral salts, glycosides, some alkaloids, most alkaloidal salts, and a hint of essential oils (Azwanida, 2015).

2.2.7.3 Decoction

Decoction is a method of extracting water soluble and heat stable ingredients from crude drugs by boiling them in water for a set period of time, cooling them, filtering them out, and adding enough water to get the desired volume (Tiwari *et al.*, 2011). Decoction extraction has traditionally been employed for dry drugs that do not produce any juice. The drug's coarse powder is cooked in 8 or 16 times its volume of water, reduced to a quarter, and then taken as juice. This method allows for a higher extraction of water-soluble extractives than other methods. This process allows for oxidation, hydrolysis, and the production of new

components, which can be advantageous, but it also has certain drawbacks, such as deterioration and the loss of volatile components (Shingadiya *et al.*, 2016).

The time that is usually left to boil to prepare a decoction varies a lot, since it depends on the characteristics of the plant and the part, we use of it, such as roots, leaves, stems, seeds, and bark; roughly, we can say that it ranges from three minutes to half an hour (Khajehei *et al.*, 2017).

2.2.7.4 Percolation

Percolation is an exhaustive extraction procedure, by which all of the soluble constituents are completely removed from a comminuted plant material, by extracting the crude drug by fresh solvent (Visht and Chaturvedi, 2012).

The rate of solvent elution is controlled using a percolator, which is a tapered glass or metallic container with a stopcock at the bottom. The percolation process has various unique characteristics, which include: It takes less time and does not require as much manipulation. The percolation procedure uses coarsely fragmented samples that have been passed through a 3 mm sieve. Otherwise, a PS that is excessively large is incompatible with the extraction equilibrium (Visht and Chaturvedi, 2012).

This method is better for an exhaustive extraction because the sample is continually exposed to new solvent. It is possible to employ a hot or cold solvent. This method cannot extract very tiny particles, resins, or powders that expand or produce a viscous eluent (Mukherjee, 2019). According to analysis on literatures, solvent composition, impregnation time, percolation speed, and solvent consumption are considered as the important factors of percolation processes (Wang *et al.*, 2020).

2.2.7.5 Soxhlet extraction

Soxhlet extraction is one of several sample preparation methods that can be used to transfer targeted analytes from a sample matrix into a solvent, to permit subsequent analysis. With Soxhlet extraction, samples are repeatedly extracted with solvent regenerated by distillation through a periodic siphoning process (Sander, 2017). Regarding Soxhlet extraction method the pulverized samples are placed in thimbles (made of cellulose) and positioned in the extraction chamber just over the collecting flask under a reflux condenser. The solvent,

which has previously been put to the heating bottle, is then heated to form vapor, which condenses under cool running water and falls back into the sample thimbles. The reflux is maintained several times before returning the aqueous extract to the heating flask. Soxhlet extraction is advantageous since it is a continuous process that takes less time and solvent than percolation and maceration procedures (Azwanida, 2015). Among the generally utilized solvents for extracting polyphenols are methanol, water, chloroform, n-hexane, ethanol, propanol, ethyl acetate, and acetone (Zhang *et al.*, 2018).

2.2.7.6 Microwave assisted Extraction (MAE)

Microwave assisted extraction employs microwave energy to break down the cell membrane, releasing intracellular lipids into an organic solvent. Polar compounds align themselves in the direction of the electric field and rotate at a high-speed during microwave treatment, causing heat and cell rupture. When compared to traditional heating methods, MAE has a significant advantage in terms of energy efficiency. The partition coefficient, solubility of target chemicals in the solvent, dielectric characteristics of the solvent and oilseeds/biomass, and mass diffusivity are all factors that influence MAE efficiency (Kumar *et al.*, 2021). It involves the application of electromagnetic radiation in frequencies between 300 MHz and 300 GHz and wavelength between 1 cm and 1 m. The microwaves applied at frequency of 2450 Hz yielded energy between 600 and 700 W. The technique uses microwave radiation to bombard an object, which can absorb electromagnetic energy and convert it into heat. Subsequently, the heat produced facilitates movement of solvent into the drug matrix (Abubakar and Haque, 2020).

2.2.7.7 Ultrasound assisted Extraction (UAE) or Sonication

Ultrasound assisted extraction (UAE) is a non-thermal technique, which uses frequencies equal or above 20 kHz. Ultrasound in the solvent cause cavitation, which speeds up solute dissolution and diffusion as well as heat transfer, improving extraction efficiency. The extraction mechanism by ultrasound process involves two main types of physical phenomena: 1. the diffusion across the cell wall and 2. rinsing the contents of cell after breaking the walls (Mason *et al.*, 1996).

Low solvent and energy usage, as well as a reduction in extraction temperature and time, are other advantages of UAE. The extraction of thermolabile and unstable chemicals is

possible with UAE. UAE is widely used in the extraction of a wide range of natural compounds (Zhang *et al.*, 2018).

2.2.7.8 Accelerated Solvent Extraction

Accelerated solvent extraction (ASE) is also known as pressurized fluid extraction (PFE) or pressurized liquid extraction (PLE). Accelerated solvent extraction is a closed system that uses a higher temperature and pressure to extract a substance. Closed systems are intended to reduce volatile loss, improve efficiency, and boost throughput. The increased temperature improves the solubility of the analyte. With ASE, a solid sample is encased in a sample cartridge that is filled with an extraction fluid and used to statically extract the sample for brief periods of time at elevated temperature (50-200 °C) and pressure (500-3000 psi) (5-10 min). To expel the sample extract from the cell into a collection vessel, compressed gas is employed. Accelerated solvent extraction (ASE) gives recoveries comparable to those obtained with Soxhlet and other techniques in use while spending only a fraction of the time and solvents needed for those techniques. ASE shows good potential for the recovery of volatile as well as semi volatile compounds (Richter *et al.*, 1996).

2.2.7.9 Supercritical Fluid Extraction

The SFE sample preparation procedure is actually a series of operations that include a) sample extraction, b) extract collecting in a trap, and c) trap rinsing to produce a processed sample that can then be used for chromatographic analysis or other sample preparation steps. Other extraction techniques, such as the separation of the solvent containing the recovered components from the residual matrix, necessitate additional steps in addition to the extraction (Moldoveanu and David, 2002). The extraction solvent in supercritical fluid extraction (SFE) is supercritical fluid (SF). SF has a solubility and diffusivity similar to liquid and can dissolve a wide range of natural compounds. Due to small pressure and temperature changes, their solvating characteristics altered substantially around their critical points. Because of its appealing qualities, such as low critical temperature (31°C), selectivity, inertness, low cost, non-toxicity, and capacity to extract thermally labile chemicals, supercritical carbon dioxide (S-CO_2) was frequently utilized in SFE. Because of its low polarity, S-CO_2 is useful for extracting non-polar natural compounds including lipids and volatile oils (Zhang *et al.*, 2018). Since this method involves a heat-controlled environment,

it is suitable for heat-labile substances. That is why it finds great applications in various industries (Vicky, 2022).

2.3 Microbial spoilage of meat and meat products

The shelf-life of meat and meat products refers to the amount of time that food can be stored while maintaining its qualitative attributes until spoiling occurs. The shelf-life of items is closely linked to their deterioration, resulting in a borderline between an acceptable and unacceptable bacterial concentration, which determines off-odors, off-flavors, and an unappealing sight. These sensory changes are linked to the amount and types of microorganisms present at the start, as well as their continuing growth. For meat products, the starting total micro biota is approximately -10^2,-10^3 cfu g^{-1}, consisting of a huge variety of species (Paudel, 2020).

Lower refrigeration temperatures inhibit bacterial development and alter the microbiota composition on meat: psychrotrophic bacteria, such as LAB, and Gram-negative bacteria, such as Pseudomonas spp., could flourish at chill temperatures (Doulgeraki *et al.*, 2012). Bacteria from the genera Acinetobacter, Pseudomonas, Brochothrix, Flavobacterium, Psychrobacterium, Moraxella, Staphylococcus, and Micrococcus, as well as lactic acid bacteria and different Enterobacteriaceae species, are the most common bacteria found on fresh meat. The survival and proliferation of these bacteria is determined, to a great extent, by the makeup of the environment surrounding the meat (Pennacchia *et al.*, 2011).

2.4 Moringa Oleifera

Moringa Oleifera can be grown in any tropical and subtropical regions of the world with a temperature around 25–35 °C (Gopalakrishnan *et al.*, 2016). It is an affordable and readily available (Saini *et al.*, 2016a). Almost each and every part of *Moringa* tree is useful for medicinal, functional food preparations, nutraceuticals, water purification, and biodiesel production; including roots, leaves, flowers, green pods, and seeds (Patel *et al.*, 2010).

The genus Moringa (Family Moringaceae) has more than 13 species, of which *M. oleifera* is considered to be the best known and widely distributed tree species among the genus.

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliophyta
Subclass:	Dilleniidae
Order:	Capparales
Family:	Moringaceae
Genus:	Moringa
Species:	Oleifera Lam.

Source: Thakur and Bajagain (2020)

2.4.1 Moringa leaves as natural antioxidants

The foliage of *M. oleifera* (MO) has been established as a rich source of phenolics and glycosylates, minerals, tocopherols, carotenoids, polyunsaturated fatty acids, ascorbic acid, and folate (Saini *et al.*, 2016b). *Moringa oleifera* extracts contain potent antioxidant compounds that have the potential to be applied in the pharmaceutical and food industries (Elazzazy *et al.*, 2021). The multiple biological activities including antiproliferation, hepatoprotective, anti-inflammatory, antinociceptive, anti-atherosclerotic, oxidative DNA damage protective, antiperoxidative, cardioprotective, as well as folk medicinal uses of M. oleifera (MO) are attributed to the presence of functional bioactive compounds, such as phenolic acids, flavonoids, alkaloids, phytosterols, natural sugars, vitamins, minerals, and organic acids (Saini *et al.*, 2016a).

The *M. oleifera* leaves extract (MLE) have been studied for its potential to be used as natural antioxidant. The results also have been quite positive. Incorporation of 0.1% extract of MOL (100 mg／100 g meat) could protect cooked goat meat patties against lipid oxidation during refrigerated storage. The MOL extract was more effective than BHT in maintaining

low TBARS number of precooked chilled goat meat patties (Das *et al.*, 2011). The inclusion of MLE at a level of 600 mg equivalent phenolics/100 g meat inhibited ($P < 0.05$) lipid oxidation in raw and cooked pork patties to a much greater extent compared to control during refrigerated storage. Phytochemicals extracted from natural herbs like *M. oleifera* leaves may be used as a potential source of antioxidants to protect meat products against oxidative rancidity without any adverse effects on sensory attributes (Muthukumar *et al.*, 2014). The application of *M. oleifera* extracts in ground beef revealed that this plant is a promising candidate as natural antimicrobial agent in meat industry (Falowo *et al.*, 2016). M. oleifera leaf extracts have substantial amounts of phytochemicals with significant free radical scavenging activity. The application of the *M. oleifera* can delay the formation of lipid oxidation in meat products during refrigerated storage. MLE could be used as a potential source of antioxidants to replace synthetic antioxidant in meat industry (Falowo *et al.*, 2017a).

2.4.1.1 Extraction of Moringa leaves extract (MLE)

2.4.1.1.1 Effect of solvent in extraction

The selection of the solvent is crucial for solvent extraction. Selectivity, solubility, cost and safety should be considered in selection of solvents. Based on the law of similarity and impermissibility (like dissolves like), solvents with a polarity value near to the polarity of the solute are likely to perform better and vice versa (Zhang *et al.*, 2018). Many solvents are used to extract active materials from plants, including alcohols (ethanol or methanol), diethyl ether, chloroform, ethyl acetate, *n*-butanol, and water (Seleshe and Kang, 2019).

Studies like Nweze and Nwafor (2014); Kasolo *et al.* (2010); Rocchettia *et al.* (2020);Dadi *et al.* (2018) show that different solvents has significant effect on phytochemical extraction, antioxidant capacity and antimicrobial activity in MLE.

The aqueous extract constitutes more phytochemicals than the ethanolic extract (Nweze and Nwafor, 2014). The evaluation of antimicrobial properties of leaves of *Moringa oleifera* reported that the ethanolic extract did not exhibit antimicrobial activity while aqueous extract inhibited almost all in the test organisms, thus rendering water a good solvent for extraction for the leaves. The antimicrobial activity of the aqueous extract was due to the

ability of water to extract more quantity of antimicrobial phytochemicals than the ethanolic extract (Oluduro, 2012).

2.4.1.1.2 Effect of temperature and time in extraction

In the study done by Ali (2012) the results illustrated that when the extraction temperature was increased, the total phenolic content (TPC) peaked at higher temperature of 90ºc than at 30ºc in MLE. Wang and Zheng (2001) also reported that temperature strongly changes antioxidant properties in strawberries. Extraction of TPC increases with the increase of temperature due increasing both solubility of solute and diffusion coefficient but after a certain temperature, phenolic compounds can be denatured (Spigno and De Faveri, 2007).

As per the study of Ali (2012), the time factor has a very low influence in the final response. Extraction time has no significant effect on the extraction of phenolic compounds. There was small increase in TPC with the increase of time. It was clear that a shorter time would extract the same amount of phenolic extracts as longer time while saving cost. Too much extraction time is not useful to extract more phenolic antioxidants (Silva *et al.*, 2007). Small increase of total phenolic contents at a longer extraction time may be due Polymers and wall-bound phenolics retained in cells that were extracted out as well as the polymerization reaction that occurs and new components produced as reported by Spigno and De Faveri (2007). It was clear that a shorter time would extract the same amount of phenolic extracts as longer time while saving cost.

2.4.2 Moringa polyphenols and flavonoid compounds

M. oleifera leaves extracts contain catechol tannins, gallic tannins, steroids and terpenoids, flavonoids, saponins, anthraquinones, alkaloids and reducing sugars which have been identified by other researchers in various plants and in different parts of plants (Kasolo *et al.*, 2010).

Plant phenolics have been reported to have a lot of biological activities including anti-carcinogenic, antioxidant and anti-mutagenic (Agbo *et al.*, 2015). Flavonoids are strong antioxidants, also found to be effective antimicrobial substances. They have been shown to have antifungal activity in vitro (Galeotti *et al.*, 2008). The potent antioxidant activity of

flavonoids reveals their ability to scavenge hydroxyl radicals, superoxide anions and lipid peroxy-radicals (Miller, 1996).

2.4.3 Total antioxidant capacity

Antioxidants are compounds capable of delaying or inhibiting the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species (Martínez-Cabanas *et al.*, 2021). The measurements of the antioxidant activity can be carried out by two methods:

1. Direct determination: a radical is used as a quantification factor (since it produces an analytical signal). In this sense, the addition of the antioxidant, before or after the generation of the radical, causes a decreasing in the signal ($ABTS^{\bullet+}$ or DPPH methods), which is proportional to the antioxidant activity of the sample.

2. Indirect determination: the presence of free radicals causes the loss or appearance of a reagent and therefore, in the presence of an antioxidant, an increasing or decreasing in the signal is caused (ORAC and FRAP methods) proportional to the antioxidant activity of the sample.

The majority of plant extracts do not contain a single phenolic component, but rather a combination of many. In this sense, the concentration of each one in the analysis matrix will affect its antioxidant activity. Because there is no one methodology for determining an extract's antioxidant capacity, different methodologies with diverse mechanisms of action must be applied to gain more thorough and full information (Pabón-Baquero *et al.*, 2018).

2.4.4 DPPH radical scavenging assay

DPPH assay measures the ability of a substance to scavenge the DPPH radical, reducing it to hydrazine. When a substance that acts as a donor of hydrogen atoms is added to a solution of DPPH, hydrazine is obtained, with a change in color from violet to pale yellow (Formagio *et al.*, 2014). The DPPH test is based on the ability of the stable 2, 2-diphenyl1-picrylhydrazyl free radical to react with hydrogen donors. The DPPH radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolorized after reduction with an antioxidant (AH) or a radical ($R^{\bullet}$) in accordance with the following scheme:

$$DPPH^{\bullet} + AH \rightarrow DPPH^{\bullet} - H + A^{\bullet},$$

$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH^{\bullet} - R$$

The $DPPH^{\bullet}$ radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available and does not have to be generated before assay. This assay is based on the measurement of the reducing ability of antioxidants toward $DPPH^{\bullet}$. The DPPH assay is considered to be mainly based on an electron transfer (ET) reaction, and hydrogen-atom abstraction is a marginal reaction pathway. The test is simple and rapid and needs only a UV-vis spectrophotometer to perform, which probably explains its widespread use in antioxidant screening (Prior *et al.*, 2005).

2.4.5 Antimicrobial potential of moringa leaves

Antimicrobial compounds are naturally occurring or synthetic compounds used for inhibiting the growth of various pathogenic (also sometimes referred to as spoilage) microorganisms. These compounds not only ensure safety of foods but also prolong their shelf life. However, some of the synthetic antimicrobials have potential side effects, such as sulphites that cause degradation of thiamine vitamin (Gutiérrez del Río *et al.*, 2018). In recent years, interest in searching for antimicrobial compounds of plant origin having ability to inhibit food borne pathogens and extend the shelf life of perishable foods has enhanced. Such compounds are considered as safe as they decompose easily and cause no toxicity to the environment. Apart from herbal plants, many fruits and vegetable have potential chemicals that can help reduce the incidence of various diseases. In particular, fruit peels contain promising novel compounds having antimicrobial activity (AMA) and antioxidant activity that can prevent food borne illnesses and food spoilage. These compounds are generally secondary metabolites (phenolic compounds, steroids and alkaloids in particular) exerting plenty of useful effects on human health (Singh *et al.*, 2018).

Data regarding antimicrobial activity confirmed an important action of Moringa extracts against different strains of pathogens, bacteria and fungi (Doria *et al.*, 2017).The antimicrobial activities of plant extracts have been linked to the presence of the bioactive compounds, such as phenol and flavonoid, which have been reported to form to complexes with extracellular and soluble proteins and bacterial cell walls, although their mode of mechanism has not been fully understood (Falowo *et al.*, 2017b).

As per the study done by Abalaka *et al.* (2012), the result reveals that chloroform and aqueous crude extracts of the leaf of *M. oleifera* were active against the test organisms. The control (ampiclox) had the highest zone of inhibition (23mm) on *E. coli* and *S. typhi* followed by aqueous leaf extract on E. coli (20mm) and chloroform extract on *Pseudomonas aeruginosa* with zone of inhibition of 18mm *P. aeruginosa* was resistant to the activity of the aqueous extract. The test also included the minimum inhibitory concentration (MIC) screening of chloroform and aqueous extract of the leaf of *M. oleifera*. The result revealed that the MIC of the chloroform and aqueous leaf extract on E. coli and S. typhi were 1.667mg/ml and 0.417mg/ml respectively. The MIC of the chloroform leaf extracts on *P. aeruginosa* was 0.417mg/ml but there was no MIC of the aqueous leaf extract on *P. aeruginosa*. The result of the minimum bactericidal (MBC) screening of chloroform and aqueous extract of the leaf of *M. oleifera*. The MBC of the chloroform and aqueous leaf extract on *E. coli* and *S. typhi* were 6.667mg/ml and 1.667mg/ml respectively. The MBC of the chloroform leaf extracts on *P. aeruginosa* was 6.667mg/ml but there was no MBC of the aqueous leaf extract on *P. aeruginosa.*

The activity of the leaf against both gram positive and gram-negative bacteria may be indicative of the presence of broad-spectrum bioactive compounds in the leaf. As a result, *M. oleifera* could be a promising natural antibacterial agent with pharmaceutical applications for suppressing the pathogenic bacteria (Abalaka *et al.*, 2012).

2.5 Chemometric analysis

The principal component analysis and correlation was carried out for multivariate analysis of data.

2.5.1 Principal Component Analysis

Principal component analysis is one of the most important and powerful methods in chemometrics as well as in a wealth of other areas. Principal component analysis provides the weights needed to get the new variable that best explains the variation in the whole dataset in a certain sense (Bro and Smilde, 2014). The objective of PCA is to reduce the dimensionality of a data set in which there are a large number of interrelated variables, while explaining as much of the data variation as possible. Another advantage of PCA is that the

reduction to fewer latent variables can increase the sensitivity of a control scheme to assignable causes (Mastrangelo *et al.*, 1996).

2.5.2 Correlation Plot Analysis

Correlation plot shows matrix chart of phytochemical content and pharmacological parameters. The upper panels show the Pearson's correlation coefficients while the lower panel report the scatter plots. *, **, *** indicates significance at $p<0.05$, <0.01 and 0.001 respectively (Batubara *et al.*, 2020).

Part III

Materials and methods

3.1 Materials

3.1.1 Raw Materials

Moringa leaves: Moringa leaves were collected from periphery of Central Campus of Technology, Dharan-14, Nepal.

Meat: Lean buffalo meat was procured from a slaughter place in Dharan after 4 hours of slaughtering and conditioned at 4±1°C refrigeration temperature before and after grinding for further experiment.

3.1.2 Chemicals and apparatus required

All the chemicals, glass wares and equipment required were used from Central Campus of Technology laboratory. List of the major apparatus, chemicals and equipment required are given in Appendix A.

3.2 Method

3.2.1 Experimental Procedure

3.2.1.1 Preparation of moringa leaves extract

Fresh leaves of moringa were collected from Dharan-14, Bijaypur then were cleaned by rinsing gently with distilled water for further studies. The extracts were prepared by following different aqueous methods:

a. Decoction

10 g of clean and fresh stem was cut in to small pieces and pounded in mortar and pestle. It was kept in a clean 500 ml glass beaker and 160 ml water (16 times) was added and boiled till the volume reaches to $1/4^{th}$ of initial volume. Then distilled water was added in order to make final volume of 150 ml and passed through Whatman filter paper. This extraction method is based on method prescribed in Ayurveda for preparation of herbal juices (Shingadiya *et al.,* 2016).

b. Hot Infusion

This extraction was done as per Ramirez *et al.* (2017) with some modifications. 10 g of clean and fresh stem was cut in to small pieces and grounded in mortar and pestle to semi-paste. Then 150 ml of hot water at 80°C was added and left for 5 minutes with few stirrings in between. Then it was filtered through Whatman filter paper.

c. Cold Infusion

This method was done as per Alo *et al.* (2012) with some modifications. 10 g of clean and fresh stem was cut in to small pieces and pounded in mortar and pestle. It was kept in a clean 500 ml conical flask and 150 ml of cold water was added. The mixture was stirred properly for some time with glass rod and then kept at room temperature for 24 hours. Then it was passed though Whatman filter paper. This extraction method is also given in Ayurveda scriptures for preparation of herbal juices (Shingadiya *et al.*, 2016).

3.2.1.2 Preparation of ground meat

Fresh, lean buffalo meat bought from the Dharan were closely trimmed of external fat, cut into strips for grinding. Prior to grinding meat, mincer was washed properly with water, 70% ethyl alcohol and finally with distilled water. After mincing, meat was divided into four parts, control without addition of moringa leaf extract, and other with different concentrations viz 1%, 1.5% and 0.01% BHT. The samples were packed in aluminum foil placed in PET containers placed for airtightness and stored in refrigerator at temperature of 4±1°C. All the analysis were done every alternate day for 16 days.

3.2.2 Analytical procedure

3.2.2.1 Determination of moisture content

Moisture content of the sample was determined by using hot air oven method as standard method of Ranganna (1986).

3.2.2.2 Determination of crude protein

The crude protein was determined by using micro Kjeldahl's method. 2 g fatless sample was digested, steam distillated after decomposing the former NaOH. Titration of entrapped NH_3 boric acid was done with standard acid as standard method of Ranganna (1986).

3.2.2.3 Determination of crude fat

The fat content was determined by Soxhlet method. Solvent extraction of 10 g sample was done by recycling hot solvent for number of times until complete extraction and fat was recovered by evaporating away the solvent as standard method of Ranganna (1986).

3.2.2.4 Determination of ash content

Ash content was determined using muffle furnaces according to Ranganna (1986). 5 g of weighed sample in silica crucible was charred in hot plate till no smoke raise from it and finally, ashing was done in muffle furnace at 550°C to the constant weight. The difference in weight was the total ash content remaining in crucible, under standardized condition (Ranganna, 1986).

3.2.2.5 Determination of crude fiber

Crude fiber was determined by using chemical process, the sample was treated with boiling dilute Sulphuric acid, boiling sodium hydroxide and then with alcohol as standard method of Ranganna (1986).

3.2.2.6 Estimation of total phenolic content

Total phenol in the moringa leaves extract (MLE) was determined by using Folin-Denis ciocalteu reagent as described in AOAC method with slight modification. 250 µL of unconcentrated extract was diluted with distilled water to 10 ml. Aliquots of 1 ml of diluted extract was mixed with 5 ml of 10-fold diluted Folin-Denis Ciocalteu reagent. After 3 min, 4 ml of 7.5% sodium carbonate was added. The mixtures were allowed to stand for 0.5 h at 30°C in incubator before the absorbance measured at 734 nm in UV visible spectrophotometer. The total polyphenol content in the extract was calculated and expressed as gallic acid equivalent (GAE; mg GAE/ g on dry basis) using a gallic acid (0-300µg/ml) standard curve.

3.2.2.7 Estimation of flavonoid content

Total flavonoid content was determined using a modified aluminium chloride assay method as described by (Lusia Barek *et al.*, 2015). 4 ml of moringa leaves extract was added to 0.4 ml 5% $NaNO_3$ and stand for 5 minutes. Again 0.4 ml of 5% $AlCl_3$ was added and let stand for 5 minutes. 2ml of 1N NaOH and volume was made up to 10ml using distilled water and let stand for 15 min before measuring absorbance at 510 nm against blank. The results were expressed as quercitin equivalent (mg QE/g extract).

3.2.2.8 Determination of DPPH radical scavenging capacity

The capacity of prepared MLE to scavenge the 'stable' free radical DPPH was monitored according to the method of Hatano *et al.* (1988) with slight modifications. Extracts (100 µL) was dissolved in 3.9 ml freshly prepared methanolic solution of DPPH (1 mM, 0.5 ml). The mixture was vortexed for 15 seconds and then left to stand at room temperature for 30 min in the dark. The absorbance of the resulting solution was read spectrophotometrically (UV/VIS spectrometer) at 517 nm. The percentage inhibition of the radicals due to the antioxidant activity of moringa leaves extracts (MLE) was calculated using the following formula.

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

$A_{control}$ is the absorbance of the DPPH solution with nothing added (control).

Asample is the absorbance of DPPH incorporated extract solution

Lower absorbance of the reaction mixture indicates higher free radical scavenging activity (Zhao *et al.*, 2008).

3.2.2.9 Determination of peroxide value

Peroxide value was determined based on the method described by KC and Rai (2007). 5 g of extract incorporated ground meat sample was weighed accurately (by difference) in the Iodine flask. 25 ml of solvent was added. 1 ml of KI solution was added and allowed and to stand for 1 min (with gentle shaking). 35 ml of distilled water was added and few drops of starch indicator was added. Appearance of blue color on addition of starch indicates presence of free iodine. Liberated iodine was titrated with 0.01N or 0.1N sodium thiosulphate until

the blue color vanished. Blank determination was carried out simultaneously. Peroxide value was calculated using the following equation,

$$\text{PV (meq/kg)} = \frac{N \times (V_S - V_B) \times 1000}{\text{Wt. of sample (g)}}$$

Where,

N = normality of sod-thiosulfate

V_S = sod-thiosulfate consumed by sample (ml)

V_B= sodium-thiosulfate consumed by blank (ml).

3.2.2.10 Determination of TBARS

Lipid oxidation was also evaluated by Thiobarbituric acid reactive substances (TBARS) according to the method described by (Ahn *et al.*, 1998) with some modifications. Briefly, 5 g of sample was homogenized with 15 ml of distilled water and centrifuged at 2000 rpm for 15 min. 2 ml of the resulting slurry was transferred into a test tube containing 4 ml of trichloroacetic acid/ thiobarbituric acid (TCA/TBA) solution consisting of 15% TCA (w/v) and 0.375% TBA (w/v) in 0.25 M HCl and 6 ml 2% butylated hydroxytoluene (w/v) prepared in absolute ethanol in a test tube and, the tube content was immediately vortexed. Following water bath treatment at 100 °C for 15min, the tube content was cooled rapidly down to room temperature and centrifuged at 1000 rpm for 10 min. Then, absorbance was measured at 531 nm with a spectrophotometer against blind (2 ml distilled water and 4 ml TCA-TBA-HCl solution). TBARS were calculated using 1,1,3,3-tetraethoxypropane standard curve and expressed as mg MDA/kg meat. TBARS was calculated using the formula given below as µM/g of the sample:

$$\text{TBARS (µM/g)} = (Ac \times V)/W$$

where Ac is the amount determined from the calibration curve and W is the weight of the sample taken while V is volume in ml or dilution factor of the total extract prepared.

3.2.2.11 Determination of total plate count

Total plate count was determined by Pour plate method as described by Harrigan and McCane (1979) using plate count agar and distilled water as diluent. Molten plate count agar and other glassware to be used were autoclaved prior to research.

1. 5g of meat sample was added to 45 ml of distilled water to make it 10^{-1} dilution and homogenized using vortex mixer.
2. With the help of sterile pipette, serial dilution of sample was carried out. 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution was carried out.
3. 1ml of each sample was withdrawn with the help of sterile pipette and transferred to each sterile Petri plate.
4. To each plate, 10 ml of molten Plate count agar (PCA) at 45°C was added and medium and inoculum was mixed immediately by gently swirling the plate.
5. The plates were allowed to set and incubated at 37°C for 24 to 48 hrs.

3.2.3 Statistical analysis

Experiment was conducted in three replications. Analysis of variance was done and Tukey's honesty test was performed by JMP version 14 to check the significant relationship between the mean values of the samples at $p< 0.05$. R version 4.02 was used for multivariate analyses using correlation and principal component analysis.

Part IV

Results and discussion

Leaves of *M. oleifera* variety was collected from Dharan-14, Sunsari and buffalo meat was bought from local slaughter place in Dharan. Leaves were cleaned and powdered and meat was minced and proximate analysis was carried out. Three different Moringa leaves extract by Decoction, Hot Infusion and Cold Infusion were prepared and quantitatively analyzed for phenol, flavonoid and DPPH radical scavenging capacity. Moringa leaf extract with best result was then incorporated in minced buffalo meat and TBARS, Total plate count and Peroxide value was analyzed every alternate day during storage at refrigerated temperature for 16 days.

4.1 Proximate analysis of Moringa leaves

Proximate analysis of Moringa leaves was carried out and the result is tabulated in table 4.1

Table 4.1 Proximate analysis of Moringa leaves

Components	Values (%)
M.C.	71.47±0.003
Protein	22.19±0.0005
Fat	7.82±0.0002
Crude Fiber	8.77±0.0008
Ash content	8.39±0.0003

* Values are the means of triplicate ± standard deviation. (All values are expressed in dry basis expect for moisture).

Values thus obtained were slightly different from result obtained as per Yadav (2018) which were 73.35% for moisture content, 5.52% for crude fat, 21.65% for crude protein, 8.72% for ash content, and 8.19% for crude fiber.

4.2 Effect of methods on phytochemicals and antioxidant assay of moringa leaves

Total phenol, flavonoid content and DPPH radical scavenging capacity of the extracts prepared by three different methods were found out. Gallic acid standard curve was used for

the determination of phenols and quercetin standard curve for flavonoid (Appendix B). The results are presented in table 4.2

Table 4.2 Total phenol, flavonoid and DPPH radical scavenging capacity of different extracts

Methods	Total phenol content (TPC) in dry basis (mg GAE/g of extract)	Total flavonoid content (TFC) in dry basis (mg QE/g extract)	DPPH radical scavenging capacity
Decoction	1.17 ± 0.04^{a}	30.37 ± 0.1^{a}	76.86 ± 0.62^{a}
Hot Infusion	0.83 ± 0.04^{b}	26.62 ± 0.45^{b}	75.24 ± 1.15^{a}
Cold Infusion	0.64 ± 0.04^{c}	29.7 ± 0.29^{a}	68 ± 1.87^{b}

*Values are the means of triplicate ± standard deviation. Different alphabets in the superscript represent significant difference ($p<0.05$) from each other (Appendix D).

Statistical analysis at 5% level of significance shows that the total phenol content is significantly different with methods used. Significantly highest value was obtained for Decoction method (1.17±0.04 mg GAE/g) followed by Hot Infusion (0.83±0.04 mg GAE/g) and Cold Infusion (0.64±0.04 mg GAE/g). The high value of phenolic content in the Decoction extract may be the result of improved phytochemical extraction and greater hot water penetration into the plant matrix. Higher solvent system penetration capability has a significant impact on the extraction of phenolics from plants since the majority of soluble plant phenolics are thought to be found in plant cell vacuoles whereas the majority of insoluble plant phenolics are thought to be found in the cell wall (Kronholm *et al.*, 2007) (Mokgadi *et al.*, 2013).

Statistical analysis at 5% level of significance shows that the total flavonoid content of MLE obtained by Decoction method (30.37±0.1 mg QE/g) is significantly different from that obtained from Hot infusion (26.62±0.45 mg QE/g). In the other hand, Decoction and Cold Infusion (29.7±0.29 mg QE/g) are not significantly different whereas Hot Infusion and Cold Infusion have significant difference. In study conducted by Margarida *et al.* (2019) on *A. deliciosa* and *A. arguta*, Decoction had higher value than Infusion similar to our result.

This fact, as previously observed for the TPC, may be due to the contact time and the temperature at which the fruit is submitted in the decoction, which causes a greater extraction.

Statistical analysis at 5% level of significance shows that the highest value of DPPH radical scavenging capacity was obtained for Decoction method (76.86±0.62). The value is significantly different from the value of Cold Infusion (68±1.87) whereas it is not significantly different from Hot Infusion (75.24±1.15). Hot Infusion and Cold Infusion have values that are significantly different. The highest value in the Decoction extract might be due to its corresponding high TPC in the extract which are responsible for antioxidant activity. Also, the extraction by boiling at high temperature might have helped in the extraction of alkaloid compounds present in the plant which are also known to possess antioxidant activity (Mokgadi *et al.*, 2013). While lower temperature in cold infusion gave lower values in comparison. Different extraction conditions and methods used can highly affect the antioxidant potential of extracts (Dhanani *et al.*, 2017; Waleed *et al.*, 2014).

4.2.2 Cluster dendrogram of different parameters

The cluster dendrogram of different parameters which are TPC, TFC and DPPH radical scavenging assay is given in fig. 4.2

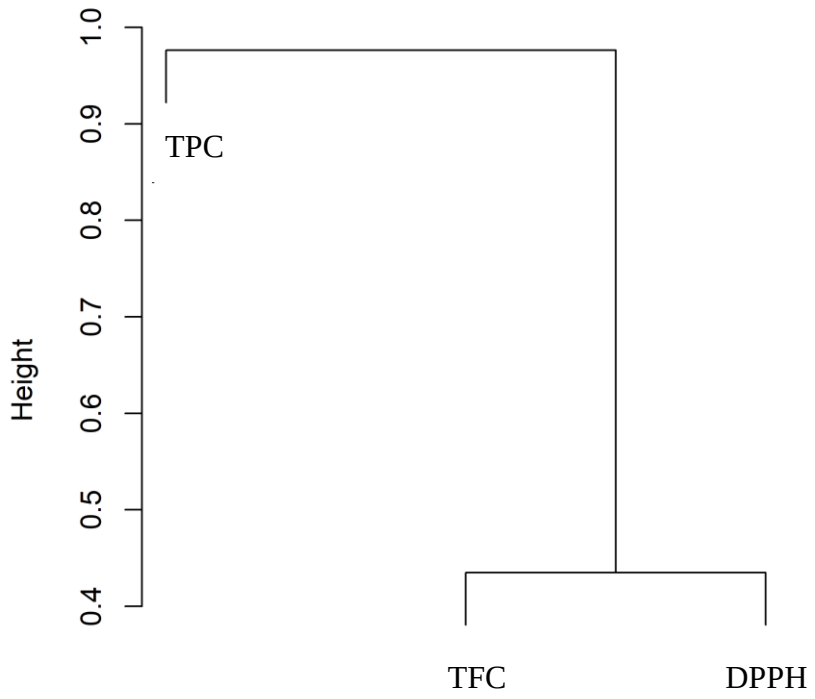

Fig. 4.2 Cluster dendrogram of different parameters

The above diagram is indicative of the fact that TFC and DPPH are similar to each other as these two are joined together by a single clade and height of the branch is nearly equal. We also can see that the TPC chunk is completely separate from the others interpreting that its distribution is substantially different from that of remaining chunks.

4.2.1 Principal component analysis

The principal component analysis for different extracted varieties of samples is shown in the fig 4.1. As we can see TPC and TFC are located in the same quadrant which indicates that there is positive correlation between phenolic and flavonoid content. The closest proximity of green circles i.e., Decoction method with TFC and TPC shows that they possess high value of these parameters. Cold infusion (red circle) is closer to TFC than TPC and DPPH whereas Hot infusion (blue circle) has closer proximity to DPPH than TPC and TFC. Hence, Decoction method is regarded best as it shows good correlation with all the given parameters in comparison to other methods.

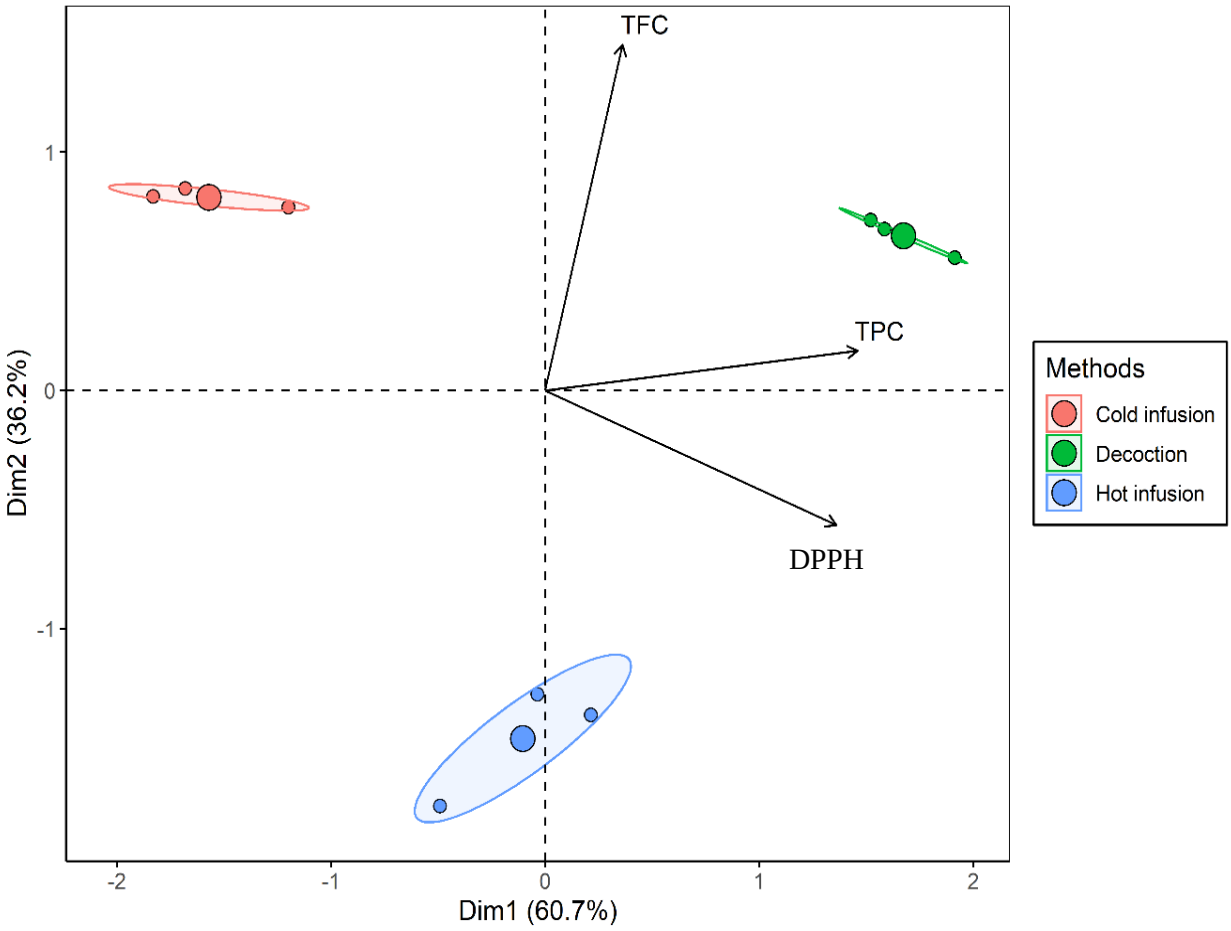

Fig. 4.1 PCA bi-plot of different extracted varieties of samples

4.1.3 Correlation between different parameters

The correlation between TPC, TFC and DPPH radical scavenging assay is shown in the fig. 4.3.

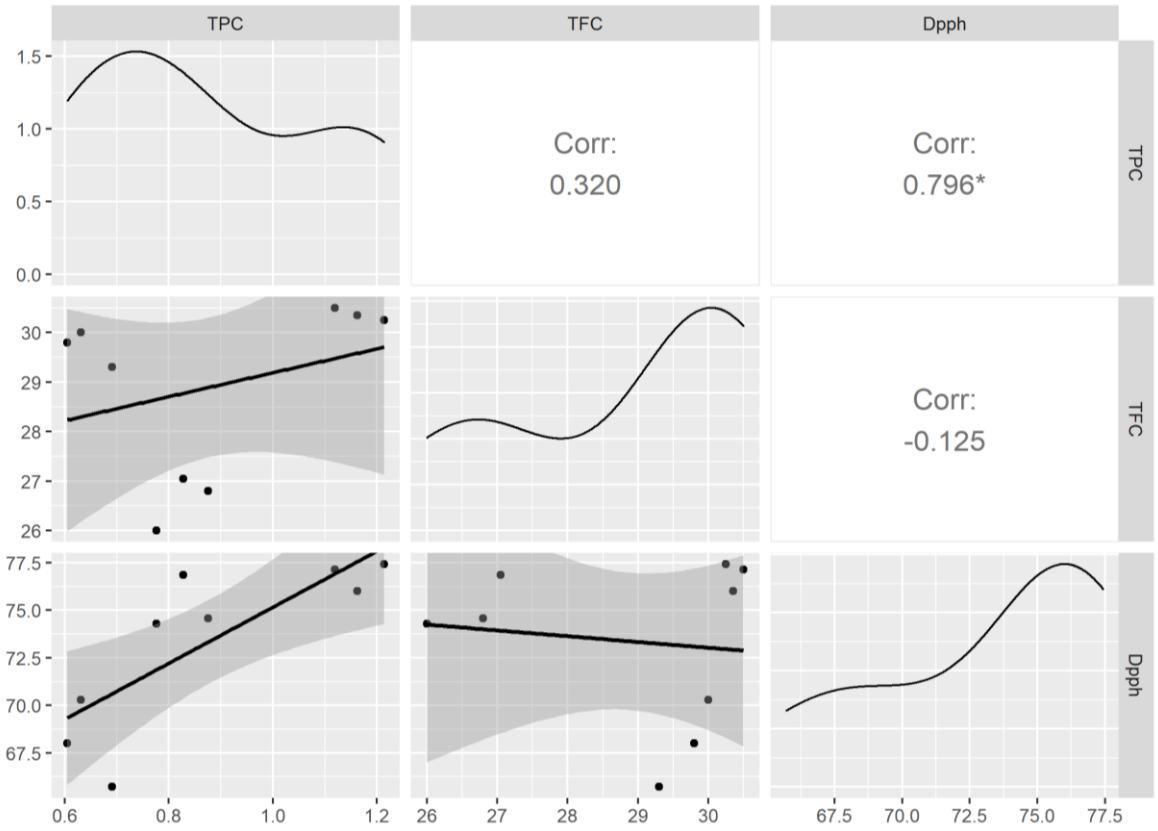

Fig. 4.3 Correlation between TPC, TFC and DPPH

4.1.3.1 Correlation between TPC and DPPH

The correlation between TPC and DPPH was found to be 0.796 which indicates them having a good positive correlation. This result showed that with increase in TPC of extracts the scavenging activity also increased indicating increase in antioxidant activity. Similar to our work Patel *et al.* (2018) has found strong positive correlation (r=0.797) between the TPC and DPPH. Kim (2012) has also reported good positive correlation (R^2=0.8623) between TPC and DPPH.

Plant polyphenols have long been cited as a source of plant-based natural antioxidants. We can understand that polyphenols present in our extracts can be good DPPH radical

scavengers and can therefore likely be a useful source of natural antioxidants and the strong correlation between them as found from our study indicates the same.

4.1.3.2 Correlation between TFC and DPPH radical scavenging activity

The correlation between TFC and DPPH is seen to be -0.125 indicating that there is weak downhill linear relationship between these two parameters which is coherent with value of Moein *et al.* (2008) (r=-0.172). The molecular structure of the different groups of flavonoids, notably the number and position of hydroxyl groups, heavily affects their antioxidant potential or capacity (Ioannou *et al.*, 2015). Different flavonoid compound can show different affinity towards DPPH radical scavenging activity (Hirano *et al.*, 2001). Therefore, the amount and presence of certain specific flavonoids in plants and plant extracts are crucial factors when determining the antioxidant effect, they have on certain compounds in various antioxidant activity assays (Hirano *et al.*, 2001; Firuzi *et al.*, 2004).

4.1.3.3 Correlation between TPC and TFC

TPC and TFC had the weak correlation with each other (r=0.32) according to our study. This can be related to the presence of some chemical groups such as amino acids and proteins that can also react with Folin-Ciocalteu reagent. Moein *et al.* (2008) had similar findings. Such low correlation was also found by other researchers such as Meda *et al.* (2005). Presence of other chemical groups such as amino acids and proteins can also interfere with TPC determination by FC reagent method thus resulting in higher content/concentration (Moein *et al.*, 2008). Although flavonoids constitute the majority of dietary polyphenols and are a subclass of polyphenols, a strong correlation between TPC and TFC levels is normally expected (Gonzalez *et al.*, 2013). However, the amount of these phytochemicals in the extracts can be significantly impacted by the various solvents used, the extraction technique, and processing (Rababah et al., 2010; Dhanani et al., 2013; Waleed et al., 2014).

4.2 Selection of best method for incorporation in the ground meat

From the above observation, Decoction method had the highest amount of phenol, flavonoid content and radical scavenging capacity. Therefore, for incorporation in ground meat, extract obtained from Decoction method was selected. The MLE was incorporated in the concentration of 1% and 1.5% in the ground meat.

4.3 Change in total peroxide value of the ground meat over time

In the four samples of ground meat incorporated with 0.01% BHT, 1% MLE, 1.5% MLE and control (without addition of MLE and BHT), peroxide value was recorded over time. The obtained values are tabulated in Appendix D. The trend of change in peroxide value is presented in fig. 4.4.

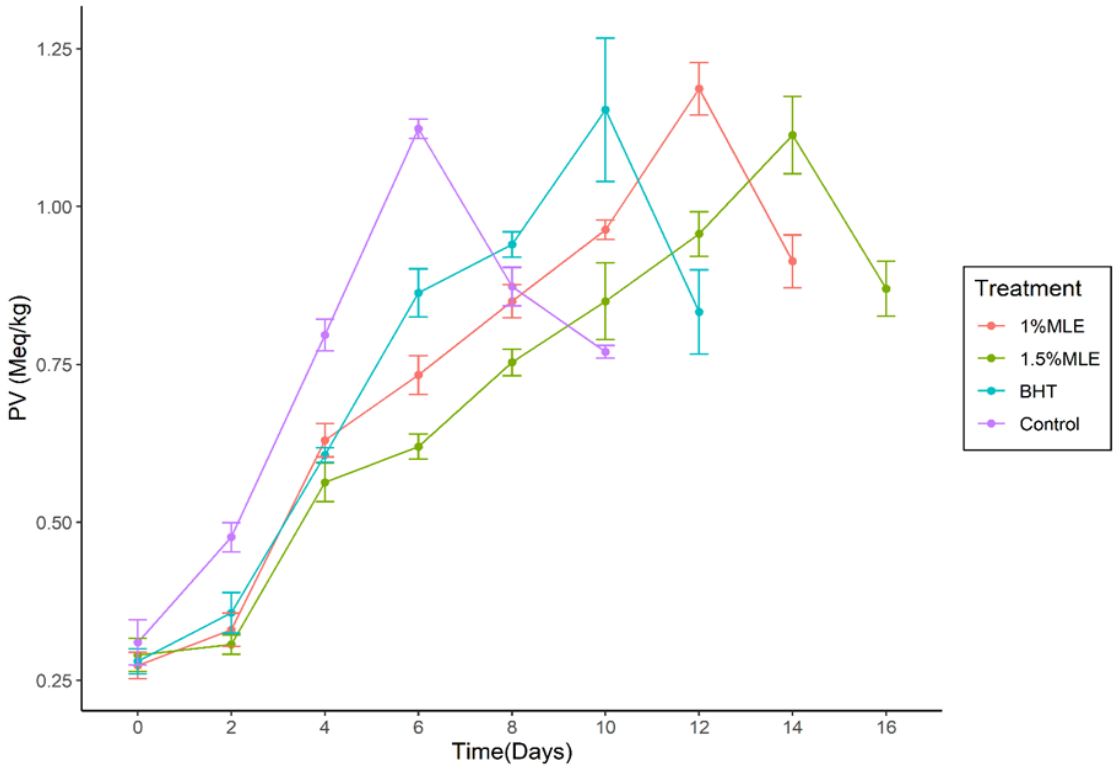

Fig. 4.4 Change in peroxide value over time for different samples

The data points are the mean values of triplicate observations. Error bars represent standard deviation from the mean. Significance testing results are presented in Appendix D.

Both antioxidant addition and storage time had significant effects on PV. The highest initial PV was observed in control samples with no antioxidant additives where the lowest result was in ground meat with 1.5% MLE.

At 5% level of significance, there is a significant difference between the peak values of PV obtained for control, 0.01% BHT, 1% MLE and 1.5% MLE treated samples. The control sample reached the peak of its value (1.12 Meq peroxide/kg fat) at 6^{th} day of observation. For BHT, peak PV value (1.153 Meq peroxide/kg fat) reached at 10^{th} day of observation.

Similarly, the maximum PV value (1.187 Meq peroxide/kg fat) for 1% MLE incorporated sample reached at 12th day of observation. For 1.5% MLE incorporated sample, maximum PV value of 1.113 Meq peroxide/kg fat reached at 14th day of observation. Higher antioxidative effect on peroxide value was observed in *M. oleifera* leaf extract treated samples than control and BHT treated samples. The result obtained agreed with Rahman *et al.* (2020) findings on MLE treated goat meat nuggets.

4.4 Estimation of change in TBARS value of the ground meat over time

In the four samples of ground meat incorporated with 0.01% BHT, 1% MLE, 1.5% MLE and control, TBARS value was recorded over time. The obtained values are tabulated in Appendix E. The trend of change in TBARS value is presented in fig. 4.5.

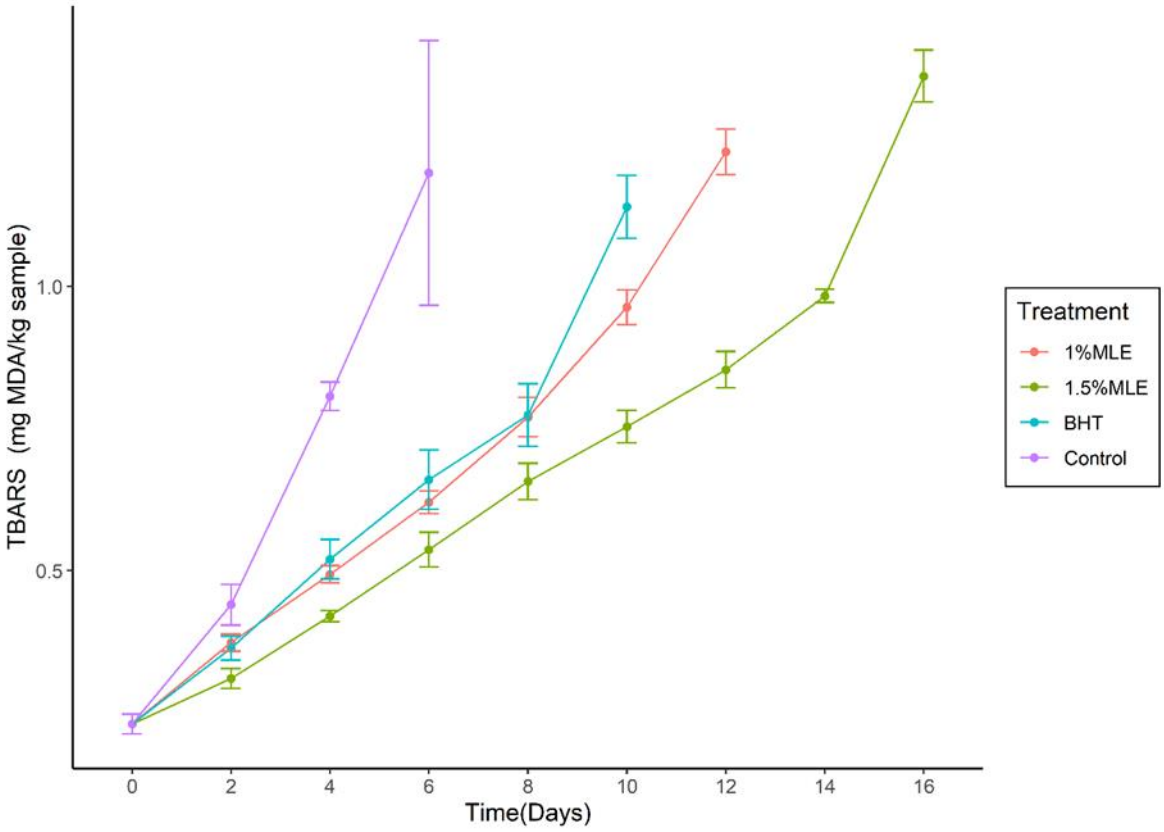

Fig. 4.5 Change in TBARS value over time for different samples

The data points are the mean values of triplicate observations. Error bars represent standard deviation from the mean. Significance testing results are presented in Appendix E.

For control, BHT incorporated sample, 1% MLE added sample and 1.5% MLE added samples, the TBARS values reached the threshold of 1 mg MDA/kg sample at 6th, 10th, 12th and 16th days respectively.

At 5% level of significance, both antioxidant and the storage time significantly affected TBARS values of ground meat. The lowest TBARS value was observed in ground meat with 1.5% MLE. Prolonged storage time resulted in high TBARS value. Addition of antioxidant sources reduced TBARS values significantly. Our result is in line with the findings of Falowo *et al.* (2017b) and Shah *et al.* (2015) who reported a lower TBARS values in beef patties treated with Moringa plant extract. These results show that the use of natural antioxidant sources could be effective in preventing meat products against lipid oxidation at refrigerated storage.

4.5 Estimation of total plate count of the ground meat over time

In the four samples of ground meat incorporated with 0.01% BHT, 1% MLE, 1.5% MLE and control, Total plate count was recorded over time. The obtained values are tabulated in Appendix F. The trend of change in total plate count is presented in fig. 4.3

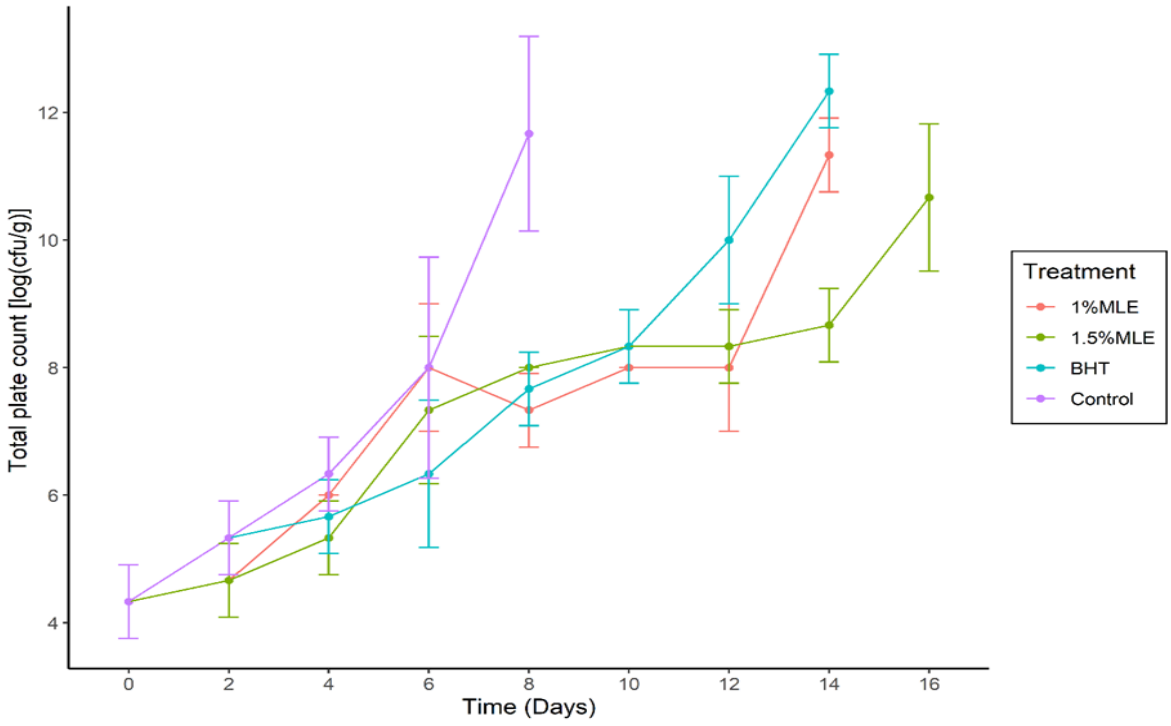

Fig. 4.3 Change in total plate count over time for different samples

The data points are the mean values of triplicate observations. Error bars represent standard deviation from the mean. Significance testing results are presented in Appendix F.

The control sample exceeded the legal threshold (10^7) in 8 days while the samples with 0.01% BHT, 1% MLE and 1.5% MLE and incorporated samples exceeded the limit in 10, 14 and 16 days. The findings are coherent with that of Hazra *et al.* (2012) done on cooked ground buffalo meat treated with MLE. Jayawardana *et al.* (2015) study on herbal chicken sausages also had significant lower value of TPC on addition of Moringa leaves powder. Phytochemicals derived from various plant sources have been shown to be a good alternative to synthetic chemical substances in preventing growth of several pathogenic bacteria (Kanatt *et al.*, 2010).

Part V

Conclusions and recommendations

5.1 Conclusions

As per the objectives, methodologies stated in the methods were carried out for the results. Based on the obtained results, the following conclusions have been drawn:

1. Extract from Decoction method was found to have highest number of total phenols, total flavonoids and antioxidant activity.
2. The leaves extract inhibited lipid oxidation significantly which was studied in terms of PV and TBARS number when compared with BHT of 0.01%.
3. The leaves extract exhibited significant antimicrobial activity studied in terms of Total plate count of the samples.
4. Significant difference between sample treated with 1% and 1.5% MLE was found indicating that increase in concentration enhances antioxidant and antimicrobial activity.

5.2 Recommendations

1. Moringa leaves extract can be used as natural antioxidant in enhancing shelf life of meat as a replacement to synthetic antioxidants.
2. Further studies can be progressed by using advanced methods for extraction.

Part VI

Summary

Moringa oleifera is known as “miracle tree” as various scientific studies have proven its antioxidant and nutritional values. Its inclusion as leaves extract in meat and meat products in place of synthetic antioxidants is a huge discovery. Buffalo meat being a promising market because of certain properties like, reduced fat and cholesterol, low connective tissue, desirable texture, high protein, water-holding capacity and emulsifying capacity is an important topic for study.

Moringa leaves were collected from Dharan, Nepal and proximate analysis was carried out and leaves were extracted by Decoction, Hot infusion and Cold infusion. The extracts were analyzed for total phenols, total flavonoids and total radical scavenging capacity. The richest value in all these properties was selected and incorporated in the ground meat at the concentration of 1% and 1.5% and compared with 0.01% BHT incorporated sample and control. Four samples were stored in refrigerated storage and Peroxide value, TBARS and TPC were recorded every alternate day for 16 days.

Phenol content of leaves extracted from decoction, hot infusion and cold infusion were found to be 1.17 ± 0.04^{a}, 0.83 ± 0.04^{b} and 0.64 ± 0.04^{c} mg GAE/g in dry basis respectively. Values of total flavonoid content were 30.37 ± 0.1^{a}, 26.62 ± 0.45^{b} and 29.7 ± 0.29^{a} mg QE/g for decoction, hot infusion and cold infusion respectively in dry basis. DPPH radical scavenging activity of extract from decoction, hot infusion and cold infusion were found to be 76.86 ± 0.62^{a}, 75.24 ± 1.15^{a} and 68 ± 1.87^{b}. From the above observation, Decoction method had the highest amount of phenol, flavonoid content and radical scavenging capacity hence was selected from incorporating it with ground meat.

PV reached peak of its value for control, BHT, 1% MLE and 1.5% MLE treated samples at 6^{th}, 10^{th}, 12^{th} and 14^{th} day respectively. Threshold TBARS number of 1 mg MDA/kg sample reached at 6^{th}, 10^{th}, 12^{th} and 16^{th} days day respectively for control, BHT, 1% MLE incorporated sample and 1.5% MLE incorporated sample. TPC recorded overtime shows that Legal threshold (10^{7}) was exceeded in 8^{th} and 12^{th} days for control and BHT incorporated sample while for 1% MLE and 1.5% MLE incorporated sample, threshold reached at 14^{th} and 16^{th} days.

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Appendices

Appendix A

Chemicals and equipment used

Chemicals:

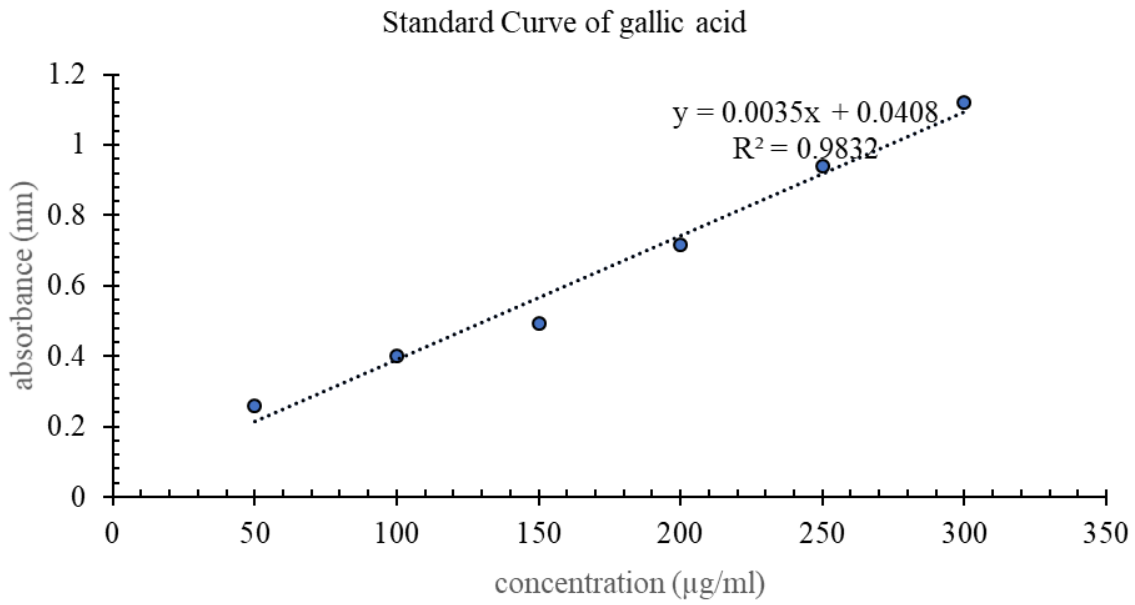
1. Sodium Carbonate
2. Gallic acid
3. Sulphuric acid
4. Sodium Phosphate
5. Ammonium Molybdate
6. Ascorbic acid
7. Phosphate buffer
8. Potassium Fericyanide
9. Trichloro acetic acid
10. Potassium Iodide
11. Sodium Thiosulphate
12. Folin-Denis Ciocalteu reagent

Apparatus:

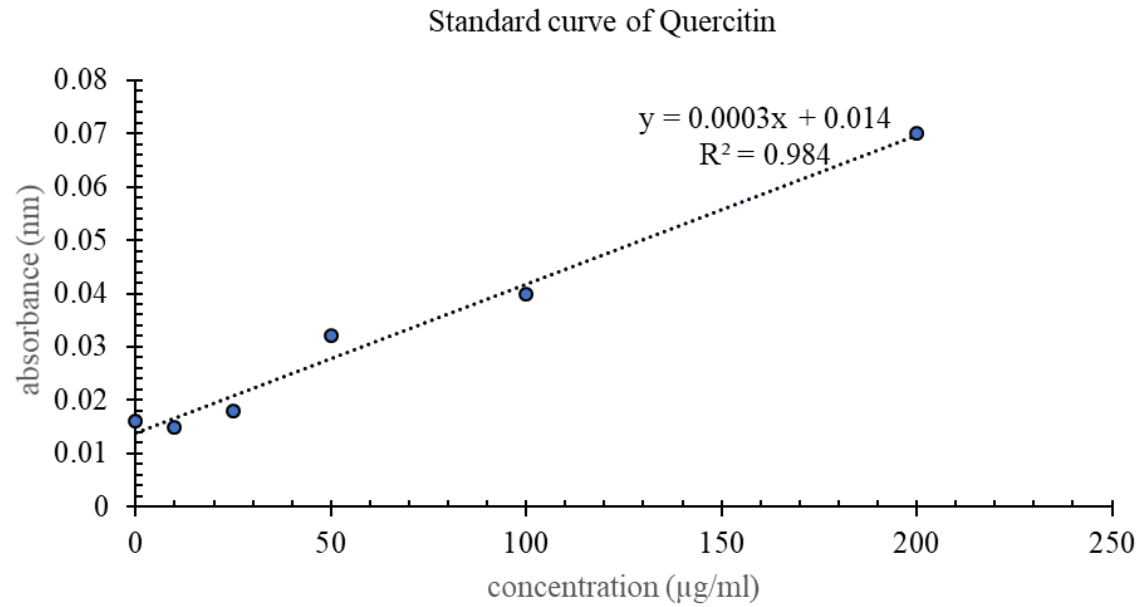
1. Refrigerator
2. Water bath
3. Spectrophotometer
4. Glassware
5. Weighing Balance
6. Grinder
7. Mincer
8. Meat chopper
9. Hot air oven
10. Incubator
11. Centrifuge machine
12. Vacuum evaporator

Appendix B

B.1 Standard curve for gallic acid



B.2 Standard curve for Quercetin



Appendix C

C.1 Significance test for phenol

Dependent variable: phenol

Table C.1 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Methods	2	0.42197143	0.210986	95.9916	<.0001*
Error	6	0.01318776	0.002198		
C. Total	8	0.43515918			

Connecting Letters Report

Level				Mean
Decoction	A			1.1651429
Hot infusion		B		0.8265714
Cold infusion			C	0.6422857

*Levels not connected by same letter are significantly different.

C.2 Significance test for flavonoids

Dependent variable: phenol

Table C.2 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Methods	2	24.013889	12.0069	80.6437	<.0001*
Error	6	0.893333	0.1489		
C. Total	8	24.907222			

Connecting Letters Report

Level			Mean
Decoction	A		30.366667
Cold infusion	A		29.700000
Hot infusion		B	26.616667

*Levels not connected by same letter are significantly different.

C.3. Significance test for DPPH radical scavenging capacity

Dependent variable: DPPH radical scavenging capacity

Table C.3 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Methods	2	133.46032	66.7302	25.7238	0.0011*
Error	6	15.56463	2.5941		
C. Total	8	149.02494			

Connecting Letters Report

Level			Mean
Decoction	A		76.857143
Hot infusion	A		75.238095
Cold infusion		B	68.000000

*Levels not connected by same letter are significantly different.

Appendix D

D. Significance test for effect of various treatments of sample to Peroxide Value with time

D.1 Within treatments

Table no. D.1.1 One-way analysis of Day 0 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.00230000	0.000767	1.0824	0.4101
Error	8	0.00566667	0.000708		
C. Total	11	0.00796667			

Connecting Letters Report

Level		Mean
Control	A	0.31000000
1.5%MLE	A	0.29000000
BHT	A	0.28000000
1%MLE	A	0.27333333

*Levels not connected by same letter are significantly different.

Table no. D.1.2 One-way analysis of Day 2 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.05142500	0.017142	27.4267	0.0001*
Error	8	0.00500000	0.000625		
C. Total	11	0.05642500			

Connecting Letters Report

Level			Mean
Control	A		0.47666667
BHT		B	0.35666667
1%MLE		B	0.33000000
1.5%MLE		B	0.30666667

*Levels not connected by same letter are significantly different.

Table no. D.1.3 One-way analysis of Day 4 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.09389167	0.031297	52.1620	<.0001*
Error	8	0.00480000	0.000600		
C. Total	11	0.09869167			

Connecting Letters Report

Level				Mean
Control	A			0.79666667
1%MLE		B		0.63000000
BHT		B	C	0.60666667
1.5%MLE			C	0.56333333

*Levels not connected by same letter are significantly different.

Table no. D.1.4 One-way analysis of Day 6 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.42150000	0.140500	187.3333	<.0001*
Error	8	0.00600000	0.000750		
C. Total	11	0.42750000			

Connecting Letters Report

Level				Mean
Control	A			1.1233333
BHT		B		0.8633333
1%MLE			C	0.7333333
1.5%MLE			D	0.6200000

*Levels not connected by same letter are significantly different.

Table no. D.1.5 One-way analysis of Day 8 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.05375833	0.017919	29.0586	0.0001*
Error	8	0.00493333	0.000617		
C. Total	11	0.05869167			

Connecting Letters Report

Level				Mean
BHT	A			0.94000000
Control		B		0.87333333
1%MLE		B		0.85000000
1.5%MLE			C	0.75333333

*Levels not connected by same letter are significantly different.

Table no. D.1.6 One-way analysis of Day 10 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.24875833	0.082919	19.5488	0.0005*
Error	8	0.03393333	0.004242		
C. Total	11	0.28269167			

Connecting Letters Report

Level				Mean
BHT	A			1.1533333
1%MLE		B		0.9633333
1.5%MLE		B	C	0.8500000
Control			C	0.7700000

*Levels not connected by same letter are significantly different.

Table no. D.1.7 One-way analysis of Day 12 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	2	0.19295556	0.096478	39.1126	0.0004*
Error	6	0.01480000	0.002467		
C. Total	8	0.20775556			

Connecting Letters Report

Level			Mean
1%MLE	A		1.1866667
1.5%MLE		B	0.9566667
BHT		B	0.8333333

*Levels not connected by same letter are significantly different.

Table no. D.1.8 One-way analysis of Day 14 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	0.06000000	0.060000	21.9512	0.0094*
Error	4	0.01093333	0.002733		
C. Total	5	0.07093333			

HSD Threshold Matrix

Abs (Dif)-HSD

	1.5%MLE	1%MLE
1.5%MLE	-0.11852	0.08148
1%MLE	0.08148	-0.11852

*Positive values show pairs of means that are significantly different.

Table no. D.1.9 One-way analysis of Day 16 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	0	0.00000000	.	.	.
Error	2	0.00380000	0.001900		
C. Total	2	0.00380000			

D.2 Within treatments

Table no. D.2.1 One-way analysis of Control by Days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	5	1.2651833	0.253037	406.6661	<.0001*
Error	12	0.0074667	0.000622		
C. Total	17	1.2726500			

Connecting Letters Report

Level						Mean
6	A					1.1233333
8		B				0.8733333
4			C			0.7966667
10			C			0.7700000
2				D		0.4766667
0					E	0.3100000

*Levels not connected by same letter are significantly different.

Table no. D.2.2 One-way analysis of BHT by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	6	1.8240476	0.304008	102.4746	<.0001*
Error	14	0.0415333	0.002967		
C. Total	20	1.8655810			

Connecting Letters Report

Level						Mean
10	A					1.1533333
8		B				0.9400000
6		B				0.8633333
12		B				0.8333333
4			C			0.6066667
2				D		0.3566667
0				D		0.2800000

*Levels not connected by same letter are significantly different.

Table no. D.2.3 One-way analysis of 1%MLE by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	7	2.0680667	0.295438	329.7914	<.0001*
Error	16	0.0143333	0.000896		
C. Total	23	2.0824000			

Connecting Letters Report

Level							Mean
12	A						1.1866667
10		B					0.9633333
14		B	C				0.9133333
8			C				0.8500000
6				D			0.7333333
4					E		0.6300000
2						F	0.3300000
0						F	0.2733333

*Levels not connected by same letter are significantly different.

Table no. D.2.4 One-way analysis of 1.5% MLE by Days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	8	1.9163852	0.239548	162.5075	<.0001*
Error	18	0.0265333	0.001474		
C. Total	26	1.9429185			

Connecting Letters Report

Level						Mean
14	A					1.1133333
12		B				0.9566667
16		B				0.8700000
10		B	C			0.8500000
8			C			0.7533333
6				D		0.6200000
4				D		0.5633333
2					E	0.3066667
0					E	0.2900000

*Levels not connected by same letter are significantly different

Appendix E

E. Significance test for effect of various treatments of sample to TBARS Value with time

E.1 Within treatments

Table no. E.1.1 One-way analysis of Day 0 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.00000000	0.000000	0.0000	1.0000
Error	8	0.00240000	0.000300		
C. Total	11	0.00240000			

Connecting Letters Report

Level		Mean
1%MLE	A	0.23000000
1.5%MLE	A	0.23000000
BHT	A	0.23000000
Control	A	0.23000000

*Levels not connected by same letter are significantly different.

Table no. E.1.2 One-way analysis of Day 2 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.02563333	0.008544	15.0784	0.0012*
Error	8	0.00453333	0.000567		
C. Total	11	0.03016667			

Connecting Letters Report

Level				Mean
Control	A			0.44000000
1%MLE		B		0.37333333
BHT		B	C	0.36333333
1.5%MLE			C	0.31000000

*Levels not connected by same letter are significantly different.

Table no. E.1.3 One-way analysis of Day 4 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.25946667	0.086489	159.6718	<.0001*
Error	8	0.00433333	0.000542		
C. Total	11	0.26380000			

Connecting Letters Report

Level				Mean
Control	A			0.80666667
BHT		B		0.52000000
1%MLE		B		0.49333333
1.5%MLE			C	0.42000000

*Levels not connected by same letter are significantly different.

Table no E.1.4 One-way analysis of Day 6 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.81882500	0.272942	18.7160	0.0006*
Error	8	0.11666667	0.014583		
C. Total	11	0.93549167			

Connecting Letters Report

Level				Mean
Control	A			1.2000000
BHT			B	0.6600000
1%MLE			B	0.6200000
1.5%MLE			B	0.5366667

*Levels not connected by same letter are significantly different.

Table no E.1.5 One-way analysis of Day 8 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	2	0.02646667	0.013233	7.5380	0.0231*
Error	6	0.01053333	0.001756		
C. Total	8	0.03700000			

Connecting Letters Report

Level			Mean
BHT	A		0.77333333
1%MLE	A		0.77000000
1.5%MLE		B	0.65666667

*Levels not connected by same letter are significantly different.

Table no. E.1.6 One-way analysis of Day 10 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	2	0.22482222	0.112411	69.2945	<.0001*
Error	6	0.00973333	0.001622		
C. Total	8	0.23455556			

Connecting Letters Report

Level				Mean
BHT	A			1.1400000
1%MLE		B		0.9633333
1.5%MLE			C	0.7533333

*Levels not connected by same letter are significantly different.

Table no. E.1.7 One-way analysis of Day 12 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	0.22041667	0.220417	165.3125	0.0002*
Error	4	0.00533333	0.001333		
C. Total	5	0.22575000			

HSD Threshold Matrix

Abs (Dif)-HSD

	1%MLE	1.5%MLE
1%MLE	-0.08278	0.30056
1.5%MLE	0.30056	-0.08278

*Positive values show pairs of means that are significantly different.

Table no. E.1.8 One-way analysis of Day 14 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	0	0.00000000	.	.	.
Error	2	0.00026667	0.000133		
C. Total	2	0.00026667			

Table no. E.1.9 One-way analysis of Day 16 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	0	0.00000000	.	.	.
Error	2	0.00420000	0.002100		
C. Total	2	0.00420000			

E.2 Within treatments

Table no. E.2.1 One-way analysis of Control by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	3	1.6382250	0.546075	38.6374	<.0001*
Error	8	0.1130667	0.014133		
C. Total	11	1.7512917			

Connecting Letters Report

Level				Mean
6	A			1.2000000
4		B		0.8066667
2			C	0.4400000
0			C	0.2300000

*Levels not connected by same letter are significantly different.

Table no. E.2.2 One-way analysis of BHT by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	5	1.5699111	0.313982	174.9746	<.0001*
Error	12	0.0215333	0.001794		
C. Total	17	1.5914444			

Connecting Letters Report

Level					Mean
10	A				1.1400000
8		B			0.7733333
6		B			0.6600000
4			C		0.5200000
2				D	0.3633333
0				E	0.2300000

*Levels not connected by same letter are significantly different.

Table no. E.2.3 One-way analysis of 1% MLE by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	6	2.1974286	0.366238	519.66	<.0001*
Error	14	0.0098667	0.000705		
C. Total	20	2.2072952			

Connecting Letters Report

Level								Mean
12	A							1.2366667
10		B						0.9633333
8			C					0.7700000
6				D				0.6200000
4					E			0.4933333
2						F		0.3733333
0							G	0.2300000

*Levels not connected by same letter are significantly different.

Table no E.2.4 One-way analysis of 1.5% MLE by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	8	3.0948519	0.386856	514.5382	<.0001*
Error	18	0.0135333	0.000752		
C. Total	26	3.1083852			

Connecting Letters Report

Level		Mean
16	A	1.3700000
14	B	0.9833333
12	C	0.8533333
10	D	0.7533333
8	E	0.6566667
6	F	0.5366667
4	G	0.4200000
2	H	0.3100000
0	I	0.2300000

*Levels not connected by same letter are significantly different

Appendix F

F. Significance test for effect of various treatments of sample to TPC Value with time

F.1 Between Treatments

Table no. F.1.1 One-way analysis of Day 0 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	0.0000000	0.000000	0.0000	1.0000
Error	8	2.6666667	0.333333		
C. Total	11	2.6666667			

Connecting Letters Report

Level		Mean
1%MLE	A	4.3333333
1.5%MLE	A	4.3333333
BHT	A	4.3333333
Control	A	4.3333333

*Levels not connected by same letter are significantly different.

Table no. F.1.2 One-way analysis of Day 2 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	1.3333333	0.444444	1.3333	0.3300
Error	8	2.6666667	0.333333		
C. Total	11	4.0000000			

Connecting Letters Report

Level		Mean
BHT	A	5.3333333
Control	A	5.3333333
1%MLE	A	4.6666667
1.5%MLE	A	4.6666667

*Levels not connected by same letter are significantly different.

Table no. F.1.3 One-way analysis of Day 4 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	1.6666667	0.555556	2.2222	0.1631
Error	8	2.0000000	0.250000		
C. Total	11	3.6666667			

Connecting Letters Report

Level		Mean
Control	A	6.3333333
1%MLE	A	6.0000000
BHT	A	5.6666667
1.5%MLE	A	5.3333333

*Levels not connected by same letter are significantly different.

Table no. F.1.4 One-way analysis of Day 6 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	5.583333	1.86111	1.1167	0.3979
Error	8	13.333333	1.66667		
C. Total	11	18.916667			

Connecting Letters Report

Level		Mean
1%MLE	A	8.0000000
Control	A	8.0000000
1.5%MLE	A	7.3333333
BHT	A	6.3333333

*Levels not connected by same letter are significantly different.

Table no. F.1.5 One-way analysis of Day 8 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	36.666667	12.2222	16.2963	0.0009*
Error	8	6.000000	0.7500		
C. Total	11	42.666667			

Connecting Letters Report

Level			Mean
Control	A		11.666667
1.5%MLE		B	8.000000
BHT		B	7.666667
1%MLE		B	7.333333

*Levels not connected by same letter are significantly different.

Table no. F.1.6 One-way analysis of Day 10 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	2	0.2222222	0.111111	0.5000	0.6297
Error	6	1.3333333	0.222222		
C. Total	8	1.5555556			

Connecting Letters Report

Level		Mean
1.5%MLE	A	8.3333333
BHT	A	8.3333333
1%MLE	A	8.0000000

*Levels not connected by same letter are significantly different.

Table no. F.1.7 One-way analysis of Day 12 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	2	6.888889	3.44444	4.4286	0.0659
Error	6	4.666667	0.77778		
C. Total	8	11.555556			

HSD Threshold Matrix

Abs (Dif)-HSD

	BHT	1.5%MLE	1%MLE
BHT	-2.2093	-0.5427	-0.2093
1.5%MLE	-0.5427	-2.2093	-1.8760
1%MLE	-0.2093	-1.8760	-2.2093

*Positive values show pairs of means that are significantly different.

Table no. F.1.8 One-way analysis of Day 14 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	2	21.555556	10.7778	32.3333	0.0006*
Error	6	2.000000	0.3333		
C. Total	8	23.555556			

HSD Threshold Matrix

Abs (Dif)-HSD

	BHT	1%MLE	1.5%MLE
BHT	-1.4463	-0.4463	2.2203
1%MLE	-0.4463	-1.4463	1.2203
1.5%MLE	2.2203	1.2203	-1.4463

*Positive values show pairs of means that are significantly different.

Table no. F.1.9 One-way analysis of Day 16 by treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	0	0.0000000	.	.	.
Error	2	2.6666667	1.33333		
C. Total	2	2.6666667			

F.2 Within treatments

Table no. F.2.1 One-way analysis of control by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	4	99.06667	24.7667	19.5526	0.0001*
Error	10	12.66667	1.2667		
C. Total	14	111.73333			

Connecting Letters Report

Level					Mean
8	A				11.666667
6		B			8.000000
4		B	C		6.333333
2		B	C		5.333333
0			C		4.333333

*Levels not connected by same letter are significantly different.

Table no. F.2.2 One-way analysis of BHT by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	7	149.33333	21.3333	39.3846	<.0001*
Error	16	8.66667	0.5417		
C. Total	23	158.00000			

Connecting Letters Report

Level						Mean
14	A					12.333333
12		B				10.000000
10		B	C			8.333333
8			C	D		7.666667
6			C	D	E	6.333333
4				D	E	5.666667
2					E	5.333333
0					E	4.333333

*Levels not connected by same letter are significantly different.

Table no. F.2.3 One-way analysis of 1% MLE by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	7	105.29167	15.0417	36.1000	<.0001*
Error	16	6.66667	0.4167		
C. Total	23	111.95833			

Connecting Letters Report

Level					Mean
14	A				11.333333
6		B			8.000000
10		B			8.000000
12		B			8.000000
8		B	C		7.333333
4			C	D	6.000000
2				D	4.666667
0				D	4.333333

*Levels not connected by same letter are significantly different.

Table no. F.2.4 One-way analysis of 1.5% MLE by days

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Days	8	106.29630	13.2870	25.6250	<.0001*
Error	18	9.33333	0.5185		
C. Total	26	115.62963			

Connecting Letters Report

Level					Mean
16	A				10.666667
14	A	B			8.666667
10		B			8.333333
12		B			8.333333
8		B			8.000000
6		B	C		7.333333
4			C	D	5.333333
2				D	4.666667
0				D	4.333333

*Levels not connected by same letter are significantly different.

Appendix G

List of plates

Plate no G.1 Moringa leaves collected for study

Plate no G.2 Mincing of lean buffalo meat

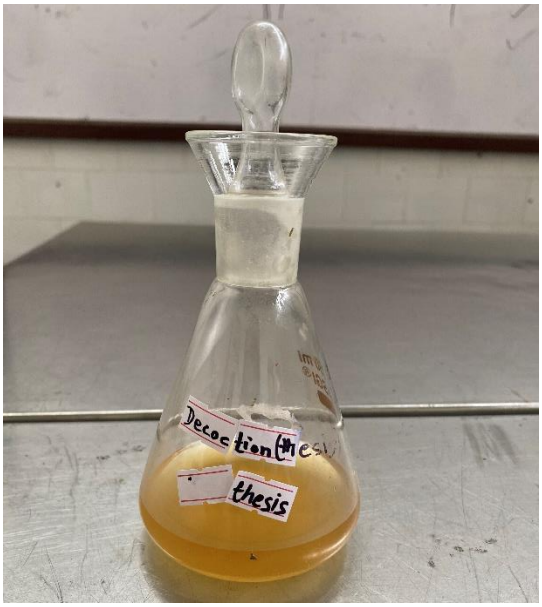


Plate no G.3 Extract obtained from decoction method

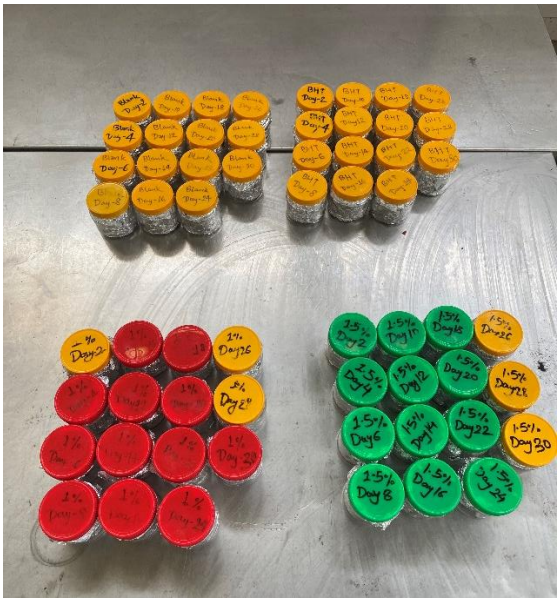


Plate no G.4 Extract incorporated samples ready for storage

Plate no G.5 Total plate count for microbiological analysis

Plate no G.6 Performing analysis in laboratory